Ki16425, a Subtype-Selective Antagonist for EDG-Family Lysophosphatidic Acid Receptors

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ABSTRACT

Lysophosphatidic acid (LPA) exerts a variety of biological responses through specific receptors: three subtypes of the EDG-family receptors, LPA₁, LPA₂, and LPA₃ (formerly known as EDG-2, EDG-4, and EDG-7, respectively), and LPA₄/GPR23, structurally distinct from the EDG-family receptors, have so far been identified. In the present study, we characterized the action mechanisms of 3-(4-[4-(1-[2-chlorophenyl]ethoxy)carbonyl amino)-3-methyl-5-isoxazolyl] benzylsulfanyl) propanoic acid (Ki16425) on the EDG-family LPA receptors. Ki16425 inhibited several responses specific to LPA, depending on the cell types, without any appreciable effect on the responses to other related lipid receptor agonists, including sphingosine 1-phosphate. With the cells overexpressing LPA₁, LPA₂, or LPA₃, we examined the selectivity and mode of inhibition by Ki16425 against the LPA-induced actions and compared them with those of diocetyl glycerol pyrophosphate (DGPP 8:0), a recently identified antagonist for LPA receptors. Ki16425 inhibited the LPA-induced response in the decreasing order of LPA₁ ≫ LPA₃ ≫ LPA₂, whereas DGPP 8:0 preferentially inhibited the LPA₃-induced actions. Ki16425 inhibited LPA-induced guanosine 5′-O-(3-thiotriphosphate binding as well as LPA receptor binding to membrane fractions with a same pharmacological specificity as in intact cells. The difference in the inhibition profile of Ki16425 and DGPP 8:0 was exploited for the evaluation of receptor subtypes involved in responses to LPA in A431 cells. Finally, Ki16425 also inhibited LPA-induced long-term responses, including DNA synthesis and cell migration. In conclusion, Ki16425 selectively inhibits LPA receptor-mediated actions, especially through LPA₁ and LPA₂; therefore, it may be useful in evaluating the role of LPA and its receptor subtypes involved in biological actions.

Lysophosphatidic acid (LPA) has been shown to elicit diverse biological actions, including Ca²⁺ mobilization, change in cAMP accumulation, change in cell shape and motility in association with actin rearrangement, and proliferation in a variety of cell types (Moolenaar, 1999; Contos et al., 2000; Ye et al., 2002). Extracellular LPA has also been shown to be involved in certain diseases, such as atherosclerosis and cancer (Xu et al., 1995, 2001; Siess et al., 1999; Maschberger et al., 2000). LPA was first thought to be released from activated platelets; however, a major part of extracellular LPA has been shown to be produced from lysophosphatidylcholine by lysosphospholipase D, which was previously called autotaxin (Sano et al., 2002). LPA increases low-density lipoprotein during its oxidation, activates endothelial cells (Siess et al., 2002). The concentration of plasma LPA is about 100 nM, and its serum concentration can be as high as 5 μM (Sano et al., 2002). LPA increases low-density lipoprotein during its oxidation, activates endothelial cells (Siess et al., 2002). This work was supported in part by a research grant grants-in-aid for scientific research from the Japan Society for the Promotion of Science and by research grants from The Mitsubishi Foundation, Pusan National University and Yamanouchi Foundation for Research on Metabolic Disorders. H.O. and K.S. contributed equally to this work.

ABBREVIATIONS: LPA, 1-oleoyl-sn-glycero-3-phosphate or lysophosphatidic acid; Ki16425, 3-(4-[4-{[1-(2-chlorophenyl)ethoxy]carbonyl amino}-3-methyl-5-isoxazolyl] benzylsulfanyl) propanoic acid; DGPP 8:0, diocetyl glycerol pyrophosphate; PAF, platelet activating factor; S1P, sphingosine 1-phosphate; SPC, sphingosylphosphorylcholine; EDG, endothelial differentiation gene; BSA, bovine serum albumin; AM, acetoxymethyl ester; PTX, pertussis toxin; GTPγS, guanosine 5′-O-(3-thiotriphosphate; Bp, base pair(s); RT, reverse transcription; PCR, polymerase chain reaction; CHO, Chinese hamster ovary; HEK, human embryonic kidney; FBS, fetal bovine serum; Ro 20-1724, 4-[3-butoxy-4-methoxyphenyl]-methyl]-2-imidazolidinone; MOPS, 3-(N-morpholino)propanesulfonic acid; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; VPC12249, N-oleoyl-2-(benzyly-4-oxobenzyl) ethanolamide phosphoric acid.
some proliferator-activated receptor although a novel intracellular mechanism through peroxi-

Although a novel intracellular mechanism through peroxisome proliferator-activated receptor γ was recently demonstrated (McIntyre et al., 2003). These LPA receptor subtypes are expressed and function in a variety of cell types; however, the precise role of each LPA receptor subtype has not yet been fully characterized.

Receptor antagonists are very useful tools for evaluating the role of LPA and its receptors in biological actions and for controlling specific diseases (Tigyi, 2001). Based on their ability to inhibit Ca2+ response to LPA in A431 cells or LPA-responsive cells, we have screened 150,000 low-molecular-weight compounds developed by the Kirin Brewery Co. Ltd, for LPA receptor antagonists, and found that some isoxazole derivatives showed such an inhibitory activity against the LPA action. We therefore synthesized several isoxazole derivatives and finally selected Ki16425 as the best candidate compound (Ueno et al., 2001). In the present study, we examined the pharmacological properties of Ki16425 and compared it with that of DGPP 8:0, a recently identified LPA antagonist (Fischer et al., 2001). We found that Ki16425 inhibited LPA-induced actions in a manner highly specific to LPA and LPA receptor subtypes. Such differences in the selectivity of Ki16425 and DGPP 8:0 were successfully applied to discriminate the receptor subtypes in A431 cells. Thus, the novel antagonist Ki16425 seems to be a useful tool for investigating physiological and pathophysiological roles of LPA and its receptors.

Materials and Methods

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**Materials.** 1-Oleoyl-sn-glycero-3-phosphate (LPA), 1,α-l-lysophosphatidylcholine palmitoyl (C16:0), 1,α-l-lysophosphatidylcholine β-acetyl-γ-O-alkyl (PAF), and 1,β-d-galactosylsphingosine (psychosine) were purchased from Sigma-Aldrich (St. Louis, MO); sphingosine 1-phosphate (SIP) was from Cayman Chemical Co. (Ann Arbor, MI); sphingosylphosphorylcholine (SPC) was from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA); 2-arachidonylglycerol and fatty acid-free BSA (Fraction V) were from Calbiochem-Novabiochem Co. (San Diego, CA); diocyl glycerol pyrophosphate (DGPP 8:0) was from Avanti Polar Lipids, Inc. (Alabaster, AL); Fura 2/AM was from Dojindo (Tokyo, Japan); PTX was from List Biological Laboratories, Inc. (Campbell, CA); [myo-2-H]inositol (23.0 Ci/mmol) was from American Radiolabeled Chemicals, Inc. (St. Louis, MO); [35S]GTPγS (1.200 Ci/mmol) was from Amersham Biosciences, Inc. (Piscataway, NJ); and [3H]LPA (48 Ci/mmol) was from PerkinElmer Life Science Products, Inc. (Boston, MA). Ki16425 was synthesized by Kirin Brewery Co. (Takasaki, Japan), and its chemical structure is shown in Fig. 1.

**Preparation of LPA Receptor cDNA Plasmids and Expression.** The entire coding regions of LPA1 (1,095 bp, GenBank accession number Y09479), LPA2 (1,149 bp, GenBank accession number AF011466), and LPA3 (1,148 bp, GenBank accession number AF127138) were amplified from human cDNA library by RT-PCR. The respective amplified fragment was cloned into the EcoRI site of pEFeNeo eukaryotic expression vector (Kon et al., 1999; Sato et al., 2000), and each DNA sequence was confirmed. The primers used for the RT-PCR were as follows. The 5′-primers contained an EcoRI site, a Kozak sequence (CCACC), and the N-terminal region of the respective receptor. The 3′-primers contained an EcoRI site and a stop codon in addition to the C-terminal region of the respective receptor. CHO cells or RH7777 cells were transfected with pEFeNeo empty vector alone or the pEFeNeo vector containing human LPA1, human LPA2, or human LPA3 by electroporation, and the neomycin-resistant cells (G418 sulfate at 1 mg/ml for CHO cells and 0.3 mg/ml for RH7777 cells) were selected. CHO cells resistant to G418 were used in bulk, whereas RH7777 cells expressing the different LPA receptor subtypes were cloned by limiting dilution. Subsequently, clonal RH7777 cells expressing human LPA1 (C3) and human LPA2 (C5) were isolated. However, in the early stages of the experiments, we were unable to obtain stable human LPA1, RH7777 cells; therefore, we used a stable mouse LPA1-expressing clone of RH7777 cells, which was provided by Prof. Kevin R. Lynch of the University of Virginia School of Medicine (Im et al., 2000). Human LPA1 cDNA was also subcloned into the HindIII/BamHI site of pcDNA3. Human and mouse orthologs of LPA1 subcloned into pcDNA3 vector (Im et al., 2000), provided by Prof. Kevin R. Lynch, were transfected into HEK293T cells.

**Cell Culture.** THP-1, HL-60, and A431 cells were cultured in RPMI 1640 media supplemented with 10% (v/v) FBS (Sigma) (Okajima and Kondo, 1995); Swiss 3T3 fibroblasts, SIP receptor-transfected CHO cells (Kon et al., 1999), and LPA receptor-transfected CHO cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) FBS; and LPA receptor-transfected RH7777 cells and HEK293T cells were cultured in minimum essential medium supplemented with 10% (v/v) FBS. FRTL-5 thyroid cells and PC-12 cells were cultured as described previously (Sho et al., 1999; Maschberger et al., 2000), and has also been identified as a growth-promoting factor for cancer cells in malignant ascites of patients suffering from ovarian cancer (Xu et al., 1995; Xu et al., 2001). These cellular responses to LPA are mediated through G-protein-coupled receptors, and several subtypes of LPA receptors, including LPA1/EDG-2, LPA2/EDG-4, LPA3/EDG-7, and LPA4/GPR23, a non-EDG-family LPA receptor, have been identified (Hecht et al., 1996; An et al., 1998a,b; Bandoh et al., 1999; Moolenaar, 1999; Contos et al., 2000; Im et al., 2000; Ye et al., 2002; Noguchi et al., 2003), although a novel intracellular mechanism through peroxi-

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**TABLE 1**

<table>
<thead>
<tr>
<th>Receptor types</th>
<th>IP Response</th>
<th>GTPγS Binding μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse LPA1</td>
<td>0.34</td>
<td>0.25</td>
</tr>
<tr>
<td>Human LPA1</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>Human LPA2</td>
<td>6.50</td>
<td>5.80</td>
</tr>
<tr>
<td>Human LPA3</td>
<td>0.93</td>
<td>0.36</td>
</tr>
</tbody>
</table>

The Ki values of Ki16425 were estimated from inositol phosphate response shown in Fig. 2 (left column) or from the GTPγS binding assay shown in Fig. 3 (right column), in a manner similar to that for the inositol phosphate response for LPA1, and based on the Schild regressions for other receptor subtypes. See more detail under Materials and Methods.

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**Fig. 1.** Chemical structure of Ki16425.
Membranes were prepared as described previously (Kon et al., 1999). The cells were maintained for about 6–10 h in fresh medium supplemented with 10% (v/v) FBS and cells were cultured for an additional 24 h. The medium was changed to fresh minimum essential medium supplemented with 0.1% (v/v) BSA (fraction V). Where indicated, PTX (100 ng/ml) or its solvent was added to the medium. S1P1-transfected CHO cells were similarly incubated with test agents. In this case, the incubation medium contained 10 μM forskolin and 0.5 mM 3-isobutyl-1-methylxanthine. After a 10-min incubation, the reaction was terminated by adding 100 μl 1 N HCl, and cyclic AMP radioactivity incorporated into the cellular inositol lipids, and the net [Ca^{2+}]_i change (peak value – basal value) at around 15 s was calculated.

### Inositol Phosphate Production

Assays were performed essentially as described previously (Murata et al., 2000). The cells were incubated for 1 min with or without test agents, and the inositol phosphates (sum of inositol bisphosphate and inositol triphosphate) were measured. The results were normalized to 10^6 dpm of the total radioactivity incorporated into the cellular inositol lipids, and the radioactivity of trichloroacetic acid (5%)-insoluble fraction was considered as the total radioactivity.

### cAMP Accumulation

The cells were washed once and preincubated for 10 min at 37°C in HEPES-buffered medium. FRTL-5 thyroid cells were incubated with LPA or other agonists in the presence or absence of Ki16425. Forskolin (10 μM), 100 μM Bo20-1724, and 0.5 units/ml adenosine deaminase were added to the medium. S1P1-transfected CHO cells were similarly incubated with test agents. In this case, the incubation medium contained 10 μM forskolin and 0.5 mM 3-isobutyl-1-methylxanthine. After a 10-min incubation, the reaction was terminated by adding 100 μl 1 N HCl, and cyclic AMP was measured as described previously (Sho et al., 1991).

### GTPγS Binding

Binding assay was performed by incubating the membranes with 0.1 nM GTPγS (2 × 10^6 dpm/ml) for 30 min at 30°C, as described previously (Im et al., 2000).

### [3H]LPA binding

Membrane protein (40 μg) was incubated in 0.4 ml of the binding buffer (50 mM HEPES, 100 mM NaCl, 5 mM MgCl2, pH 7.5) containing 25 μg/ml of saponin, 0.6 mM BSA, 0.5 mM Na3VO4, 10 μM [3H]LPA (48 Ci/mmol), and different concentrations of Ki16425 and LPA for 60 min at 4°C. The reaction was terminated and binding activity was measured as for GTPγS binding assay. Nonspecific binding was determined in the presence of 10 μM unlabeled LPA.

### RNA Extraction and Northern Blot Analysis

Total RNA was prepared from the cells using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Total RNA (20 μg) was electrophoresed in a 1% agarose gel containing 3.7% formaldehyde in 20 mM MOPS buffer, and blotted to a nylon membrane (Hybond-N) with 20× standard saline citrate. The probes (20 ng) were labeled with [α-32P]dATP by random oligonucleotide priming and added to the blots at a concentration of about 5 × 10^6 dpm in 5 ml of hybridization buffer. The hybridization was carried out at 60°C. After hybridization, the blots were washed at 60°C with 0.2× standard saline citrate and 0.1% SDS, as described previously (Sato et al., 1999).

### RT-PCR Analysis

Single-strand DNA was synthesized using the SUPERSCRIPT II kit (Invitrogen). To ensure that no genomic DNA contamination was present, the reaction was performed in parallel without reverse transcriptase. The product was then subjected to amplification by PCR. Degenerate DNA primers were based on the cDNA sequences of the human LPA1 (GenBank accession number Y09479), human LPA3 (GenBank accession number AF011466), and

### TABLE 2

<table>
<thead>
<tr>
<th>Cell Type and LPA Receptor Expression</th>
<th>Ligand</th>
<th>Ca^{2+} Response</th>
<th>Ca^{2+} Response to LIGAND + 10 μM Ki16425</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1</td>
<td>LPA(nM)</td>
<td>432 ± 4</td>
<td>24 ± 5*</td>
</tr>
<tr>
<td>LPA1</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPA3</td>
<td>300</td>
<td>231 ± 26</td>
<td>221 ± 27</td>
</tr>
<tr>
<td>PAF</td>
<td>10</td>
<td>317 ± 32</td>
<td>295 ± 3</td>
</tr>
<tr>
<td>SPC</td>
<td>30</td>
<td>74 ± 8</td>
<td>65 ± 3</td>
</tr>
<tr>
<td>3T3</td>
<td>LPA(nM)</td>
<td>906 ± 60</td>
<td>151 ± 20*</td>
</tr>
<tr>
<td>LPA1</td>
<td>10</td>
<td>419 ± 43</td>
<td>429 ± 7</td>
</tr>
<tr>
<td>LPA2</td>
<td>400</td>
<td>793 ± 50</td>
<td>776 ± 2</td>
</tr>
<tr>
<td>S1P</td>
<td>10</td>
<td>451 ± 76</td>
<td>397 ± 49</td>
</tr>
<tr>
<td>100</td>
<td>913 ± 80</td>
<td>820 ± 103</td>
<td></td>
</tr>
<tr>
<td>A431</td>
<td>LPA(nM)</td>
<td>10</td>
<td>711 ± 8</td>
</tr>
<tr>
<td>LPA1</td>
<td>10</td>
<td>39 ± 3</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>LPA2</td>
<td>10</td>
<td>41 ± 44</td>
<td>47 ± 8</td>
</tr>
<tr>
<td>LPC</td>
<td>10</td>
<td>116 ± 23</td>
<td>111 ± 22</td>
</tr>
<tr>
<td>SPC</td>
<td>30</td>
<td>305 ± 8</td>
<td>298 ± 51</td>
</tr>
<tr>
<td>LPC</td>
<td>3</td>
<td>34 ± 8</td>
<td>34 ± 10</td>
</tr>
<tr>
<td>30</td>
<td>171 ± 33</td>
<td>157 ± 29</td>
<td></td>
</tr>
<tr>
<td>Pef</td>
<td>3</td>
<td>57 ± 5</td>
<td>63 ± 9</td>
</tr>
<tr>
<td>100</td>
<td>176 ± 17</td>
<td>163 ± 2</td>
<td></td>
</tr>
<tr>
<td>2AG</td>
<td>26</td>
<td>39 ± 8</td>
<td>31 ± 6</td>
</tr>
</tbody>
</table>

*The effect of Ki16425 is significant.

1 LPC, lysophosphatidylcholine; Pef, peychoine; 2AG, 2-arachidonoylglycerol.
human LPA_3 (GenBank accession number AF127138), according to the previous study (Pages et al., 2001) with slight modifications. The primers for LPA_2 were synthesized according to the cDNA sequence of the human GRP29 (GenBank accession number NM002596). The primers used were: LPA_2: forward, 5′-GAGCCTGGTCTCTCGGTAGC-3′; reverse, 5′-AGGCTGAGTGGCCTACCTCTCCATGTTCCA-3′; LPA_3: forward, 5′-AAGTGTCGACTATGACAGCCTAGGCC-3′; reverse, 5′-TGAGGTGTCAGAGGCACACATGCGAC-3′. The primer sequences in these regions were identical for the human and mouse, except for the reverse primer of mouse LPA_2, which was 5′-AGGCTGAGTGGCCTACCTCTCCATGTTCCA-3′. Primers for the human orthologs were effective for amplification of mouse (3T3 fibroblasts) and rat (PC-12 cells) LPA cDNA. Each PCR was carried out using 2.5 units of Taq DNA polymerase and 1 μl of reverse transcriptase reaction product in a 50-μl reaction mixture containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl_2, 200 μM dNTP, and 10 pmol of each primer. An extended denaturation at 94°C for 1 min was followed by cycles consisting of denaturation at 94°C for 45 s, annealing at 50°C (LPA_1), 50°C (LPA_3), 47°C (LPA_2), and 52°C (LPA_4) for 30 s, and elongation at 72°C for 1 min. After final extension at 72°C for 10 min, the PCR products were analyzed by agarose gel electrophoresis.

**DNA Synthesis.** Swiss 3T3 fibroblasts were pretreated for 10 min with Ki16425 in fresh Dulbecco’s modified Eagle’s medium containing 0.1% BSA and then cultured for 20 h with LPA, S1P, or PDGF, and with [3H]thymidine (0.5 μCi in 1 ml) for another 4 h. The radioactivity in trichloroacetic acid-insoluble fraction was measured as described previously (Kimura et al., 2000).

**Cell Migration.** The migration experiment was performed using a Boyden chamber apparatus (Neuro Probe Inc., Gaithersburg, MD) as described previously (Tamama et al., 2001). Cells remaining on the upper surface of the filters were removed, and the filters were then fixed in methanol and stained with Diff-Quik Solution II (International Reagents Corp., Kobe, Japan). The number of cells that had migrated to the lower surface was determined by counting the cells in four microscopic fields at 400× magnification.

**Calculation of K_i Value.** The K_i values for Ki16425 or DGPP 8:0 were estimated from inositol phosphate responses or Ca²⁺ responses, based on the following equation: $K_i = K_{D_{50}} \times B/(EC_{50i} – EC_{50})$, where B is the concentration of Ki16425 or DGPP 8:0, and EC_{50i} and EC_{50} are the half-maximal effective concentration of LPA in the absence and presence of the antagonist, respectively. The half-maximal effective concentration was estimated as a value that graphically gives a 50% maximal response, unless otherwise specified. With regard to the Schild regressions, the half-maximal effective concentration was estimated from the Scatchard plots. The $K_i$ value was determined by plotting the log of Dose Ratio-1 at each concentration of Ki16425 against the log concentration of Ki16425. The x-intercept of the linear transformation equals the inverse log of the $K_i$.

**Statistical Analysis.** All experiments were performed in duplicate or triplicate, and the results of multiple observations were presented as means ± S.E. of at least three independent experiments, unless otherwise stated. Statistical significance was assessed by Student’s t test and values were considered significant at p < 0.05.

**Results**

**Pharmacological Characterization of Ki16425.** From 150,000 low-molecular weight compounds screened for selective antagonist action on LPA receptors, Ki16425 was identified as the best candidate (Fig. 1). When we started this study, EDG-family LPA receptors, but not LPA_2/GPR23, had been identified. To study pharmacological characteristics of the compound, EDG-family LPA receptor subtypes were transfected into RH7777 cells, which have previously been shown to express none of these LPA receptors in a detectable level (Im et al., 2000). No significant increase in [Ca²⁺]_i, increase in inositol phosphate production, or inhibition of cAMP accumulation in response to LPA could be found in wild-type or vector-transfected RH7777 cells (data not shown). LPA induced inositol phosphate production in a dose-dependent manner with different efficacy and potency in cells transfected with the different receptor subtypes. In the case of LPA_1 and LPA_3, 1 μM Ki16425 treatment slightly but significantly suppressed the LPA action, and the inhibitory effect became pronounced at 10 μM. On the other hand, the inhibitory effect of 10 μM Ki16425 in LPA_2-expressing cells was marginal, although significant, in the −0.1 to 1 μM concentration range of LPA. In all cases, Ki16425 showed a
competitive inhibition pattern, and $K_i$ values were estimated to be 0.34 $\mu M$ for LPA$_1$, 6.5 $\mu M$ for LPA$_2$, and 0.93 $\mu M$ for LPA$_3$, as shown in Table 1. A similar selectivity of Ki16425 was observed to the Ca$^{2+}$ responses in RH7777 cells expressing the different receptor subtypes (data not shown).

To further characterize the actions of Ki16425, we performed GTP$\gamma$S binding assays in membranes prepared from HEK293T cells transfected with cDNAs encoding the LPA receptors and G-protein subunits. In this experiment, we also compared the effects of Ki16425 on the mouse and human receptors and G-protein subunits. In this experiment, we also presented in Table 1. These values were similar to those estimated from the inositol phosphate response in intact RH7777 cells; the small difference probably reflects the differences in assay conditions, including temperature, reaction time, and cell type used. Thus, Ki16425 was more effective in the membranes that expressed LPA$_1$ orthologs in their sensitivity to Ki16425 (Fig. 3, A and B).

Next, we examined the effect of Ki16425 on LPA receptor subtype, and $K_i$ values were calculated from Schild regressions and presented in Table 1. These values were similar to those estimated from the inositol phosphate response in intact RH7777 cells; the small difference probably reflects the differences in assay conditions, including temperature, reaction time, and cell type used. Thus, Ki16425 was more effective in the membranes that expressed LPA$_1$ orthologs in their sensitivity to Ki16425 (Fig. 3, A and B).

The GTP$\gamma$S binding activity was increased to about 3,000 dpm ($\approx$LPA$_1$) by 0.1 $\mu M$ or absence (○) of Ki16425. Schild analysis was performed, as shown in the inset, and the estimated $K_i$ values are expressed as percentages of the response to 10 $\mu M$ LPA. Ki16425 treatment decreased the basal activity in the absence of LPA from approximately 2,000 dpm to 3,000 (LPA$_2$ and LPA$_3$) $\approx$4,000 dpm (any LPA$_1$). This activity was increased to about 3,000 (vector), 8,000 (LPA$_2$ and LPA$_3$), and 11,000 dpm (any LPA$_1$) by 10 $\mu M$ LPA. Ki16425 treatment decreased the basal activity in the absence of LPA to about 1,500 dpm in all cases regardless of the receptor transfection. Thus, Ki16425 seems to inhibit the basal activity without LPA receptor stimulation. LPA-induced activities in the absence or presence of indicated concentrations of Ki16425 are expressed as percentages of the response to 10 $\mu M$ LPA in the absence of Ki16425. Shild analysis was performed, as shown in the inset, and the estimated $K_i$ values are shown in Table 1.
this experiment, 3T3 fibroblasts were incubated with or without 10 μM Ki16425 for the last 1 or 10 min during labeling time with Fura2/AM, the cells were washed with fresh medium three times, and the Ca²⁺ response to LPA was mea-

Fig. 4. Effect of Ki16425 on LPA receptor binding. A, effect of receptor transfection on the LPA-specific binding. The same HEK293T cell membranes as those in Fig. 3 were used. The membranes from the cells that were transfected with mLPA₁ or hLPA₂ were incubated with 5 nM [³H]LPA. LPA-specific binding without (control) or with 10 μM Ki16425 was measured by subtracting the nonspecific binding activity (6,000 dpm); this value remained unchanged by receptor transfection. The specific binding was increased by the transfection of LPA₁ or LPA₂ (Fig. 4A) but not by the transfection of LPA₃, possibly because of the lower potency of LPA in LPA₃-expressing cell membranes. The results are shown as LPA specific binding ± S.E. B, the increase in the binding activity caused by receptor transfection was attributed to an LPA receptor-dependent binding activity, and the effect of Ki16425 is expressed as a percentage of this activity. From the LPA receptor binding data, the \( K_i \) value can be theoretically estimated from the following equation: 

\[
K_i = \frac{IC_{50} \times K}{K + A},
\]

where \( A \) is the concentration of [³H]LPA used for the binding experiment (5 nM), \( IC_{50} \) is the half-maximal inhibitory concentration of Ki16425 (0.72 μM for LPA₁ and 22 μM for LPA₂, estimated from Fig. 4B), and \( K \) is the dissociation constant of LPA for the receptor. However, an accurate \( K \) value could not be determined (see text). Instead, we used an \( EC_{50} \) value of LPA for GTPγS binding (72 nM for LPA₁ and 148 nM for LPA₂, estimated from Fig. 4B) as the \( K \) value and \( K_i \) values were estimated to be 0.67 and 21.3 μM for mouse LPA₁ and human LPA₂, respectively. * the effect of Ki16425 was significant in A and the activity was significant between LPA₁ and LPA₂ in B.

Fig. 5. Effect of Ki16425 on [Ca²⁺]i increase induced by LPA in THP-1 cells and 3T3 fibroblasts. Ca²⁺ response to indicated concentrations of LPA was measured in the presence (●) or absence (○) of Ki16425 (1 μM) in THP-1 cells in (A) and Swiss 3T3 fibroblasts (B). The results are expressed as percentages of the maximal [Ca²⁺]i increase (404 ± 27 nM in THP-1 cells and 1,256 ± 87 nM in Swiss 3T3 fibroblasts) by 10 μM LPA in the absence of Ki16425. Inset, Ca²⁺ response to 100 nM LPA was measured in the presence of indicated concentrations of Ki16425. The results are expressed as percentages of the [Ca²⁺]i increase (217 ± 36 nM in THP-1 cells and 759 ± 73 nM in Swiss 3T3 fibroblasts) by 100 nM LPA in the absence of Ki16425. C, the reversibility of the Ki16425 effect was examined in Swiss 3T3 fibroblasts. The cells were incubated for 1 or 10 min with or without 10 μM Ki16425 during labeling time with Fura 2/AM and washed three times with the HEPES-buffered medium. The [Ca²⁺]i increase by 100 nM LPA was then examined after the 1- or 10-min preincubation with or without 10 μM Ki16425.
sured in the presence or absence of Ki16425. There was no significant effect of the pretreatment with Ki16425 on the LPA action, indicating that the compound could be washed out without leaving residual inhibition.

In PC-12 cells, Ki16425 had only a small, although significant, inhibitory effect on the Ca\textsuperscript{2+} response to LPA. In HL-60 cells, a small but significant [Ca\textsuperscript{2+}], increase by LPA was insensitive to Ki16425 (Table 2). Thus, Ki16425 inhibited the LPA-induced Ca\textsuperscript{2+} response in THP-1 cells, 3T3 fibroblasts, and A431 cells, but had only a marginal effect in PC-12 cells and HL-60 cells. On the other hand, the Ca\textsuperscript{2+} responses to ATP, PAF, and SPC in THP-1 cells, PDGF and S1P in 3T3 fibroblasts, EGF and PAF in A431 cells, and SPC, lysophosphatidylcholine, psychosine, and 2-arachidonoylglycerol in HL-60 cells were not significantly inhibited by Ki16425 (Table 2).

Figure 6 shows the effect of Ki16425 on the inositol phosphate response. This LPA-induced response in 3T3 fibroblasts (Fig. 6A) and A431 cells (Fig. 6C) was inhibited by Ki16425, and the mode of Ki16425 inhibition was consistent with competitive inhibition in 3T3 fibroblasts (the \(K_i\) value was 0.68 \(\mu\)M). In contrast, the responses to S1P in 3T3 fibroblasts (Fig. 6A, right), S1P\textsubscript{3}-expressing CHO cells, and S1P\textsubscript{2}-expressing CHO cells (Fig. 6B) were insensitive to Ki16425. Similarly, the response to PAF and bradykinin was not significantly affected by the inhibitor (Fig. 6C).

Figure 7 shows the effects of Ki16425 on the inhibition of the forskolin-induced cAMP accumulation in response to LPA and phenylisopropyladenosine, an A\textsubscript{1} adenosine receptor agonist, and S1P. LPA inhibited cAMP accumulation in FRTL-5 thyroid cells in a PTX-sensitive manner (Fig. 7A), suggesting a G\textsubscript{i}-mediated inhibition of the LPA action. This inhibition by LPA was significantly suppressed by Ki16425 even at 0.1 \(\mu\)M and almost completely at 10 \(\mu\)M. However, the phenylisopropyladenosine-induced inhibition was insensitive to even 10 \(\mu\)M Ki16425, although the inhibition was PTX-sensitive in the same cells (Fig. 7B), thus ruling out the possibility that Ki16425 might act on G\textsubscript{i}-proteins. Likewise, the inhibition of cAMP accumulation by S1P in S1P\textsubscript{1}-expressing CHO cells was hardly affected by Ki16425 (Fig. 7C), and
this S1P action was also reversed by PTX treatment (data not shown).

In summary, Ki16425 was found to specifically inhibit LPA-induced response without any appreciable effect on responses to other receptor agonists. However, the extent of the LPA-induced inhibition seemed to depend on the cell types; the inhibition was marginal in PC-12 cells and undetectable in HL-60 cells.

**Differential Expression of LPA Receptor Subtypes May Explain the Susceptibility to Ki16425.** mRNA expression of LPA receptor subtypes in the cells used (Figs. 5–7) was examined by Northern blotting and RT-PCR methods. Some of the results are summarized in Table 2. As seen in Fig. 8, A431 cells express all subtypes of EDG-family LPA receptor mRNAs but not LPA4/GPR23 mRNA; 3T3 fibroblasts and THP-1 cells express LPA1, LPA2, and LPA4 mRNAs; HL-60 cells express LPA2 and LPA4 mRNAs; FRTL-5 cells express LPA1 mRNA as a major band; and PC-12 cells express LPA4 mRNA as a major band. Although the size of LPA2 mRNA differed among the cell types, as shown by Northern blotting (Fig. 8A), a single band with a similar size was amplified by RT-PCR (Fig. 8B). This suggests the heterogeneity of LPA2 mRNA, possibly because of alternative splicing (An et al., 1998a,b). In the case of LPA3, a trace band with almost the same size as the band in A431 cells was detected in HL-60 and THP-1 cells by Northern blotting (Fig. 8A); however, no specific band was amplified by RT-PCR. Thus, the expression of LPA3 mRNA in HL-60 and THP-1 cells could not be confirmed. LPA1 mRNA was detected in Ki16425-sensitive A431 cells, FRTL-5 cells, 3T3 fibroblasts, and THP-1 cells. On the other hand, LPA2 and/or LPA4 were detected in Ki16425-insensitive or less-sensitive HL-60 cells.
and PC-12 cells as major bands (Fig. 8 and Table 2). Thus, cell sensitivity to Ki16425 seems to reflect the differential expression of LPA receptor subtypes, especially LPA₁, and Ki16425 seems to be a useful tool for evaluating the involvement of specific LPA receptors in the short-term response to LPA.

**Ki16425 Inhibits Long-Term DNA Synthesis and Cell Migration As Induced by LPA.** We next examined whether Ki16425 was applicable for inhibiting long-term responses including DNA synthesis (24 h) and cell migration (4 h). As shown in Fig. 9A, the LPA-induced DNA synthesis in 3T3 fibroblasts was markedly inhibited by 1 μM Ki16425, but those induced by S1P and PDGF were not appreciably affected at this concentration of the compound. Significant inhibition of the LPA-induced action by Ki16425 was observed at ~0.01 to 0.1 μM, but DNA synthesis induced by S1P or PDGF was not affected even at 3 μM (Fig. 9B). LPA maximally stimulated the migration of 3T3 fibroblasts at 100 nM, and the activity declined as the LPA concentration increased. The bell-shaped migration response to LPA was also observed in glioma cells (Manning et al., 2000) and lymphocytes (Zheng et al., 2001), although the mechanism has not yet been well characterized. One possible mechanism might be desensitization caused by high agonist concentration. Ki16425 at ~0.1 to 1 μM significantly inhibited the peak migration activity (Fig. 9C), but PDGF-induced cell migration was not inhibited under these conditions (Fig. 9D), similar to the case of DNA synthesis (Fig. 9B).

**Evaluation of LPA Receptor Subtypes Involved in LPA-Induced Actions.** DGPP 8:0 has recently been re-

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**Fig. 10.** Subtype selectivity of DGPP 8:0 on LPA receptors. LPA₁ (A), LPA₂ (B), or LPA₃ (C)-RH7777 cells were incubated with the indicated concentrations of LPA in the presence (●) or absence (○) of 30 μM DGPP 8:0. The results are expressed as percentages of the value obtained at 10 μM LPA in the absence of Ki16425. The 100% values were 511 ± 4 nM for LPA₁-expressing cells, 688 ± 40 nM for LPA₂-expressing cells, and 908 ± 38 nM for LPA₃-expressing cells.

**Fig. 11.** Evaluation of the subtype of LPA receptors involved in inositol phosphate response in A431 cells. A, the effect of PTX on the LPA-induced action. Normal A431 cells not treated with PTX or A431 cells treated with the toxin were incubated with the indicated concentrations of LPA. The basal activity was 1,163 ± 53 dpm for control cells and 1,174 ± 58 dpm for PTX-treated cells. B, normal cells; C, PTX-treated cells. The LPA-induced activity was measured in the absence or presence of 1 μM Ki16425, 10 μM Ki16425, or 30 μM DGPP 8:0. The results are expressed as percentages of the response at 10 μM LPA in the normal or PTX-treated cells shown in A.
ported as a selective antagonist of LPA₁ and LPA₃ (Fischer et al., 2001). Therefore, we attempted to confirm the pharmacological specificity of DGPP 8:0 for LPA receptor subtypes. As shown in Fig. 10, this compound clearly inhibited the LPA₄-mediated Ca²⁺ response and slightly but significantly inhibited the LPA₁-mediated one. The competitive inhibition by DGPP 8:0 was also observed in the inositol phosphate response in LPA₂-expressing CHO cells (data not shown). However, as originally proposed (Fischer et al., 2001), this agent was ineffective for the LPA₂-mediated Ca²⁺ response (Fig. 10B) and the inositol phosphate response (data not shown). Based on the ability to inhibit Ca²⁺ response, the Kᵢ values of DGPP 8:0 were estimated to be 19.9 μM for LPA₁ and 3.33 μM for LPA₃. Thus, DGPP 8:0 was more effective for LPA₃ than LPA₁, whereas Ki16425 was slightly more effective for LPA₁ than LPA₃ (Table 1).

Finally, we explored the differences in the receptor selectivity of DGPP 8:0 and Ki16425 for the evaluation of the lipid receptor subtypes involved in the LPA response in A431 cells. A431 cells express three EDG-family LPA receptor subtypes but not LPA₄ (Fig. 8 and Table 2). As shown in Fig. 11A, LPA induced inositol phosphate production in a dose-dependent manner and was partially inhibited by PTX. When the effects of Ki16425 were compared in A431 cells (Fig. 11B) and PTX-treated cells (Fig. 11C), 1 μM Ki16425 was inhibitory at the lower concentrations (~10–100 nM) of LPA in normal cells; the Kᵢ value was 0.73 μM, which is comparable with that of the mean for LPA₁ or LPA₃ (Table 1). However, DGPP 8:0 was found to be ineffective (Fig. 11B, right). These results suggest that the LPA-induced inositol phosphate response is predominantly mediated by LPA₁ in normal A431 cells. On the other hand, in the PTX-treated A431 cells, Ki16425, even at 10 μM, hardly affected the LPA-induced action (Fig. 11C), suggesting that LPA₂, but neither LPA₁ nor LPA₃, might be a major receptor subtype in the PTX-treated A431 cells. Thus, in normal A431 cells, both LPA₁ as a major route and LPA₃ as a minor route may mediate the LPA-induced inositol phosphate response.

**Discussion**

In the present study, we showed that Ki16425 inhibited LPA-induced actions, including short-term (~10 min) (i.e., Ca²⁺, inositol phosphate response, and cAMP responses) and long-term responses (~4–24 h) (i.e., DNA synthesis and cell migration). Ki16425 did not exhibit any significant effects on the other receptor agonist-induced actions, including S1P, SPC, psychosine, 2-arachidonoylglycerol, PAF, lysophosphatidylcholine, ATP, A₁-adenosine receptor agonist, bradykinin, EGF, and PDGF. Among the EDG-family LPA receptor subtypes analyzed in the present study, Ki16425 preferentially inhibited LPA₁- and LPA₃-mediated actions but only weakly inhibited those mediated by LPA₂. Because GPR23 was reported as a fourth LPA receptor during the review process of this article, it remains to be elucidated whether Ki16425 affects LPA₂/GPR23. The inhibition of the LPA-induced actions by Ki16425 showed a competitive pattern; Ki16425 least inhibited LPA binding to LPA₂, but with a high potency, whereas to LPA₃, it was inhibitory with a low potency, suggesting a competitive antagonist for LPA receptors with a preference for LPA₁ and LPA₃ over LPA₂.

Recent studies suggest that ligands loosely classified as antagonists can be divided into at least two classes: neutral antagonists, which possess no intrinsic activity, and inverse agonists, which possess negative intrinsic activity (de Ligt et al., 2000). Thus, inverse agonists can inhibit the basal activity without agonist stimulation. Ki16425 inhibited the basal activity of GTPγS binding (Fig. 3), suggesting that Ki16425 might be an inverse agonist. The basal activity in the inositol phosphate assay, however, was not changed by Ki16425 (Fig. 2). It remains to be elucidated whether the discrepancy of the results is caused by the differences in the time employed in these assays (i.e., 30 min in the GTPγS binding assay and 1 min in the inositol phosphate assay). Increasing the incubation time of inositol phosphate assay and the effects of other LPA receptor antagonists (mentioned below) on the Ki16425-induced GTPγS binding may help to conclude that Ki16425 is a neutral antagonist or an inverse agonist.

Recently, several agents have been shown to inhibit LPA-induced actions, although their effects on LPA binding have not yet been reported. DGPP 8:0 has been shown to have a preferential competitive property for LPA₁ over LPA₃ and was ineffective on LPA₂ (Fischer et al., 2001). Consistent with this, we also observed in the present study that this agent competitively inhibited the LPA-induced Ca²⁺ response; the Kᵢ value was estimated to be 3.33 μM for LPA₁ and 19.9 μM for LPA₃, but no inhibitory effect was detected for LPA₂. Based on the GTPγS binding assay in HEK293 cells (Heise et al., 2001), VPC12249 has been shown to exert a selective and competitive inhibitory effect on LPA₁ (Kᵢ value of 0.14 μM) and LPA₃ (Kᵢ value of 0.43 μM), but not on LPA₂. Thus, DGPP 8:0 is selective for LPA₁, whereas VPC12249 is selective for LPA₁ and LPA₃. Ki16425 seems to have a pharmacological specificity similar to that of VPC12249, but the effect of the former on LPA₂ is not negligible. Thus, Ki16425 has a broad selectivity on LPA receptor subtypes compared with the previously described LPA antagonists, although its interaction on the recently identified LPA₄ remains uncharacterized. Such a broad selectivity might be beneficial to the evaluation of the participation of LPA in the biological responses compared with strictly subtype-selective LPA antagonists.

The differential subtype selectivity of Ki16425 and DGPP 8:0 was successfully applied for the evaluation of the LPA receptor subtypes involved in biological responses. In the present study, we analyzed in detail the inositol phosphate response to LPA in A431 cells, which seem to express all three EDG-family LPA receptor subtypes but not LPA₄ (Fig. 8). The LPA-induced action was sensitive to Ki16425 in normal cells (Fig. 11B) but insensitive to the antagonist in the PTX-treated cells (Fig. 11C). The change of susceptibility to Ki16425 may be explained as follows. In normal A431 cells, both LPA₁ and LPA₂ may couple to G₁ and Gₛ, respectively, thereby mediating the LPA-induced inositol phosphate response; however, the minor LPA₁/Gₛ pathway may be masked by the major LPA₂/Gₛ pathway. When Gₛ was inactivated by PTX, however, the LPA₂/Gₛ pathway was unmasked as a major route for the activation of phospholipase C. The Ca²⁺ and inositol phosphate responses to LPA in THP-1 cells and 3T3 fibroblasts seem to be mediated mainly by LPA₁, as evidenced by their susceptibility to Ki16425 (Fig. 5) but not to DGPP 8:0 (data not shown). Although the effect of Ki16425 on LPA₄ has not yet been examined, LPA₄ might be insensitive to Ki16425, because the Ca²⁺ response to LPA
in LPA₁-expressing HL-60 cells was barely affected by Ki16425 (Table 2). The slight difference in the Ki value between THP-1 cells (0.17 μM for Cu²⁺ response) and 3T3 cells (0.54 μM for the Ca²⁺ response and 0.68 μM for the inositol phosphate response) might reflect the difference in the contribution of LPA₂ or LPA₃ to these responses. Concerning the inhibition of the cAMP response in FRTL-5 cells, it is likely that LPA₁ is the major receptor subtype in this cell line.

First, LPA-induced inhibition of cAMP accumulation was sensitive to Ki16425 (Fig. 7A), but not to DGP (8:0) (data not shown). Second, only LPA₁ among the subtypes was effective for inhibiting cAMP accumulation when transfected (Im et al., 2000; Noguchi et al., 2003). Finally, only LPA₁ mRNA was detected in FRTL-5 cells (Fig. 8). Thus, these antagonists are potentially useful for discerning receptor subtypes involved in the given response, even when more than one LPA receptor subtype is expressed.

Ki16425 was also effective in the long-term responses, including DNA synthesis and cell migration in Swiss 3T3 fibroblasts (Fig. 9). Although LPA-induced DNA synthesis is one of the well known actions of the lipid mediator, its mechanism has not been fully characterized. In NIH 3T3 cells and one of the well known actions of the lipid mediator, its mechanism has not been fully characterized. In NIH 3T3 cells and one of the well known actions of the lipid mediator, its mechanism has not been fully characterized. In NIH 3T3 cells and

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