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 LPA/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 dioctyl glycerol pyrophosphate (DGPP 8:0), a recently identified antagonist for LPA receptors. Ki16425 inhibited the LPA-induced response in the decreasing order of LPA1 $\geq$ LPA3 $\Rightarrow$ 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.

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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, dioctyl 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; GTP\(\gamma\)S, guanosine 5\(^{-}\)-O-(3-thio)triphosphate; 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-0leyl-2-benzyl-4-oxobenzyl ethanolamidle phosphoric acid.
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 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

**Materials.** 1-Oleoyl-sn-glycero-3-phosphate (LPA), 1-α-lysophosphatidylcholine palmitoyl (C16:0), 1-α-lysophosphatidylcholine β-acetyl-γ-O-alkyl (PAF), and 1 β-α-galactosylsphingosine (psychosine) were purchased from Sigma-Aldrich (St. Louis, MO); sphingosine 1-phosphate (SIP) was from Cayman Chemical Co. (Ann Arbor, MI); sphingosyllphosphorylcholine (SPC) was from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA); 2-arachidonoylglycerol and fatty acid-free BSA (Fraction V) were from Calbiochem-Novabiochem Co. (San Diego, CA); dioctyl glycerol pyrophosphate (DPGPP 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-2H₂]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); and mouse orthologs of LPA₁ subcloned into pcDNA3 vector (Im et al., 2000), 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) (Oka-jima 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., 1987).

**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 subcloned into the EcoRI site of pEFneo 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 pEFneo empty vector alone or the pEFneo vector containing human LPA₁, human LPA₂, or human LPA₃ by electroporation, and the neonycin-resist-ant cells (G418 sulfate at 1 mg/ml for CHO cells and 0.3 mg/ml for RH7777 cells) were selected. CHO cells resistant to G-418 were used in bulk, whereas RH7777 cells expressing the different LPA receptor subtypes were cloned by limiting dilution. Subsequently, clonal RH7777 cells expressing human LPA₂ (C3) and human LPA₃ (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.

**TABLE 1**

<table>
<thead>
<tr>
<th>Kᵢ Values of Ki16425</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IP Response</strong></td>
</tr>
<tr>
<td><strong>GTPγS Binding</strong></td>
</tr>
<tr>
<td><strong>µM</strong></td>
</tr>
<tr>
<td>Receptor types</td>
</tr>
<tr>
<td>Mouse LPA₁</td>
</tr>
<tr>
<td>Human LPA₁</td>
</tr>
<tr>
<td>Human LPA₂</td>
</tr>
<tr>
<td>Human LPA₃</td>
</tr>
</tbody>
</table>
TABLE 2
Correlation of LPA receptor mRNA expression and Ki16425 inhibition of Ca²⁺ responses to LPA and other agonists in different cell types
Effects of Ki16425 on [Ca²⁺]i, increase induced by LPA and other receptor agonists in various cell types, including THP-1, 3T3 fibroblasts, A431 cells, PC-12 cells, and HL-60 cells. These cells were incubated in the presence or absence of Ki16425 (10 μM). LPA receptor expression was assessed from Northern blotting and PCR methods shown in Fig. 8.

<table>
<thead>
<tr>
<th>Cell Type and LPA Receptor Expression</th>
<th>Ligand</th>
<th>Ca²⁺ Response</th>
<th>Ca²⁺ Response to Ligation + 10 μM Ki16425</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1</td>
<td>LPA (nM)</td>
<td>10</td>
<td>432 ± 4</td>
</tr>
<tr>
<td></td>
<td>LPA₂</td>
<td>10</td>
<td>371 ± 32</td>
</tr>
<tr>
<td></td>
<td>ATP (μM)</td>
<td>10</td>
<td>74 ± 8</td>
</tr>
<tr>
<td></td>
<td>PAF (μM)</td>
<td>10</td>
<td>74 ± 8</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>906 ± 60</td>
<td>151 ± 20*</td>
</tr>
<tr>
<td>3T3</td>
<td>LPA (nM)</td>
<td>10</td>
<td>419 ± 43</td>
</tr>
<tr>
<td></td>
<td>PDGF (μM)</td>
<td>100</td>
<td>793 ± 50</td>
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<tr>
<td></td>
<td>EGF (nM)</td>
<td>100</td>
<td>913 ± 80</td>
</tr>
<tr>
<td></td>
<td>FGF (μM)</td>
<td>10</td>
<td>459 ± 32</td>
</tr>
<tr>
<td>PC-12</td>
<td>LPA (nM)</td>
<td>10</td>
<td>64 ± 6</td>
</tr>
<tr>
<td></td>
<td>LPA₂</td>
<td>100</td>
<td>362 ± 17</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>315 ± 35</td>
<td>333 ± 22</td>
</tr>
<tr>
<td>HL-60</td>
<td>LPA (μM)</td>
<td>10</td>
<td>39 ± 3</td>
</tr>
<tr>
<td></td>
<td>LPA₂</td>
<td>10</td>
<td>41 ± 4</td>
</tr>
<tr>
<td></td>
<td>ATP (μM)</td>
<td>100</td>
<td>116 ± 23</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>305 ± 8</td>
<td>298 ± 51</td>
</tr>
<tr>
<td></td>
<td>LPC (μM)</td>
<td>3</td>
<td>34 ± 8</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>171 ± 33</td>
<td>157 ± 29</td>
</tr>
<tr>
<td></td>
<td>PEF (μM)</td>
<td>3</td>
<td>57 ± 5</td>
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<tr>
<td></td>
<td>30</td>
<td>176 ± 17</td>
<td>163 ± 2</td>
</tr>
<tr>
<td></td>
<td>2AG (μM)</td>
<td>26</td>
<td>39 ± 8</td>
</tr>
</tbody>
</table>

* The effect of Ki16425 is significant.

1991; Sato et al., 1997). For cAMP assay, RH7777 cells were plated to dishes coated with rat tail collagen (400 μg/ml). Twenty-four hours before experiments, the medium was replaced with serum-free medium as specified containing 0.1% (w/v) BSA (fraction V). In the case of the inositol phosphate response, the medium was changed to TCM199 medium containing 20 μCi of [³²P]inositol (in 6 ml) and 0.1% (w/v) BSA (fraction V). Where indicated, PTX (100 ng/ml) or its agonists in the presence or absence of Ki16425. Forskolin (10 μM), 100 μM Ro20-1724, 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 of 1 N HCl, and cAMP accumulation was measured as described previously (Sho et al., 1991).

**GTPγ[S] Binding.** Binding assay was performed by incubating the membranes with 0.1 nM GTPγ[S] (~2 × 10⁶ dpm/ml) for 30 min at 30°C, as described previously (Im et al., 2000).

**[³²P]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, 0.5 μM BSA, 0.5 μM Na₂VO₃, and different concentrations of Ki16425 and LPA for 60 min at 4°C. The reaction was terminated and binding activity was measured as for GTPγ[S] binding assay. Nonspecific binding was determined in the presence of 10 μM unlabelled 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 20X 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 × 10⁶ 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 0.2X 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 LPA₂ (GenBank accession number AF011466) and
human LPA3 (GenBank accession number AF127138), according to the previous study (Pages et al., 2001) with slight modifications. The primers for LPA1 were synthesized according to the cDNA sequence of the human GRP23 (GenBank accession number NM005296). The primers used were: LPA1 forward, 5'-ATCCTTGGCTTAGTGCGCAAC272-3'; reverse, 5'-GGCTGGAAGCTCCACGGCA258-3'; LPA2 forward, 5'- TGGCTGGAAGCTCCACGGCA258-3'; reverse, 5'-GGCTGGAAGCTCCACGGCA258-3'; LPA3 forward, 5'-GAGCGTGGTCTCTCGGTAGC-3'; reverse, 5'-GGATGATGGTTCTCTCGGTAGC-3'; LPA4 forward, 5'-567TGAAGGCTTCTCCAAACGTGTCTG590-3; reverse, 5'-909GTTCAGAGTTGCAAGGCACAAGGT886-3. The primer sequences in these regions were identical for the human and mouse, except for the reverse primer of mouse LPA2, which was 5'-AGGCTGAGTGTTGCTCTCCGTTAGC-3'. Primers for the human orthologs were effective for amplification of mouse (3T3 fibroblasts) and rat (PC-12 cells) LPA1 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 MgCl2, 200 µM dNTP, and 10 µM of each primer. An extended denaturation at 94°C for 4 min was followed by 35 cycles, consisting of denaturation at 94°C for 45 s, annealing at 50°C (LPA1), 50°C (LPA2), 47°C (LPA3), and 52°C (LPA4) 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 Ki Values. The Ki values for Ki16425 or DGPP 8:0 were estimated from inositol phosphate responses or Ca²⁺ responses, based on the following equation: 

\[ \text{Ki} = \left( \frac{\text{EC}_{50} \times B}{\left(\text{EC}_{50} - \text{EC}_{i\theta}\right)} \right) \]

where \( B \) is the concentration of Ki16425 or DGPP 8:0, and \( \text{EC}_{50} \) and \( \text{EC}_{i\theta} \) 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 the 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 LPA4/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⁺], 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 LPA1 and LPA3, 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 LPA2-expressing cells was marginal, although significant, in the −0.1 to 1 µM concentration range of LPA. In all cases, Ki16425 showed a

![Fig. 2. LPA receptor subtype selectivity of Ki16425. The production of inositol phosphate (IP) by indicated concentrations of LPA was measured in the presence (A, 1 µM); B, 10 µM) or absence (C) of Ki16425 in RH7777 cells expressing LPA1 (A), LPA2 (B), or LPA3 (C). The results are expressed as percentages of the maximal response to 10 µM LPA in the absence of Ki16425. The basal activity and the activity at 10 µM LPA were 553 ± 47 and 1,547 ± 84 dpm for LPA1 cells, 527 ± 43 and 2,589 ± 39 dpm for LPA2 cells, and 743 ± 120 and 2,813 ± 130 dpm for LPA3 cells, respectively. Ki16425 did not significantly change the basal activity. * indicates the effect of Ki16425 was significant.](molpharm.aspetjournals.org/doi/fig/10.1124/mol.997)
orthologs of LPA1. As shown in Fig. 3, LPA receptor trans-
compared the effects of Ki16425 on the mouse and human
receptors and G-protein subunits. In this experiment, we also
HEK293T cells transfected with cDNAs encoding the LPA
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
orthologs and human orthologs of LPA1. As shown in Fig. 3, LPA receptor trans-
fection introduced an increase in GTP\(\gamma\)S binding activity in
response to LPA. Consistent with a previous study (Im et al.,
2000), the potency of LPA was slightly lower for LPA3 than
for LPA1 or LPA2. Ki16425 competitively inhibited the LPA-
induced action with different potencies, depending on the
LPA receptor subtype, and \(K_i\) values were calculated from the
Schild regressions and presented in Table 1. These values
were similar to those estimated from the inositol phosphate
responses in intact RH7777 cells; the small difference proba-
ably reflects the differences in assay conditions, including
assay conditions, including
assay conditions, including
assay conditions, including

Next, we examined the effect of Ki16425 on LPA receptor
ligand binding. Because of the high nonspecific binding to the
membranes and uncharacterized (specific) binding of LPA,
Ki16425 is a potent and reversible antagonist (Fig. 5C). In
the increment in specific binding resulting from the receptor
transfection to LPA receptor-dependent binding activity. The
dose-dependent effect of Ki16425 on LPA receptor-dependent
binding is shown in Fig. 4B. Ki16425 was 20 to 30 times more
potent for LPA1 than for LPA2, and the \(K_i\) values were
estimated to be 0.67 \(\mu\)M for LPA1 and 21.4 \(\mu\)M for LPA2,
which were close to those estimated from the inositol phos-
phate responses and GTP\(\gamma\)S binding (Table 1). In summary,
Ki16425 preferentially inhibited LPA1- and LPA2-mediated
responses but had only a moderate effect on LPA3.

**Ki16425 Inhibition Is Selective for LPA Receptors.** To
investigate the compound’s specific and usefulness as an
antagonist of LPA receptor, we applied Ki16425 to a variety
of cell types and measured several cellular responses (Table
2 and Figs. 5–7). The dose-dependent \([Ca^{2+}]\), increase eli-
ited by LPA in THP-1 cells (Fig. 5A) and in 3T3 fibroblasts
(Fig. 5B) was inhibited by Ki16425 in a competitive manner:
The \(K_i\) value was estimated to be 0.17 \(\mu\)M in THP-1 and 0.54
\(\mu\)M in 3T3 fibroblasts. Likewise, in A431 cells, the LPA
response was markedly inhibited by Ki16425 (Table 2). Thus,
Ki16425 is a potent and reversible antagonist (Fig. 5C).

![Fig. 3. LPA-induced GTP\(\gamma\)S binding to cell membranes expressing different LPA receptors.](image-url)
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 Ca\(^{2+}\) 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 mLPA\(_1\) or hLPA\(_2\) were incubated with 5 nM \(^{[3}\)H\]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 LPA\(_1\) or LPA\(_2\) (Fig. 4A) but not by the transfection of LPA\(_3\), possibly because of the lower potency of LPA in LPA\(_3\)-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: 

\[
K_i = \left(\frac{IC_{50} \times K_A}{K + A}\right)
\]

where \(A\) is the concentration of \(^{[3}\)H\]LPA used for the binding experiment (5 nM), \(IC_{50}\) is the half-maximal inhibitory concentration of Ki16425 (0.72 μM for LPA\(_1\) and 22 μM for LPA\(_2\), 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 for GTP\(\gamma\)S binding (72 nM for LPA\(_1\) and 148 nM for LPA\(_2\), estimated from Fig. 3) as the \(K\) value and \(K_i\) values were estimated to be 0.67 and 21.3 μM for mouse LPA\(_1\) and human LPA\(_2\), respectively. * the effect of Ki16425 was significant in A and the activity was significant between LPA\(_1\) and LPA\(_2\) in B.

Fig. 5. Effect of Ki16425 on [Ca\(^{2+}\)]_i increase induced by LPA in THP-1 cells and 3T3 fibroblasts. Ca\(^{2+}\) 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 [Ca\(^{2+}\)]_i increase (404 ± 27 nM in THP-1 cells and 1,256 ± 87 nM in Swiss 3T3 fibroblasts) by 100 nM LPA in the absence of Ki16425. Inset, Ca\(^{2+}\) response to 100 nM LPA was measured in the presence of indicated concentrations of Ki16425. The results are expressed as percentages of the [Ca\(^{2+}\)]_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 [Ca\(^{2+}\)]_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\(^{2+}\) response to LPA. In HL-60 cells, a small but significant \([Ca^{2+}]\) increase by LPA was insensitive to Ki16425 (Table 2). Thus, Ki16425 inhibited the LPA-induced Ca\(^{2+}\) 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\(^{2+}\) responses to ATP, PAF, and SPC in THP-1 cells, PDGF and S1P in 3T3 fibroblasts, EGF and PAF in A431 cells, and SPC, lysocephatidylcholine, 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\(_{3}\)-expressing CHO cells, and S1P\(_{2}\)-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\(_1\) adenosine receptor agonist, and S1P. LPA inhibited cAMP accumulation in FRTL-5 thyroid cells in a PTX-sensitive manner (Fig. 7A), suggesting a G\(_i\)-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\(_i\)-proteins. Likewise, the inhibition of cAMP accumulation by S1P in S1P1-expressing CHO cells was hardly affected by Ki16425 (Fig. 7C), and...
K16425 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 LPA4 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.

**Fig. 8.** Northern blot (A) and RT-PCR (B) analyses of LPA receptor mRNAs in various cell types. As a positive control for LPA4/GPR23, rat ovary was used. It should be noted that even though the size of LPA2 mRNA determined by Northern blotting differed among cell types, a single band with a similar size was amplified by RT-PCR analysis for LPA2.

**Fig. 9.** Effects of Ki16425 on DNA synthesis and migration of Swiss 3T3 fibroblasts. A, the cells were incubated with indicated concentrations of agonists in the presence or absence of Ki16425 (1 μM). The net increase in thymidine incorporation by the respective agonist is shown. The basal activity without agonists was 89,000 ± 1,500 dpm in the absence of Ki16425 and 60,000 ± 8,000 dpm in the presence of 1 μM Ki16425. B, the cells were incubated with 10 μM LPA, 10 μM S1P, or 400 pM PDGF in the presence of the indicated concentration of Ki16425. The results are expressed as percentages of the net activity induced by the respective agonist as shown in A. C and D, the cells were treated with the indicated concentrations of Ki16425 and loaded into the upper wells of the Boyden chamber, and then cell migration for 4 h was measured. The lower wells were filled with the indicated concentrations of LPA or PDGF. The number of cells that migrated into the lower surface of the membrane filter was counted.
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 LPA\(_1\), 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 \(\mu\)M 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 \(0.1\) \(\mu\)M, but DNA synthesis induced by S1P or PDGF was not affected even at 3 \(\mu\)M (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 \(1\) \(\mu\)M 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-
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 LPA₁-mediated Ca²⁺ response and slightly but significantly inhibited the LPA₃-mediated one. The competitive inhibition by DGPP 8:0 was also observed for the inositol phosphate response in LPA₁-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 Kᵵ 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 LPA₂ 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 Kᵵ 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 LPA₃ over LPA₁ 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 Kᵵ 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 (Kᵵ value of 0.14 µM) and LPA₃ (Kᵵ value of 0.43 µM), but not on LPA₂. Thus, DGPP 8:0 is selective for LPA₃ 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 LPA₃ may couple to G₁ and G₉, respectively, thereby mediating the LPA-induced inositol phosphate response; however, the minor LPA₂/G₉ pathway may be masked by the major LPA₁/G₁ pathway. When G₁ was inactivated by PTX, however, the LPA₂/G₉ 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 LPA-expressing HL-60 cells was barely affected by Ki16425 (Table 2). The slight difference in the K_i value between THP-1 cells (0.17 μM for Cu^2+ response) and 3T3 cells (0.54 μM for the Ca^2+ response and 0.68 μM for the inositol phosphate response) might reflect the difference in the contribution of LPA_2 or LPA_3 to these responses. Concerning the inhibition of the cAMP response in FRTL-5 cells, it is likely that LPA_1 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 DGPp 8:0 (data not shown). Second, only LPA_1 among the subtypes was effective for inhibiting cAMP accumulation when transfected (Im et al., 2000; Noguchi et al., 2003). Finally, only LPA_1 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 other cell types, LPA-induced DNA synthesis seems to be mediated through a tyrosine phosphorylation of EGF receptors (Daub et al., 1997; Cunnick et al., 1998). Thus, it is possible that Ki16425 inhibited the LPA-induced action by interacting with the EGF receptor. This mechanism is unlikely, however, because EGF was not a significant stimulator of LPA-induced EGF receptor-mediated responses, as shown in FRTL-5 cells (0.17 μM for Ca^2+ response and 0.68 μM for the inositol phosphate response) might reflect the difference in the contribution of LPA_2 or LPA_3 to these responses. Concerning the inhibition of the cAMP response in FRTL-5 cells, it is likely that LPA_1 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 DGPp 8:0 (data not shown). Second, only LPA_1 among the subtypes was effective for inhibiting cAMP accumulation when transfected (Im et al., 2000; Noguchi et al., 2003). Finally, only LPA_1 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.

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In conclusion, Ki16425 selectively inhibited the EDG-family LPA receptor-mediated actions with preference for LPA_1 and LPA_2 in vitro. This compound might also potentially serve as a therapeutic drug for diseases that involve LPA and its receptors in their initiation or progression (e.g., atherosclerosis and cancer).

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References

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acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. J Biol Chem 277:39436–39442.

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