Lysophosphatidic acid (LPA) receptors of the EDG family are differentially activated by LPA species

Structure–activity relationship of cloned LPA receptors

Koji Bandoh[a,b], Junken Aoki[a,*], Akitsu Taira[a], Masafumi Tsujimoto[b], Hiroyuki Arai[a], Keizo Inoue[a]

[a] *Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[b] *Laboratory of Cellular Biochemistry, The Institute of Physical and Chemical Research (RIKEN), 2-1, Hirosawa, Wako-shi, Saitama 351-0198, Japan*

Received 6 June 2000; accepted 19 June 2000

Edited by Marco Baggiolini

Abstract We examined the structure–activity relationship of cloned lysophosphatidic acid (LPA) receptors (endothelial cell differentiation gene (EDG) 2, EDG4, and EDG7) by measuring $[Ca^{2+}]_i$ in Sf9 insect cells expressing each receptor using LPA with various acyl chains bound at either the *sn*-1 or the *sn*-2 position of the glycerol backbone. For EDG7 the highest reactivity was observed with LPA with Δ9-unsaturated fatty acid (oleic (18:1), linoleic (18:2), and linolenic (18:3)) at *sn*-2 followed by 2-palmitoleoyl (16:1) and 2-arachidonoyl (20:4) LPA. In contrast, EDG2 and EDG4 showed broad ligand specificities, although EDG2 and EDG4 discriminated between 14:0 (myristoyl) and 16:0 (palmitoyl), and 12:0 (lauroyl) and 14:0 LPAs, respectively. EDG7 recognizes the *cis* double bond at the Δ9 position of octadecanoyl residues, since 2-elaidoyl (18:1, *trans*) and 2-petroselinoyl (18:1, *cis*-Δ12) LPA were poor ligands for EDG7. In conclusion, the present study demonstrates that each LPA receptor can be activated differentially by the LPA species.

Key words: Lysophosphatidic acid; Endothelial cell differentiation gene family; EDG2; EDG4; EDG7

1. Introduction

Lysophosphatidic acid (LPA, 1- or 2-acyl-*sn*-glycero-3-phosphate) is a lipid mediator with diverse biological properties [1,2]. It activates platelet aggregation, induces contraction of smooth muscle cells and retraction of neurites, and stimulates cell chemotaxis. It also has a mitogenic effect on the cell cycle. LPA also elicited various cellular responses including stimulation of phospholipases, mobilization of intracellular Ca^{2+}, stimulation or inhibition of adenylate cyclase, activation of the mitogen-activated protein (MAP) kinase cascade, and cytoskeleton rearrangement by activation of small GTPase Rho [1,2].

It has been demonstrated that specific G protein-coupled receptors (GPCRs) present in many types of cells mediate the cellular effects of LPA. So far, at least three GPCRs belonging to the EDG (endothelial cell differentiation gene) family, EDG2/LPA1/*vzg-1* [3], EDG4/LPA2 [4], and EDG7/LPA3 [5], have been identified as cellular receptors for LPA. These GPCRs share 50–54% identical amino acids, and may couple with different G proteins. EDG2 is most associated with activation of the G_i pathway that leads to inhibition of adenylate cyclase [3], while EDG4 and EDG7 are associated most prominently with the $G_{q/11}$ pathways mediating Ca^{2+} signaling (EDG4 and EDG7) [4–6], and activation of a serum response element reporter gene and MAP kinase (EDG4) [4,5].

LPA occurs naturally as an albumin-bound serum factor generated during blood clotting from activated platelets [7]. It also accumulates in various biological fluids including serum, aged plasma, and ascites induced by ovary tumors [8]. In activated platelets, several molecular species of LPA with saturated (stearoyl (18:0), palmitoyl (16:0)) or unsaturated (oleoyl (18:1), linoleoyl (18:2), arachidonoyl (20:4)) acyl chains have been detected [9,10]. These diverse fatty acid moieties may be linked to either the *sn*-1 or the *sn*-2 position of the glycerol backbone. In addition, a small portion of natural glycerophospholipids contain an ether linkage at the *sn*-1 position rather than an ester bond (1-alkyl-2-lyso-*sn*-glycero-3-phosphate and 1-alkenyl-2-lyso-*sn*-glycero-3-phosphate (alkyl- and alkenyl-GP)) [11,12]. Thus, various molecular species of LPA or LPA-like lipids (LPA analogs) are present in vivo. It appears that the structure–activity relationship of these LPA and LPA analogs depends on cells and tissues [13–19]. In these studies, the length, degree of unsaturation, and linkage to the glycerol backbone of fatty acyl chains modulate the activity of LPA in a different way when comparing various biological responses including platelet aggregation [13,14], calcium mobilization in A431 cells [15], proliferation of rat-1 fibroblasts [16] and cultured vascular smooth muscle cells [17], activation of serum response element-regulated genes [18], and vasoactive effects of LPA [19]. These differential responses can be explained by the differential expression of each LPA receptor, since our previous study clearly indicated that EDG4 and EDG7 had quite distinct ligand specificities [5]. In this study, as a part of our continuing studies to characterize each LPA receptor, we investigated the structure–ac-

*Corresponding author. Fax: (81)-3-3818 3173.
E-mail: jaoki@mol.f.u-tokyo.ac.jp

Abbreviations: EDG, endothelial cell differentiation gene; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; alkyl-GP, 1-alkyl-2-lyso-*sn*-glycero-3-phosphate; alkenyl-GP, 1-alkenyl-2-lyso-*sn*-glycero-3-phosphate; GPCR, G protein-coupled receptor; $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+} ion; 1-acyl-LPA, 1-acyl-2-lysophosphatidic acid; 2-acyl-LPA, 2-acyl-1-lysophosphatidic acid

0014-5793/00/$20.00
PII: S0014-5793(00)01827-5

tivity relationship of various LPA and LPA analogs using LPA non-responding Sf9 insect cells expressing each cloned LPA receptor by measuring intracellular Ca^{2+} mobilization.

2. Materials and methods

2.1. Lipids

L-α-Dioleoyl-phosphatidylcholine (PC) (18:1, *cis*-Δ^9-octadecenoic acid), L-α-dilinoleoyl-PC (18:2, *cis,cis*-Δ^9,Δ^{12}-octadecadienoic acid), L-α-dilinolenoyl-PC (18:3, all *cis*-Δ^9,Δ^{12},Δ^{15}-octadecatrienoic acid), L-α-diarachidonoyl-PC (20:4, all *cis*-Δ^5,Δ^8,Δ^{11},Δ^{14}-eicosatetraenoic acid), L-α-dimyristoyl-PC (14:0), L-α-dipalmitoyl-PC (16:0), L-α-distearoyl-PC (18:0), L-α-palmitoleoyl-PC (16:1, *cis*-Δ^9-hexadecenoic acid), L-α-dipetroselinoyl-PC (18:1, *cis*-Δ^{12}-octadecenoic acid), L-α-dielaidoyl-PC (18:1, *trans*-Δ^9-octadecenoic acid), and egg yolk phosphatidic acid (PA) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Platelet-activating factor (PAF, C18:1 *cis*) was purchased from Biomol Research Laboratories, Inc. Bovine brain phosphatidylethanolamine (PE) plasmalogen was purchased from Doosan Serdary Research Laboratories (Toronto, Ont., Canada). PA was prepared from PC according to the method described by Satouchi et al. [20] using phospholipase D from *Actinomadura* (kindly donated by Meito Sangyo, Tokyo, Japan). LPA with various acyl chains was prepared enzymatically from the corresponding PC by reacting the lipids sequentially with phospholipase D and phospholipase A1 or A2 as previously described [5]. Alkyl-GP was prepared from PAF (C18:1 *cis*) by reacting the lipid sequentially with phospholipase D and a recombinant brain PAF acetylhydrolase (α2 subunit) [21]. Alkenyl-GP was prepared from bovine brain PE plasmalogen by using phospholipase D and phospholipase A2. All lipids were dissolved in chloroform:methanol (2:1, v:v) and dissolved in MBS (see below) containing 0.1% fatty acid-free bovine serum albumin (Sigma) after the organic solvent had been removed by evaporation.

2.2. Construction of cDNA for EDG2-EDG4 chimeric receptor

The carboxy-terminal intracellular domain of EDG2 (amino acids 315–355) was replaced with that of EDG4 (amino acids 298–351). The chimeric cDNA was constructed as follows: a cDNA fragment encoding the N-terminus, seven transmembrane and intracellular domains

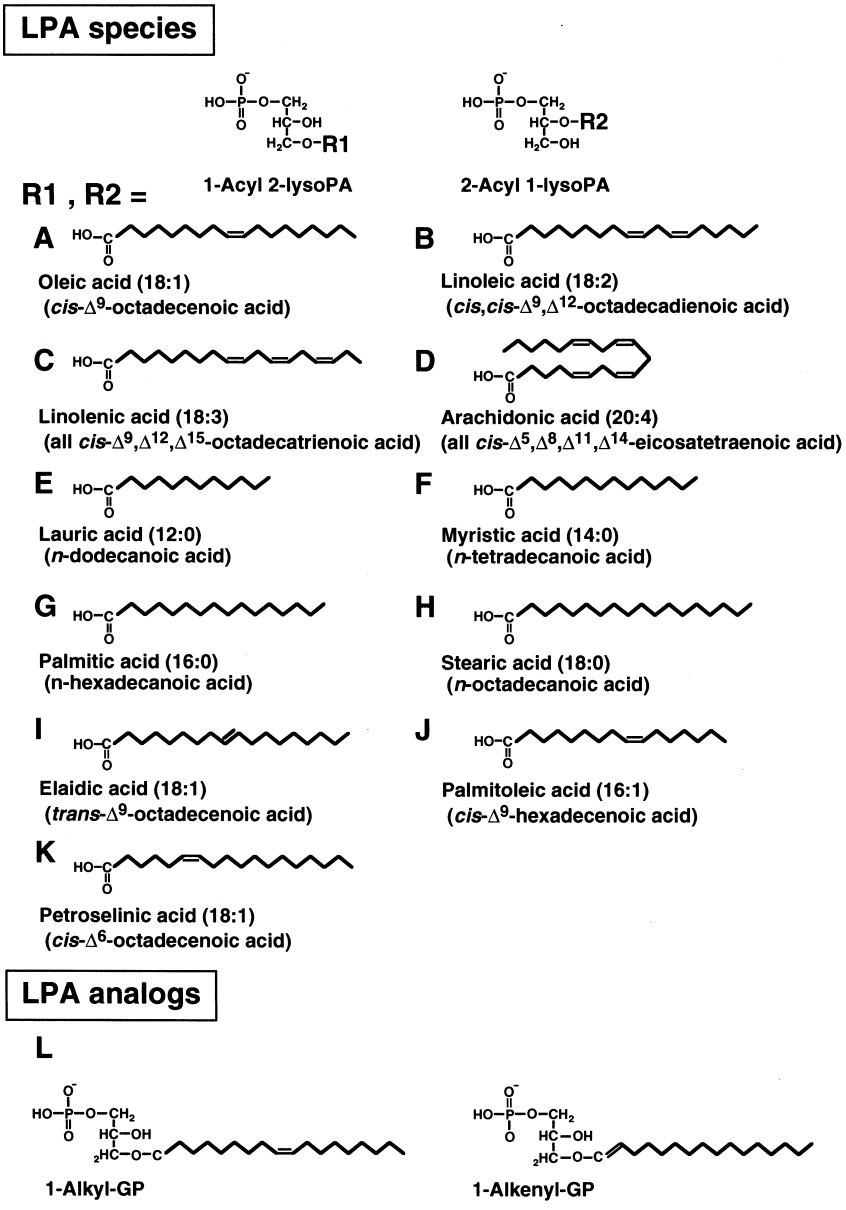

Fig. 1. Chemical structure of LPA and its structural analogs. A–K: Chemical structure of fatty acids used in this study, which are linked to either the *sn*-1 or *sn*-2 position of LPA. The chemical structure of the LPA analogs alkyl-GP and alkenyl-GP is shown in L.

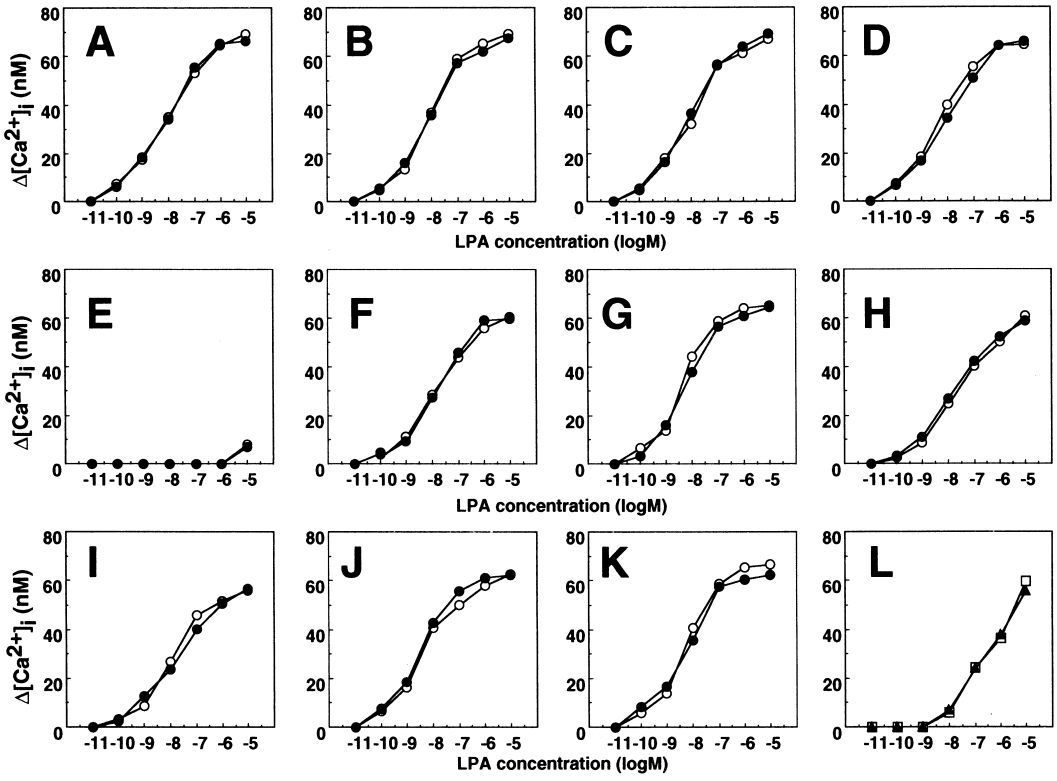

Fig. 2. The activities of LPA and its structural analogs to induce increases in $[Ca^{2+}]_i$ in EDG4-expressing Sf9 cells. EDG4-expressing Sf9 cells, loaded with Fura-2 AM, were stimulated with 22 compounds of LPA and its analogs, and changes in $[Ca^{2+}]_i$ were analyzed in CAF-110. The open and closed circles in A–K represent 1-acyl- and 2-acyl-LPA, respectively. A, Oleoyl (18:1, *cis*-Δ^9-octadecenoic acid); B, linoleoyl (18:2, *cis,cis*-Δ^9,Δ^{12}-octadecadienoic acid); C, linolenoyl (18:3, all *cis*-Δ^9,Δ^{12},Δ^{15}-octadecatrienoic acid); D, arachidonoyl (20:4, all *cis*-Δ^5,Δ^8,Δ^{11},Δ^{14}-eicosatetraenoic acid); E, lauroyl (12:0); F, myristoyl (14:0); G, palmitoyl (16:0); H, stearoyl (18:0); I, elaidoyl (18:1, *trans*-Δ^9-octadecenoic acid); J, palmitoleoyl (16:1, *cis*-Δ^9-hexadecenoic acid); K, petroselinoyl (18:1, *cis*-Δ^{12}-octadecenoic acid) LPA. L, 1-*O*-alkyl (18:1, *cis*-Δ^9-octadecenoic acid)-2-lyso-*sn*-glycero-3-phosphate (closed triangle) and 1-alkenyl-2-lyso-*sn*-glycero-3-phosphate (alkenyl-GP) from bovine brain (open square).

of EDG2 (amino acids 1–314) was generated by PCR using FLAG-EDG2 pFASTBAC as template DNA and the following two oligonucleotides: ATGCGTCGACATGGACTACAAAGACGATGACGATAAAGCTGCCATCTCTACTTCCATCCCT, and GGTGCGGCGCATCTCAGCATCTCGGTAGGAGTAAATGATGGGGTTCAT. The cDNA encoding the C-terminal intracellular domain of EDG4 was generated by PCR using FLAG-EDG4 pFASTBAC1 as template DNA and the following two oligonucleotides: ATGAACCCCATCATTTACTCCTACCGAGATGCTGAGATGCGCCGCACC, and ATGCGCGGCCGCTCAGTCCTGTTGGTTGGGTTGAGC. The resulting 1000-bp (encoding amino acids 1–314 of EDG2) and 300-bp (encoding the C-terminal domain of EDG4) PCR fragments were purified by agarose gel electrophoresis. The two DNA fragments were mixed, denatured at 94°C, and annealed on ice. Finally, using this annealed DNA as template DNA, an EDG2-EDG4 chimera receptor cDNA was generated by PCR using the following oligonucleotides: ATGCGTCGACATGGACTACAAAGACGATGACGATAAAGCTGCCATCTCTACTTCCATCCCT, and ATGCGCGGCCGCTCAGTCCTGTTGGTTGGGTTGAGC. The resulting DNA fragment (1.3 kb) was digested by *Sal*I/*Not*I and ligated into pFASTBAC1, and baculoviruses were prepared as described [5]. DNA sequences of cDNAs prepared by PCR were confirmed by DNA sequencing. Cloning and characterization of human EDG4 cDNA was first reported by An et al. [4]. We also cloned a cDNA for human EDG4 by the RT-PCR method [5]. We recently found that our cDNA has a one-base insertion in the 3′ region encoding the C-terminal domain, which results in a different amino acid sequence in the EDG4 C-terminal region. We also found that the nucleotide sequence of human EDG4 deposited in the EST database (GenBank accession number W60555) matched our sequence. The amino acid sequence of the EDG4 C-terminal region used in the chimeric receptor, in which the different amino acid from original human EDG4 [4] is shown in italics, is as follows: RDAEMRRTFRRLLCCACLRQSTRESVHYTSSAQGGASTRIMLPENGHPLM*DSTL* (amino acid positions 298–351). The nucleotide sequence data of our EDG4 have been submitted to the GenBank database under the accession number AF233092.

2.3. Baculovirus system

Sf9 insect cells were grown in serum-free EX-CELL420 insect cell medium (Nichirei, Tokyo, Japan) at 27°C. For infection, 6×10^5 cells/ml were mixed with recombinant or wild-type *Autographa californica* nuclear polyhedrosis virus to produce a multiplicity of infection of 10 and incubated for 48 h at 27°C. Expression of the FLAG-tagged protein was confirmed by immunofluorescence using an anti-FLAG monoclonal antibody (M5; Eastman Kodak Company, New Haven, CT, USA).

2.4. Ca^{2+} measurements

Sf9 cells were harvested 48 h after baculovirus infection, washed gently with morpholinoethanesulfonic acid-buffered saline (MBS) containing 10 mM NaCl, 60 mM KCl, 17 mM $MgCl_2$, 10 mM $CaCl_2$, 110 mM sucrose, 4 mM glucose, 0.1% fatty acid-free bovine serum albumin (Sigma), and 10 mM morpholinoethanesulfonic acid, pH-adjusted to 6.2 at room temperature with Tris, and loaded with 2 mM Fura-2 acetoxymethyl ester (Fura-2 AM; Molecular Probes Inc., Eugene, OR, USA) for 45 min. Free Fura-2 AM was washed out, and the cells were resuspended in the HBS buffer to produce a concentration of 10^6 cells/ml. Agonist-induced Fura-2 AM fluorescence of samples in quartz cuvettes kept at 27°C was monitored at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm using a CAF-110 spectrofluorimeter (Japan Spectroscopy, Inc., Tokyo, Japan). Fluorescence was recorded before and after addition of LPAs.

3. Results

We chose the insect Sf9 cells and expressed each LPA receptor in the cells since they did not show any Ca^{2+} responses to LPA, although in almost all types of mammalian cells tested, LPA induces a rapid increase in the $[Ca^{2+}]_i$. The EDG4 and EDG7 receptors expressed in the Sf9 cells transduced the Ca^{2+} response by LPA, whereas EDG2 did not up to 10 μM of 1-oleoyl LPA [5]. Fig. 1 gives the structure of the various LPAs and analogs used in the present study. To examine how the nature, length, linkage, and position of the acyl chain affect the potency of LPA in each cloned LPA receptor, we tested LPA species with different acyl chain lengths, varying degrees of unsaturation at either the *sn*-1 or the *sn*-2 position, and LPA analogs with different linkage of the side chain (ester or ether bonds).

3.1. Structure–activity relationship of EDG4

We first examined the ligand specificity of EDG4. From the dose–response curves in Fig. 2 and the EC_{50} values in Table 1, it is evident that all acyl-LPAs tested with saturated or unsaturated fatty acids at either the *sn*-1 or the *sn*-2 position, except for lauroyl (12:0) LPA, showed almost equal potency to EDG4. The Ca^{2+} response was detectable at as low as 0.1 nM and augmented with increased concentration of ligands. The EC_{50} values were about 10 nM. As shown in Fig. 4, alkyl-

Table 1
EC_{50} values of LPA species and its structural analogs

Compound	EDG2	EDG4	EDG7
LPA			
1-Oleoyl	~200 nM	~10 nM	~75 nM
2-Oleoyl	~200 nM	~10 nM	~10 nM
1-Linoleoyl	~200 nM	~10 nM	~80 nM
2-Linoleoyl	~200 nM	~10 nM	~10 nM
1-Linolenoyl	~200 nM	~10 nM	~80 nM
2-Linolenoyl	~200 nM	~10 nM	~10 nM
1-Arachidonoyl	~200 nM	~10 nM	~1.0 μM
2-Arachidonoyl	~200 nM	~10 nM	~100 nM
1-Lauroyl	>50 μM	>50 μM	>50 μM
2-Lauroyl	>50 μM	>50 μM	>50 μM
1-Myristoyl	>50 μM	~50 nM	>50 μM
2-Myristoyl	>50 μM	~50 nM	>25 μM
1-Palmitoyl	~400 nM	~10 nM	>50 μM
2-Palmitoyl	~400 nM	~10 nM	>25 μM
1-Stearoyl	~800 nM	~100 nM	>50 μM
2-Stearoyl	~800 nM	~100 nM	>25 μM
1-Elaidoyl	~800 nM	~50 nM	>50 μM
2-Elaidoyl	~800 nM	~50 nM	>25 μM
1-Palmitoleoyl	~200 nM	~10 nM	>25 μM
2-Palmitoleoyl	~200 nM	~10 nM	~100 nM
1-Petroselinoyl	~200 nM	~10 nM	>50 μM
2-Petroselinoyl	~200 nM	~10 nM	>25 μM
LPA analogs			
Alkyl-GP	~2 μM	~1.0 μM	~6.0 μM
Alkenyl-GP	~2 μM	~1.0 μM	>25 μM

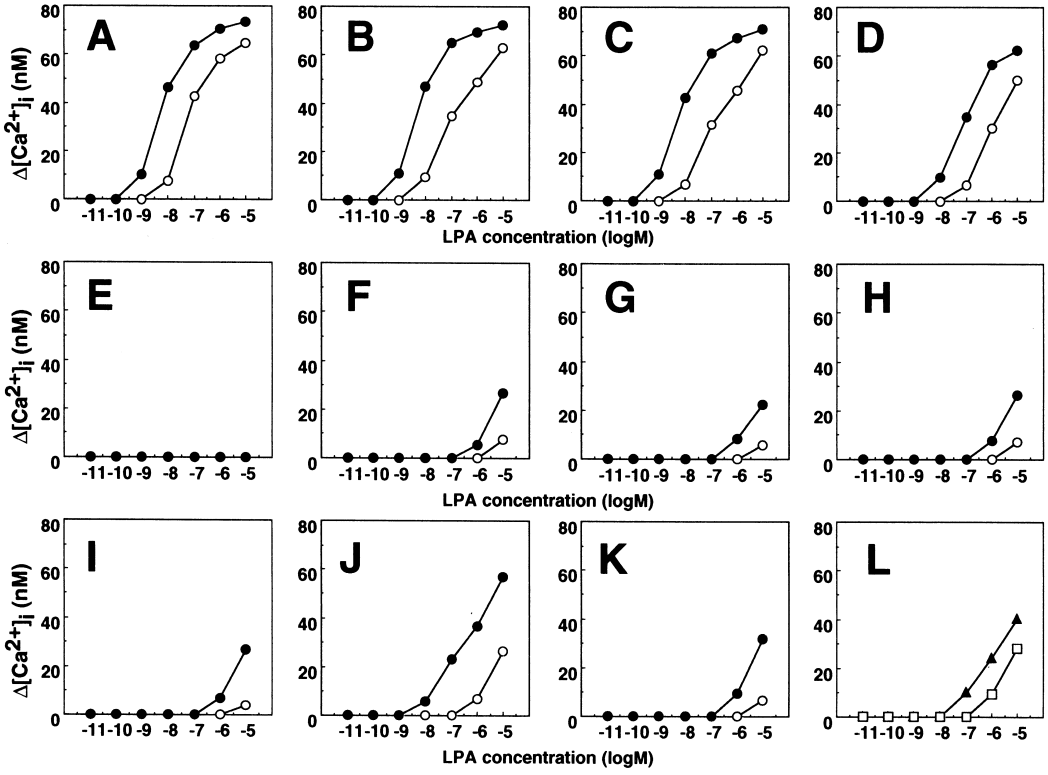

Fig. 3. The activities of LPA and its structural analogs to induce increases in $[Ca^{2+}]_i$ in EDG7-expressing Sf9 cells. EDG7-expressing Sf9 cells, loaded with Fura-2 AM, were stimulated with 22 compounds of LPA and its analogs, and changes in $[Ca^{2+}]_i$ were analyzed in CAF-110. The open and closed circles in A–K represent 1-acyl- and 2-acyl-LPA, respectively. A, Oleoyl (18:1, *cis*-Δ^9-octadecenoic acid); B, linoleoyl (18:2, *cis,cis*-Δ^9,Δ^{12}-octadecadienoic acid); C, linolenoyl (18:3, all *cis*-Δ^9,Δ^{12},Δ^{15}-octadecatrienoic acid); D, arachidonoyl (20:4, all *cis*-Δ^5,Δ^8,Δ^{11},Δ^{14}-eicosatetraenoic acid); E, lauroyl (12:0); F, myristoyl (14:0); G, palmitoyl (16:0); H, stearoyl (18:0); I, elaidoyl (18:1, *trans*-Δ^9-octadecenoic acid); J, palmitoleoyl (16:1, *cis*-Δ^9-hexadecenoic acid); K, petroselinoyl (18:1, *cis*-Δ^{12}-octadecenoic acid) LPA. L, 1-*O*-alkyl (18:1, *cis*-Δ^9-octadecenoic acid)-2-lyso-*sn*-glycero-3-phosphate (closed triangle) and 1-alkenyl-2-lyso-*sn*-glycero-3-phosphate (alkenyl-GP) from bovine brain (open square).

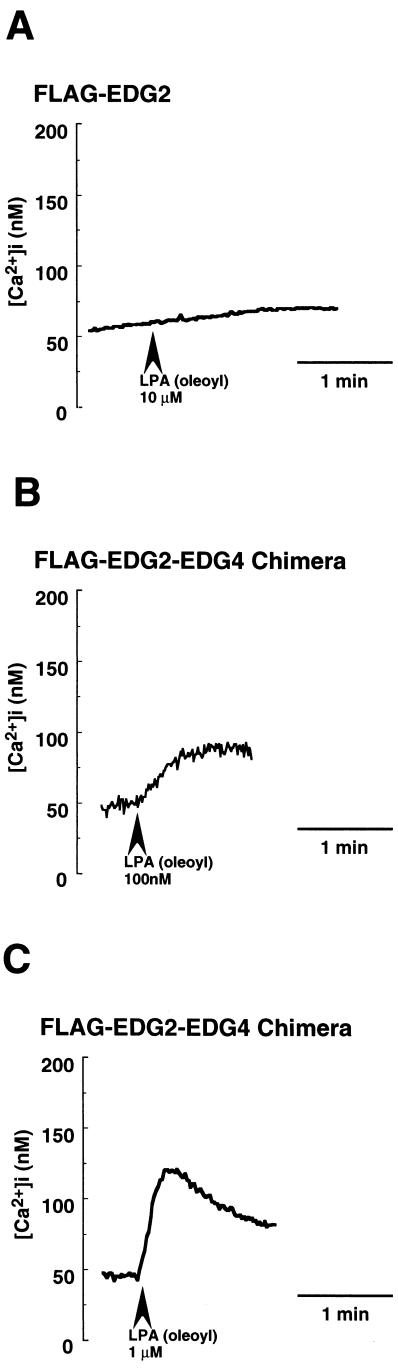

Fig. 4. EDG2-EDG4 chimeric receptor transduces the Ca^{2+} response by LPA in Sf9 cells. Ca^{2+} response of Sf9 cells infected with EDG2 (A) and EDG2-EDG4 chimeric receptor (B and C) baculoviruses by LPA. Sf9 cells were infected with each baculovirus, loaded with the fluorescent Ca^{2+} indicator Fura-2 AM, and stimulated with 1 (B) or 10 μM 1-oleoyl LPA (A and C).

and alkenyl-GP also activated EDG4. However, both of them were weaker ligands than acyl-LPAs by several tens of times.

3.2. Structure–activity relationship of EDG7

In marked contrast to EDG4, EDG7 showed quite different ligand specificity (Fig. 3, Table 1). 1- and 2-lauroyl (12:0) LPA did not induce any detectable Ca^{2+} response at concentrations up to 10 μM in Sf9 cells expressing EDG7 (Fig. 3E). In addition 1-myristoyl (14:0), 1-palmitoyl (16:0), and 1-stearoyl (18:0) LPA were also very poor agonists against EDG7. By contrast, 2-myristoyl, 2-palmitoyl, and 2-stearoyl LPA were capable of inducing a Ca^{2+} response in the cells, although a relatively higher concentration (1–10 μM) of ligands was required (Fig. 3F–H). Among 1-acyl- and 2-acyl-LPA containing unsaturated fatty acids, 2-oleoyl (18:1, *cis*-Δ^9-octadecenoic acid), 2-linoleoyl (18:2, *cis,cis*-Δ^9,Δ^{12}-octadecadienoic acid), and 2-linolenoyl (18:3, all *cis*-Δ^9,Δ^{12},Δ^{15}-octadecatrienoic acid) were the most potent ligands for EDG7 (Fig. 3A–C). The EC_{50} values of the three acyl-LPA were about 5 nM. 2-Arachidonoyl (20:4, all *cis*-Δ^5,Δ^8,Δ^{11},Δ^{14}-eicosatetraenoic acid, Fig. 3D) and 2-palmitoleoyl (16:1, *cis*-Δ^9-hexadecenoic acid, Fig. 3J) LPA followed next (EC_{50} values about 80 nM). When the activities of 1-acyl- and 2-acyl-LPA with the same acyl chain were compared, 2-acyl-LPAs always showed approximately 10 times higher activity. We next investigated which structural features of LPA are important for EDG7. As shown in Fig. 3, elaidoyl LPA (18:1, *trans*-Δ^9-octadecenoic acid, Fig. 3I) and petroselinoyl LPA (18:1, *cis*-Δ^{12}-octadecenoic acid, Fig. 3K) were very weak agonist for EDG7, although 2-acyl isomers showed higher activity than 1-acyl isomers (Fig. 3I,K). Both alkyl-GP and alkenyl-GP were again not good ligands for EDG7 (Fig. 3L).

3.3. EDG2-EDG4 chimeric receptor transduces Ca^{2+} response in Sf9 cells

We have not been able to test the ligand specificity of EDG2 using the baculovirus system, since EDG2 expressed in Sf9 cells did not mediate Ca^{2+} response (Fig. 4A), possibly due to a lack of G protein coupled to EDG2 in the insect cells [5]. Generally, the intracellular C-terminal domains of GPCR are responsible for coupling to G proteins. Accordingly, we constructed EDG2-EDG4 chimeric receptors, in which the intracellular C-terminal amino acids of EDG2 were replaced with those of EDG4, and examined the Ca^{2+} response in Sf9 cells. As shown in Fig. 4, the chimeric receptors transduced the Ca^{2+} response by LPA in Sf9 cells. The chimeric receptor may retain the ligand specificity of EDG2, because it is generally accepted that the ligand recognition domain of GPCR resides in the seventh transmembrane domain. We therefore used EDG2-EDG4 chimeric receptor to examine the structure–activity relationship of LPA for EDG2.

3.4. Structure–activity relationship of EDG2-EDG4 chimeric receptor

Apparently, the ligand specificity of the EDG2-EDG4 chimeric receptor is similar to that of EDG4, although myristoyl (14:0) LPA induced a very little response (Fig. 5F, Table 1). Lauroyl (12:0) LPA failed to activate EDG2 (Fig. 5E). Like EDG4, the EDG2-EDG4 chimeric receptor did not distinguish 1- and 2-acyl isomers. It is also evident that the affinity of the chimeric receptor to the ligand was considerably lower than the other LPA receptors (Fig. 5, Table 1). EC_{50} values of acyl-LPAs for EDG2-EDG4 chimeric receptors were about 200–800 nM. Both alkyl- and alkenyl-GP were weaker ligands toward the chimeric receptor as well (Fig. 5, Table 1).

4. Discussion

In the present study, we focused on the ligand specificity of EDG7 and further identified molecular features of LPA re-

quired for not only EDG7 but also EDG4. In addition we examined the ligand specificity of EDG2 using the EDG2-EDG4 chimeric receptor. The ligand specificity of EDG7 presented in this study strongly indicates that the endogenous ligand for EDG7 is 2-acyl LPA with unsaturated fatty acids such as oleic, linoleic, and linolenoic acids, all of which have a *cis*-double bond at the Δ^9 position (Fig. 3, Table 1). 2-Arachidonoyl (20:4, all *cis*-Δ^5,Δ^8,Δ^{11},Δ^{14}-eicosatetraenoic acid) LPA was a weaker ligand for EDG7. This is a reasonable result because the double bond at the Δ^9 position in a *cis* configuration is not present in arachidonic acid. 2-Palmitoleoyl (16:1, *cis*-Δ^9-hexadecenoic acid) is a weaker ligand than 2-oleoyl LPA. In addition 2-elaidoyl LPA was a poor agonist for EDG7. In conclusion, the double bond at the Δ^9 position in the *cis* configuration and the length of the carbon chain (C18) seem to be recognized by EDG7, and there is no strict correlation between the number of double bonds in the three octadecenoic acids (oleic (18:1), linoleic (18:2), and linolenic (18:3)) and the potency of LPA for EDG7. It should also be mentioned here that LPAs with polyunsaturated fatty acyl chains, especially 2-linoleoyl LPA, are detected in ascites from ovarian cancer patients and may account for the increasing ability to activate ovarian cancer cell lines [8]. EDG7 is expressed in human ovary [5] and to a high degree in many ovary cancer cell lines (Bandoh, Aoki, Arai and Inoue, unpublished result), indicating a possible role of EDG7 in the development of ovarian cancer. The literature indicates that among the various biological activities of LPA, contraction of smooth muscle cells [19], proliferation of cultured vascular smooth muscle cells [17], and intracellular Ca^{2+} mobilization in A431 cells [15] are dependent on the length and degree of unsaturation of fatty acids. These various responses can be explained by the differential expression of the three LPA receptors.

We showed in this study that EDG4 reacted with acyl-LPAs and LPA analogs tested although lauroyl (12:0) LPA was a very poor ligand for EDG4 (Fig. 2). Thus, it can be concluded that EDG4 discriminates between 12:0 (lauroyl) and 14:0 (myristoyl) LPAs. By contrast, the EDG2-EDG4 chimeric receptor seems to discriminate between 14:0 (myristoyl) and 16:0 (palmitoyl) LPAs (Fig. 5). Previously it has been reported that both 1-oleoyl and 1-stearoyl LPA induce EDG2-dependent cell rounding in EDG2-transfected neuronal cells [3]. In addition, the ligand specificity of EDG2 using the yeast pheromone response pathway was reported by Erickson et al. They showed that LPAs with oleic (18:1), stearic (18:0), or palmitic (16:0) acids, but not with capric (10:0) or caproic (6:0) acids, activate EDG2 [22]. These results are in good agreement with our results that LPA with both saturated and unsaturated fatty acids are active against the EDG2-EDG4 chimeric receptor and that it discriminates between 14:0 (myristoyl) and 16:0 (palmitoyl) LPAs.

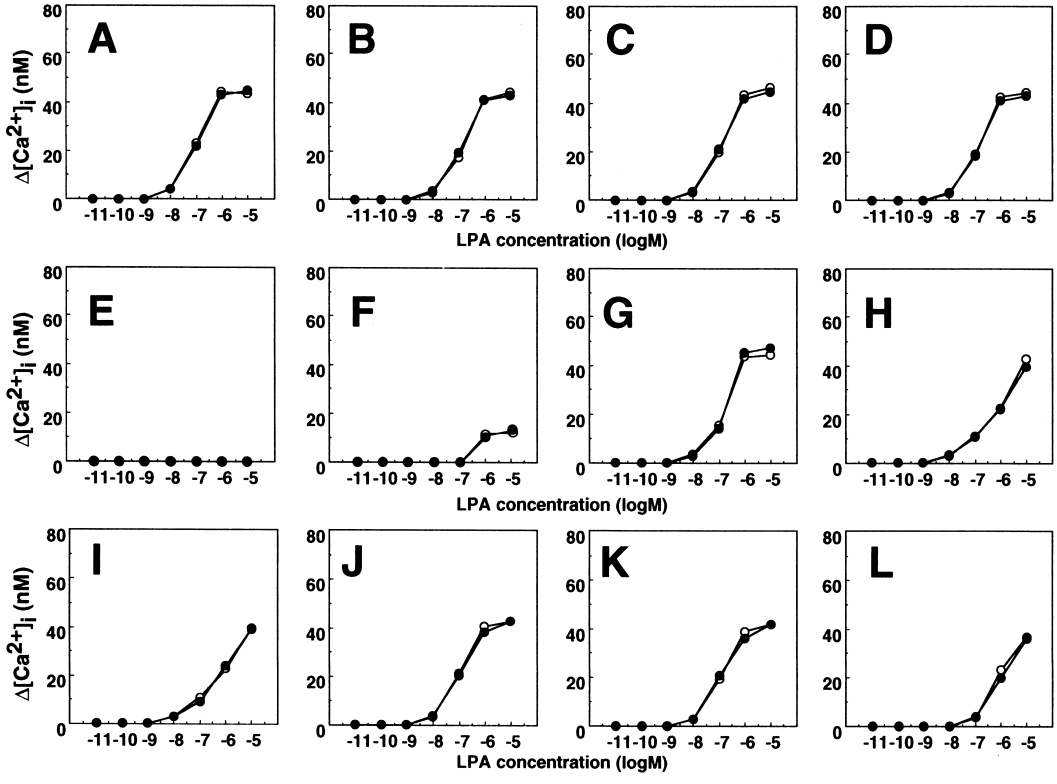

Fig. 5. The activities of LPA and its structural analogs to induce increases in $[Ca^{2+}]_i$ in EDG2-EDG4-expressing Sf9 cells. EDG2-EDG4 chimeric receptor-expressing Sf9 cells, loaded with Fura-2 AM, were stimulated with 22 compounds of LPA and its analogs, and changes in $[Ca^{2+}]_i$ were analyzed in CAF-110. The open and closed circles in A–K represent 1-acyl- and 2-acyl-LPA, respectively. A, Oleoyl (18:1, *cis*-Δ^9-octadecenoic acid); B, linoleoyl (18:2, *cis,cis*-Δ^9,Δ^{12}-octadecadienoic acid); C, linolenoyl (18:3, all *cis*-Δ^9,Δ^{12},Δ^{15}-octadecatrienoic acid); D, arachidonoyl (20:4, all *cis*-Δ^5,Δ^8,Δ^{11},Δ^{14}-eicosatetraenoic acid); E, lauroyl (12:0); F, myristoyl (14:0); G, palmitoyl (16:0); H, stearoyl (18:0); I, elaidoyl (18:1, *trans*-Δ^9-octadecenoic acid); J, palmitoleoyl (16:1, *cis*-Δ^9-hexadecenoic acid); K, petroselinoyl (18:1, *cis*-Δ^{12}-octadecenoic acid) LPA. L, 1-*O*-alkyl (18:1, *cis*-Δ^9-octadecenoic acid)-2-lyso-*sn*-glycero-3-phosphate (closed triangle) and 1-alkenyl-2-lyso-*sn*-glycero-3-phosphate (alkenyl-GP) from bovine brain (open square).

It has been reported that alkenyl-GP activates both EDG2 [22] and EDG4 [6] and that alkyl-GP activates EDG2 [22]. The present study confirmed that all LPA receptors belonging to the EDG family can be activated by both alkyl- and alkenyl-GP. This ligand specificity matches the type-3 LPA receptor which Tigyi et al. classified according to their pharmacological study [11,23,24]. In spite of this observation, we speculate that the natural ligands for EDG2, EDG4, and EDG7 are acyl-LPA rather than alkyl- or alkenyl-GP, because they were always shown to be weaker ligands than acyl-LPA for the three LPA receptors. Interestingly, alkyl-GP was found to be considerably more potent (about 30-fold) than the 1-acyl LPAs in stimulating platelet aggregation [14] and producing cardiovascular effects in cats [25]. These results, together with our present study, indicate that human platelets and carcinoma cells express different LPA receptor(s) for platelet activation, as suggested by Gueguen et al. [14].

The biological pathway for LPA formation remains to be solved, although several studies indicate that the degradation of phosphatidic acid by a phospholipase A, especially some isoforms of phospholipase A2, may be involved in part [8,26–28]. From the present study, it is easy to speculate that phospholipase A1 activity may participate in the production of 2-acyl LPA for EDG7 activation. So far, the description of phospholipase A1 in mammals is still limited [29,30], and further studies are necessary to elucidate the synthetic pathway for 2-acyl-LPA.

References

[1] Moolenaar, W.H., Kranenburg, O., Postma, F.R. and Zondag, G.C. (1997) Curr. Opin. Cell Biol. 9, 168–173.
[2] Moolenaar, W.H. (1995) J. Biol. Chem. 270, 12949–12952.
[3] Hecht, J.H., Weiner, J.A., Post, S.R. and Chun, J. (1996) J. Cell Biol. 135, 1071–1083.
[4] An, S., Bleu, T., Hallmark, O.G. and Goetzl, E.J. (1998) J. Biol. Chem. 273, 7906–7910.
[5] Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami, M.K., Tsujimoto, M., Arai, H. and Inoue, K. (1999) J. Biol. Chem. 274, 27776–27785.
[6] An, S., Bleu, T., Zheng, Y. and Goetzl, E.J. (1998) Mol. Pharmacol. 54, 881–888.
[7] Tigyi, G. and Miledi, R. (1992) J. Biol. Chem. 267, 21360–21367.
[8] Xu, Y., Gaudette, D.C., Boynton, J.D., Frankel, A., Fang, X.J., Sharma, A., Hurteau, J., Casey, G., Goodbody, A. and Mellors, A. et al. (1995) Clin. Cancer Res. 1, 1223–1232.
[9] Gerrard, J.M. and Robinson, P. (1989) Biochim. Biophys. Acta 1001, 282–285.
[10] Tigyi, G. and Miledi, R. (1992) J. Biol. Chem. 267, 21360–21367.
[11] Fischer, D.J., Liliom, K., Guo, Z., Nusser, N., Virag, T., Murakami-Murofushi, K., Kobayashi, S., Erickson, J.R., Sun, G., Miller, D.D. and Tigyi, G. (1998) Mol. Pharmacol. 54, 979–988.
[12] Sugiura, T., Nakane, S., Kishimoto, S., Waku, K., Yoshioka, Y., Tokumura, A. and Hanahan, D.J. (1999) Biochim. Biophys. Acta 1440, 194–204.
[13] Sugiura, T., Tokumura, A., Gregory, L., Nouchi, T., Weintraub, S.T. and Hanahan, D.J. (1994) Arch. Biochem. Biophys. 311, 358–368.
[14] Gueguen, G., Gaige, B., Grevy, J.M., Rogalle, P., Bellan, J., Wilson, M., Klaebe, A., Pont, F., Simon, M.F. and Chap, H. (1999) Biochemistry 38, 8440–8450.
[15] Jalink, K., Hengeveld, T., Mulder, S., Postma, F.R., Simon, M.F., Chap, H., van der Marel van Ga, B.J., van Blitterswijk, W. and Moolenaar, W.H. (1995) Biochem. J. 307, 609–616.
[16] van Corven, E.J., van Rijswijk, A., Jalink, K., van der Bend, R.L., van Blitterswijk, W.J. and Moolenaar, W.H. (1992) Biochem. J. 281, 163–169.
[17] Tokumura, A., Iimori, M., Nishioka, Y., Kitahara, M., Sakashita, M. and Tanaka, S. (1994) Am. J. Physiol. C204–210.
[18] Perkins, L.M., Ramirez, F.E., Kumar, C.C., Thomson, F.J. and Clark, M.A. (1994) Nucleic Acids Res. 22, 450–452.
[19] Tokumura, A., Fukuzawa, K. and Tsukatani, H. (1978) Lipids 13, 572–574.
[20] Satouchi, K., Pinckard, R.N., McManus, L.M. and Hanahan, D.J. (1981) J. Biol. Chem. 256, 4425–4432.
[21] Hattori, M., Adachi, H., Aoki, J., Tsujimoto, M., Arai, H. and Inoue, K. (1995) J. Biol. Chem. 270, 31345–31352.
[22] Erickson, J.R., Wu, J.J., Goddard, J.G., Tigyi, G., Kawanishi, K., Tomei, L.D. and Kiefer, M.C. (1998) J. Biol. Chem. 273, 1506–1510.
[23] Tigyi, G., Dyer, D.L. and Miledi, R. (1994) Proc. Natl. Acad. Sci. USA 91, 1908–1912.
[24] Liliom, K., Murakami-Murofushi, K., Kobayashi, S., Murofushi, H. and Tigyi, G. (1996) Am. J. Physiol. 270, C772–C777.
[25] Tokumura, A., Yoshida, J., Maruyama, T., Fukuzawa, K. and Tsukatani, H. (1987) Thromb. Res. 46, 51–63.
[26] le Balle, F., Simon, M.F., Meijer, S., Fourcade, O. and Chap, H. (1999) Adv. Enzyme Regul. 39, 275–284.
[27] Gaits, F., Fourcade, O., Le, B.F., Gueguen, G., Gaige, B., Gassama, D.A., Fauvel, J., Salles, J.P., Mauco, G., Simon, M.F. and Chap, H. (1997) FEBS Lett. 410, 54–58.
[28] Fourcade, O., Simon, M.F., Viode, C., Rugani, N., Leballe, F., Ragab, A., Fournie, B., Sarda, L. and Chap, H. (1995) Cell 80, 919–927.
[29] Sato, T., Aoki, J., Nagai, Y., Dohmae, N., Takio, K., Doi, T., Arai, H. and Inoue, K. (1997) J. Biol. Chem. 272, 2192–2198.
[30] Higgs, H.N., Han, M.H., Johnson, G.E. and Glomset, J.A. (1998) J. Biol. Chem. 273, 5468–5477.