Molecular Cloning and Characterization of a Novel Human G-protein-coupled Receptor, EDG7, for Lysophosphatidic Acid*

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Koji Bandoh‡§, Junken Aoki‡§, Hiroyuki Hosono‡, Susumu Kobayashi‡, Tetsuyuki Kobayashi**, Kimiko Murakami-Murofushi**, Masafumi Tsujimoto‡, Hiroyuki Arai‡, and Keizo Inoue‡

From the ‡Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyku, Tokyo 113-0037, Japan, the §Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12 Ichigaya-Funagawara-machi, Shinjuku-ku, Tokyo 162-8626, Japan, the **Department of Biology, Faculty of Science, Ochanomizu University, 2-1-1, Otsuka, Bunkyku, Tokyo 112-8610, Japan, and the †Laboratory of Cellular Biochemistry, The Institute of Physical and Chemical Research (RIKEN), 2-1, Hirosawa, Wako-shi, Saitama 351-0198, Japan

Lyosphosphatidic acid (LPA), together with sphingosine 1-phosphate, is a bioactive lipid mediator that acts on G-protein-coupled receptors to evoke multiple cellular responses, including Ca\(^{2+}\) mobilization, modulation of adenylyl cyclase, and mitogen-activated protein (MAP) kinase activation. In this study, we isolated a human cDNA encoding a novel G-protein-coupled receptor, designated EDG7, and characterized it as a cellular receptor for LPA. The amino acid sequence of the EDG7 protein is 53.7 and 48.8% identical to those of the human receptor for LPA. The amino acid sequence of the EDG7 protein is 53.7 and 48.8% identical to those of the human receptor for LPA. The EDG (endothelial cell differentiation gene) family of orphan receptors comprises EDG1 (8, 9), EDG3 (10), EDG4 (11), AGR16/H218 (12, 13), and EDG6 (14), and their amino acid sequences show 36–58% homology with one another. Hecht et al. (9) first reported that EDG2/Recl.3/Vzg-1 increased responsiveness to LPA in cell rounding and adenylyl-cyclase inhibition assays when overexpressed in cerebral cortical cell lines, showing that EDG2/Recl.3/Vzg-1 is a receptor for LPA. Because LPA and SIP are structurally related, this finding has enabled scientists to examine whether members of EDG family function as receptors for LPA and SIP. At present, according to their amino acid sequence homologies, ligand specificities, and genomic structures (15), these GPCRs of the EDG family fall into two major groups that interact either with LPA (EDG2 (9) and EDG4 (11)) or with SIP (EDG1 (16–18), EDG3 (19), and AGR16/H218 (20)). The ligand of EDG6, which is expressed predominantly in lymphoid tissue (14), has not been elucidated yet. A novel GPCR, named PSP24, which does not show significant sequence similarity with any member of the EDG family, has also been isolated from Xenopus oocytes as a functional receptor for LPA (21). Because some LPA-responsive cells do not express known LPA receptors (EDG2, EDG4, and PSP24), it was expected that unidentified subtypes of LPA receptors were suggested to be present in mammals (11, 22). To understand the biological functions of LPA fully, we attempted to identify novel subtypes of LPA

Lyosphosphatidic acid (LPA) and sphingosine 1-phosphate (SIP) are lipid mediators with diverse biological properties (1–3). The cellular responses elicited by LPA vary widely. The effects of LPA on the cell cycle are either mitogenic or anti-mitotic. LPA stimulates phospholipase C (PLC) activation and consequent Ca\(^{2+}\) mobilization, inhibits adenylyl cyclase, activates mitogen-activated protein (MAP) kinase, and stimulates the transcription of serum response element transcriptional reporter genes, such as c-fos, in various types of cells. It also exerts an effect on the cytoskeleton that can lead to changes in cell shapes and motility, which include inducing stress fiber production and stimulating chemotaxis, cell migration, and tumor cell invasiveness. These actions of LPA are believed to be mediated by seven-transmembrane G-protein-coupled receptor(s) (GPCR) on the cell surfaces. Some functional studies have suggested that multiple subtypes of LPA receptors with distinct signaling properties mediate the diverse cellular effects of LPA (4–6). Indeed, several subtypes of LPA receptors, which are GPCRs, were identified recently.

The EDG (endothelial cell differentiation gene) family of orphan receptors comprises EDG1 (7), EDG2/Recl.3/Vzg-1 (8, 9), EDG3 (10), EDG4 (11), AGR16/H218 (12, 13), and EDG6 (14), and their amino acid sequences show 36–58% homology with one another. Hecht et al. (9) first reported that EDG2/Recl.3/Vzg-1 increased responsiveness to LPA in cell rounding and adenylyl-cyclase inhibition assays when overexpressed in cerebral cortical cell lines, showing that EDG2/Recl.3/Vzg-1 is a receptor for LPA. Because LPA and SIP are structurally related, this finding has enabled scientists to examine whether members of EDG family function as receptors for LPA and SIP. At present, according to their amino acid sequence homologies, ligand specificities, and genomic structures (15), these GPCRs of the EDG family fall into two major groups that interact either with LPA (EDG2 (9) and EDG4 (11)) or with SIP (EDG1 (16–18), EDG3 (19), and AGR16/H218 (20)). The ligand of EDG6, which is expressed predominantly in lymphoid tissue (14), has not been elucidated yet. A novel GPCR, named PSP24, which does not show significant sequence similarity with any member of the EDG family, has also been isolated from Xenopus oocytes as a functional receptor for LPA (21). Because some LPA-responsive cells do not express known LPA receptors (EDG2, EDG4, and PSP24), it was expected that unidentified subtypes of LPA receptors were suggested to be present in mammals (11, 22). To understand the biological functions of LPA fully, we attempted to identify novel subtypes of LPA

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† To whom correspondence should be addressed. Tel.: 81-3-5841-4722; Fax: 81-3-3818-3173; E-mail: jaokic@mol.f.u-tokyo.ac.jp.

‡ The abbreviations used are: LPA, lysophosphatidic acid; GPCR, G-protein-coupled receptor; PTX, pertussis toxin; RACE, rapid amplification of cDNA ends; 2-acyl-LPA, 2-acyl-1-lysophosphatidic acid; MAP, mitogen-activated protein; PLC, phospholipase C; LPC, 1-oleoyl-lyso phosphatidylethanolamine; LPE, 1-oleoyl-lysophosphatidylethanolamine; PAF, platelet-activating factor; PCR, polymerase chain reaction; bp, base pair; AM, acetoxymethyl ester.

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receptors. In this study, we identified and characterized a novel GPCR, EDG7, the third functional LPA receptor belonging to the EDG family.

**MATERIALS AND METHODS**

**Lipids**—1-Oleoyl-LPA, 1-palmitoyl-LPA, 1-stearoyl-LPA, 1-oleoyl-lyso-phosphatidylcholine (LPC), 1-oleoyl-lyso-phosphatidylethanolamine (LPE), 1-oleoyl-lyso-phosphatidylserine (LPS), porcine liver lysophosphatidylinositol (LPI), SIP, egg yolk phosphatidic acid (PA), dioleoyl-phosphatidylserine, and platelet-activating factor (PAF C16) were purchased from Avanti Polar lipids (Alabaster, AL). 2-Acyl-1-lyso-phosphatic acid (2-acyl-LPA) was prepared from egg yolk PA (5 nmol) as follows. PA was incubated with Rhizopus delemere lipase (20 mg/ml; Seikagaku-kogyo, Tokyo, Japan) in a 50 mM Tris-malate buffer, pH 5.7, at 37 °C for 2 h in the presence of one-quarter volume of diethyl ether. After the free fatty acids had been extracted with diethyl ether/petroleum ether (1:1 v/v) four times, the remaining lysophospholipids were extracted by the method of Bligh and Dyer (23). 2-acyl-LPA contains mostly oleic acid and linoleic acid, because egg yolk PA is prepared from egg yolk phosphatidylcholine, which contains those fatty-acid chains at the sn-2 position. Because the 2-acyl-1-lyso-phospholipids were not stable, they were stored at −80 °C in chloroform/methanol (2:1 v/v) and used within 24 h after mixing with a buffer solution. Cyclic PA (cPA) and its analogs were prepared as described (24).

**Amplification of the Novel G-protein-coupled Receptor with Degenerate PCR**—cDNA was prepared from about 10⁷ Jurkat T cells, and 5.0 μg was reverse-transcribed into DNA using the cDNA Cycle Kit (Invitrogen, Carlsbad, CA) with an oligo(dT) primer. The degenerate PCR primers were designed based on the amino acid sequences of the second and sixth of the seven transmembrane regions of the G-protein-coupled receptors EDG2 and EDG4. The oligonucleotides used were: GCIGGCIGCIGACTCTTTCTTCTGCC (DP2; based on the second transmembrane region) and GCIGGCIGCIGCTTTTCTGACCA (DP6, based on the sixth transmembrane region). Each PCR was carried out using 2.5 units ExTaq DNA polymerase (Takara Shuzo Co. Ltd., Kyoto, Japan) and 25% of the reverse-transcriptase reaction mixture in a 100-μl reaction mixture containing 10 μM Tris-HCl, pH 8.3, 50 μM KCl, 2 mM MgCl₂, 200 μM dNTP, and 50 pmol of each degenerate oligonucleotide primer (DP2 and DP6). 35 PCR cycles, each consisting of 45 s at 94 °C (denaturation), 2 min at 55 °C (annealing), and 3 min at 72 °C (elongation) were performed, with the first cycle including an extended denaturation period (5 min), during which the polymerase was added. The 554-bp PCR products were purified by agarose gel electrophoresis and subcloned into a T-vector using the original TA-Cloning Kit (Invitrogen, Carlsbad, CA). Plasmid DNA was prepared using the Wizard Miniprep DNA Purification System (Promega, Madison, WI), and sequencing was performed by the dideoxy chain termination method using amplification products from a Beckman Biometra/Rotovirin-Ather Kinase (Promega, Madison, WI). 5'- and 3'-RACE—A Marathon² DNA Amplification Kit (Clontech, Palo Alto, CA) was used to perform 5'- and 3'-RACE. Double-stranded cDNA was prepared from poly(A+) RNA of human Jurkat T cells, and nested PCR was carried out using the cDNA as a template and API, AP2 (supplied in the Marathon 228 cDNA Amplification Kit), and internal oligonucleotide primers (FW1, FW2, RV1, and RV2, see below) as PCR primers under the conditions described above. The sequences of the oligonucleotides were: FW1, AACAACGCTTGGTGGTTCCTGAC (nucleotide positions 690–713); FW2, AGCTATATAGGAAGCCGGTTAGTCTG (nucleotide positions 749–772); RV1, GTCAGAAGCCGCTCTGGCAG (nucleotide positions 349–372); and RV2, AGCAATTTGGTGGACGTCC (nucleotide positions 381–404). The resulting DNA fragments were subcloned, and their DNA sequences were determined. Then the DNA fragment covering the open reading frame of EDG7 was amplified by reverse transcription-PCR using the cDNA from human Jurkat cells and oligonucleotide primers corresponding to the 5'- and 3'-noncoding regions and Pfu DNA polymerase (Toyobo, Tokyo, Japan). At least three independent reverse transcription-PCRs were carried out.

**Cell Culture**—Sf9 insect cells were grown in a serum-free ExCell-420 Cell Culture—Costar (Becton-Dickinson, Branchburg, NJ). 5'- and 3'-noncoding regions and 5′Pfu DNA polymerase (Toyobo, Tokyo, Japan). At least three independent reverse transcription-PCRs were carried out.

**Baculovirus System**—The cDNA encoding the coding region of EDG7 (nucleotide positions 38–1104) was inserted into the EcoRI/NotI site of the baculovirus transfer vector pFASTBAC1 (Life Technologies, Inc.). To achieve expression of FLAG-tagged EDG7, cDNA with a FLAG tag at its N-terminal was generated by using the PCR technique using oligonucleotides: ATGGCAATTCATTGACCACAAGACGATGAGTAAGTTAGTTAGGATTTTTA. The resulting DNA fragment was digested by EcoRI/NotI and ligated into pFastBAC1. Recombinant baculoviruses were prepared using Bac-to-Bac Express System (Becton-Dickinson, Inc.) according to the manufacturer's protocol. For infection, 6 × 10⁶ Sf9 cells 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 detected by Western blotting with an anti-FLAG monoclonal antibody (Sigma). Expression of recombinant baculoviruses was confirmed by DNA sequencing.

**Co²⁺ Measurements—**Sf9 cells were harvested 2 days after baculovirus infection, washed gently with an HBS buffer (20 mM HEPES, pH 7.4, containing 120 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl₂, 1.25 mM CaCl₂, 1.2 mM KH₂PO₄, and 10 mM glucose), and loaded with 2 μM Fura-2-acetoxymethyl ester (Fura-2 AM; Molecular Probes Inc., Eugene, OR) for 45 min. Free Fura-2 AM was washed out, and the cells were resuspended in the HBS buffer to produce a concentration of 10⁶ 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 SLM-110 spectrofluorimeter (Japan Spectroscopic Co., Ltd., Tokyo, Japan). Fluorescence was recorded before and after addition of LPA and other phospholipids, which were dissolved in phosphate-buffered saline with 0.01% (v/v) of fatty acid-free bovine serum albumin (Sigma). Pertussis Toxin and U73122 Treatment—24 h after baculovirus infection of the Sf9 cells, PTX (100 ng/ml; Calbiochem, La Jolla, CA) was added to the culture medium, and incubation was continued for an additional 24 h. Then the cells were collected, and their Ca²⁺ responses were tested as described above. The phospholipase C inhibitor U73122 (30 μM; Calbiochem, La Jolla, CA) was added to Sf9 cells 3 min before LPA was added. ([³H]LPA Binding—**Sf9 cells (5 × 10⁶)** infected by each baculovirus for 48 h were washed with a binding buffer (phosphate-buffered saline containing 0.25% bovine serum albumin) and incubated for 60 min at 0 °C in the same buffer containing various concentrations of [³H]LPA in a 96-well membrane filter plate (pore size, 65 nm; Millipore, Orland, USA). As described three times, 10 μl of each test buffer (phosphate-buffered saline containing 1% bovine serum albumin) using a Multi-screen filtration System (Millipore), and the radioactivity bound to the cells was quantified using a β-counter. Total and nonspecific binding was evaluated in the absence and presence of 10 μM nonradioactive LPA, respectively. To examine the specificity of LPA binding, the amounts of LPA bound (using 10 μM of [³H]LPA) in the presence of excess nonradioactive LPA (10 μM), LPS (10 μM), LPC (1 μM), LPI (10 μM), LPE (10 μM), SIP (10 μM), and PAF (1 μM) (10 μM of LPC or PAF caused cell lysis) were determined, and the specific binding value was calculated by subtracting the nonspecific binding value (cpm) from the total binding value (cpm).

**cAMP Measurements—**Sf9 cells were infected with recombinant baculoviruses and harvested 2 days after infection. Cells were incubated with 5 μM forskolin in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.5 μM) for 10 min followed by a 20-min stimulation with 2.5 μM LPA with various acyl chains in an HBS buffer. cAMP levels were determined using a cAMP enzyme immunoassay system (Biotrak; Amersham Pharmacia Biotech), following the instructions of the manufacturer. Assay for MAP Kinase-mediated Signal Transduction—to map kinase-mediated signal transduction, we employed the PathDetect® EtkI Trans-Reporting System (Strategene, La Jolla, CA). This assay employs a fusion protein that contains the DNA-binding domain of GAL4 and the transactivation domain of Elk1 to induce expression of a luciferase reporter driven by an artificial promoter containing five GAL4-binding sites. Phosphorylation of the transactivation domain of

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The document provides a detailed description of the methods and materials used in a study to identify and characterize a novel G-protein-coupled receptor (EDG7) for lysophosphatic acid. The study involves the amplification of cDNA from human Jurkat cells using degenerate PCR primers, followed by cloning and sequencing. The receptor is expressed in Sf9 insect cells using recombinant baculoviruses, and its expression and binding properties are analyzed using various assays including fluorescence and cAMP measurements. The results provide insights into the receptor's role and function in the context of lysophosphatidic acid signaling.
Amino acid sequences of EDG7. A, the cDNA and amino acid sequence of human EDG7. The first and second lines indicate the nucleotide and deduced amino acid sequences, respectively, with the nucleotide and amino acid positions shown on the left and right, respectively. The consensus sequences for N-linked glycosylation sites (amino acid numbers 172–174) are shown in italics, and the putative seventh transmembrane domains are underlined. B, the amino acid sequences of human EDG7, EDG2, and EDG4 were aligned by the GENETYX-MAC program. Identical amino acids in the three proteins are marked by an asterisk, the hyphens in each line show gaps, and the putative seventh transmembrane domains (TM1–7) are underlined. C, phylogenetic tree of the EDG family. The phylogenetic tree depicted was derived by the NEIGHBOR JOINING method performed, yielding a 554-bp fragment containing a GPCR-like domain to the EDG family by subjecting cDNA of human Jurkat T cells to PCR amplification. Two blocks of conserved amino acid sequences in EDG2 and EDG4, one from the second and one from the sixth transmembrane domain, were chosen for the synthesis of the degenerate oligonucleotide primers DP1 and DP6 (see "Materials and Methods"). The PCR reaction was performed, yielding a 554-bp fragment containing a GPCR-like sequence distinct from those of known members of the EDG family. Using 5' and 3' RACE, we isolated a DNA fragment that covered the entire open reading frame of this novel gene. Complete sequencing of the DNA revealed a 1059-bp open reading frame flanked by a 42-bp 5'-untranslated region and a 44-bp 3'-untranslated region. The translational initiation site (ATG) was assigned to the first methionine codon (nucleotide positions 43–45), because an in-frame stop codon was present upstream of this methionine residue and flanking sequences (CCACA) were present, fulfilling Kozak's criteria for initiation (25). An in-frame translational termination codon (TAA) was present after nucleotide 1,101. Therefore, we concluded that this new GPCR contains 353 amino acids and that its molecular mass is 40,128 Da (Fig. 1A).

A comparison of the deduced amino acid sequence with those of known EDG sequences revealed that the primary structure of the predicted protein was similar to those of the GPCRs of the EDG family, with overall sequence identities to human EDG1, human EDG2 (Vzg-1), human EDG3, human EDG4, human EDG5, human EDG6, and human EDG7 of 34.8, 53.7, 36.3, 48.8, 33.8, and 35.5%, respectively. Therefore, the protein encoded by the cloned cDNA was named EDG7 (Fig. 1B). A high degree of similarity between EDG2 and EDG4 (approximately 50%; Fig. 1B) was observed among EDG7, EDG2, and EDG4. To gain better understanding of the relationships involved in the molecular evolution of the EDG family, a phylogenetic tree was constructed using the neighbor joining method (Fig. 1C). According to this phylogenetic tree, the EDG family can be classified into two distinct groups: EDG1, EDG3, H218/AGR16, and EDG6 belong to one, and EDG2, EDG4, and EDG7 belong to the other. Because EDG1, EDG3, and H218/AGR16 have been reported to function as S1P receptors and EDG2, EDG4, and EDG7 to function as LPA receptors in almost all types of mammalian cells. Western blotting analysis using anti-FLAG antibody M5 confirmed that an approxi-
increased the \([\text{Ca}^{2+}]\). However, no such \(\text{Ca}^{2+}\) response was observed in Sf9 cells infected with wild-type baculovirus (Fig. 2B) or in uninfected control cells (data not shown), even if the cells were treated with 10\(\mu\)M LPA. The structurally related lipids 1-oleoyl-LPC, 1-oleoyl-LPE, 1-oleoyl-LPS, 2-oleoyl-LPS, 1-acyl-LPI, PAF, and S1P, each at a concentration of 1\(\mu\)M, failed to elicit significant increases in the \([\text{Ca}^{2+}]\) (Fig. 2C).

We also expressed known LPA receptors EDG2 and EDG4 in Sf9 cells and compared their \(\text{Ca}^{2+}\) response. Protein expression was confirmed by Western blotting using anti-FLAG antibody (Fig. 2A). The expression of EDG2 protein was much higher than those of EDG4 and EDG7, but it failed to transmit a detectable \(\text{Ca}^{2+}\) signal in response to LPA (oleoyl) (Fig. 2B), consistently with the observation by Zondag et al. (17). On the other hand, EDG4 mediated a \(\text{Ca}^{2+}\) signal like EDG7 in Sf9 cells (Fig. 2B). Thus, both EDG4 and EDG7 but not EDG2 mediate the \(\text{Ca}^{2+}\) response by LPA in Sf9 cells.

**LPA Binding**—We investigated the specific binding of \(^{3}\text{H}\)oleoyl-LPA to Sf9 cells expressing FLAG-EDG7, FLAG-EDG2, and FLAG-EDG4 using various doses of LPA. EDG7- and EDG4-expressing Sf9 cells increased the specific binding of \(^{3}\text{H}\)LPA in comparison with wild-type baculovirus and EDG2-infected cells (Fig. 3A). The apparent \(K_d\) values of EDG7 and EDG4 for \(^{3}\text{H}\)LPA are 206 and 73.6\(\text{nM}\), respectively (Fig. 3B). Thus, the binding affinity for LPA of EDG7 is relatively lower than that of EDG4. We also examined the competition between the binding of \(^{3}\text{H}\)ILPA and related nonradioactive lipids to FLAG-EDG7-expressing Sf9 cells. LPA (10\(\mu\)M) reduced \(^{3}\text{H}\)ILPA binding, whereas the other related lipids examined (LPC, LPE, LPS, LPI, S1P, and PAF) did not (Fig. 3C).
FIG. 2. LPA-induced increases in the intracellular \([\text{Ca}^{2+}]_i\) of Sf9 cells expressing EDG7. A, expression of FLAG-tagged EDG7, EDG2, and EDG4 proteins in Sf9 cells. Sf9 cells were infected with each baculovirus, and FLAG-tagged protein expression was examined by Western blotting with an anti-FLAG (M5) monoclonal antibody. The predicted molecular mass of FLAG-EDG7 is about 35 kDa. The size of the molecular mass marker is shown on the right. B, \(\text{Ca}^{2+}\) response of FLAG-EDG7-, FLAG-EDG2-, and FLAG-EDG4-expressing Sf9 cells to LPA. Sf9 cells were infected with each baculovirus, loaded with the fluorescent \(\text{Ca}^{2+}\) indicator Fura-2 AM, and stimulated with 1-oleoyl-LPA. A result from cells infected with the wild-type virus is also shown. C, structurally related phospholipids did not evoke the \(\text{Ca}^{2+}\) response. Fura-2 AM-loaded Sf9 cells expressing FLAG-EDG7 were stimulated sequentially with 1 \(\mu\text{M}\) each phospholipid, and the \(\text{Ca}^{2+}\) response was examined as described in the legend to B.
These data clearly demonstrate that EDG7 represents a specific receptor for LPA.

Ligand Specificity of EDG7—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 in activated platelets (26). We then investigated which structural features of LPA are important for the EDG7-dependent activation of Ca\(^{2+}\) mobilization.

1-Oleoyl-LPA is a good ligand for EDG7 (Fig. 2B). As shown in Fig. 5, however, 1-acyl-2-lysophosphatidic acids with saturated acyl chains (1-stearoyl-, 1-palmitoyl-, and 1-myristoyl-LPA), at a concentration of 10 \(\mu\)M, failed to elicit significant increases in \([\text{Ca}^{2+}]_{i}\). Furthermore, 2-acyl-LPA, which contains a mixture of oleic and linoleic acids at the sn-2 position (see “Materials and Methods”), elicited a significant increase in \([\text{Ca}^{2+}]_{i}\) (Fig. 4, A–E). No such \(\text{Ca}^{2+}\) response of Sf9 cells infected with wild-type baculovirus was induced by 2-acyl-LPA (data not shown). The ligand specificity of EDG4 was also examined in this system. In marked contrast with EDG7, 1-myristoyl-, 1-palmitoyl-, 1-stearoyl-, 1-oleoyl-, and 2-acyl-LPA equally elicited a significant increase in the \([\text{Ca}^{2+}]_{i}\) in Sf9 cells expressing EDG4 (Fig. 4, F–J).

We also examined the ability of cPA, which was first isolated from the slime mold Physarum polycephalum (27), to increase the \([\text{Ca}^{2+}]_{i}\). As shown in Fig. 5, cPA with oleoyl acid at the sn-1 position of the lipid (18:1 cPA, PHYLPA-8) increased the \([\text{Ca}^{2+}]_{i}\), whereas 10 \(\mu\)M cPAs with palmitic acid (16:0 cPA, PHYLPA-5) and cyclopropane-containing hexadecanoic acid (PHYLPA-1) were inactive. These data demonstrated that EDG7 prefers LPA with unsaturated fatty-acyl chains at the sn-1 or sn-2 position.

Characterization of the EDG7-transduced \(\text{Ca}^{2+}\) Response—We next examined the effects of PTX and the PLC inhibitor U73122 on EDG7-transduced \(\text{Ca}^{2+}\) responses. As shown in Fig. 6, pretreatment of FLAG-EDG7-expressing Sf9 cells with 100 ng/ml PTX for 24 h did not affect the \(\text{Ca}^{2+}\) response evoked by 1 \(\mu\)M LPA, whereas U73122 inhibited effectively the \(\text{Ca}^{2+}\) response transduced by EDG7, demonstrating that EDG7 is coupled to PTX-insensitive G-protein(s). The \(\text{Ca}^{2+}\) response transduced by EDG4 was also inhibited by PLC inhibitor U73122 but unaffected by PTX pretreatment (Fig. 6).

Effect on cAMP Level—It has been repeatedly demonstrated that LPA inhibits adenylyl cyclase via a \(\text{G}\)-mediated signaling event in mammalian cells. To examine whether EDG7 participates in the inhibition of adenylyl cyclase in response to LPA, Sf9 cells expressing each LPA receptor were pretreated with forskolin to raise the intracellular cAMP level and then treated with LPA. Unexpectedly, LPA did not suppress forskolin-induced cAMP accumulation but rather increased intracellular cAMP level in EDG7-expressing Sf9 cells (Fig. 7A). The in-
crease in cAMP level by LPA in EDG7-expressing Sf9 cells was insensitive to PTX (Fig. 7B) and observed only when the cells were pretreated with forskolin (data not shown). The similar response was observed in EDG4-expressing Sf9 cells (Fig. 7B).

cAMP level in EDG2-expressing Sf9 cells was unaffected upon stimulation with LPA (Fig. 7A), as described previously (17).

Effect on MAP Kinase—MAP kinase in insect Sf9 cells has not been characterized yet. In addition we could not detect MAP kinase activity using anti-human MAP kinase antibodies or substrates for MAP kinase such as oligopeptide from human EGF-receptor (data not shown). Thus we used the mammalian expression system for this experiment. To determine whether EDG7 mediates MAP kinase activation, we used the PathDetect™ Elk1 trans-Reporting System. Elk1 is a transcription factor that is phosphorylated and activated by MAP kinase (28). PC12 cells were transfected with FLAG-tagged EDG2,

Fig. 4. Substrate specificity of EDG7 and EDG4. The activities of LPA and its acyl chain analogs to induce rapid, transient increases in [Ca\textsuperscript{2+}] in Sf9 cells expressing EDG7 (A–E) or EDG4 (F–J) were measured. Cells, loaded with Fura-2 AM, were stimulated with various concentrations of LPAs, and changes in [Ca\textsuperscript{2+}] were analyzed using CAF-110 spectrofluorimeter. The means were calculated from the results of three separate experiments. A and F, 1-oleoyl-LPA; B and G, 2-acyl-LPA; C and H, 1-stearoyl-LPA; D and I, 1-palmitoyl-LPA; E and J, 1-myristoyl-LPA.

Fig. 5. Cyclic PA with oleic acid elicits Ca\textsuperscript{2+} response in Sf9 cells expressing EDG7. FLAG-EDG7-expressing Sf9 cells were loaded with the fluorescent Ca\textsuperscript{2+} indicator Fura-2 AM and stimulated sequentially with PHYLPA-1 (10 \mu M), PHYLPA-3 (palmitoyl (16:0)-cPA, 10 \mu M), PHYLPA-8 (oleoyl (18:1)-cPA, 10 \mu M), and 1-oleoyl-LPA.

Fig. 6. Effects of PTX and PLC inhibitor (U73122) on the LPA-induced Ca\textsuperscript{2+} response of Sf9 cells expressing EDG7 (A and B) and EDG4 (C and D). A and C, Sf9 cells expressing FLAG-EDG7 (A) or FLAG-EDG4 (C) were pretreated with (open circle) or without (closed circle) PTX (100 ng/ml) for 24 h before the Ca\textsuperscript{2+} response was examined using various doses of oleoyl-LPA. B and D, Sf9 cells expressing FLAG-EDG7 (B) or FLAG-EDG4 (D) were pretreated with PLC inhibitor, U73122 (3 \mu M), for 3 min before the Ca\textsuperscript{2+} response was examined. Oleoyl-LPA (1 \mu M) was used.

EDG4, or EDG7 and subjected to an assay of MAP kinase activation. Expressions of each receptor were confirmed by immunofluorescence in PC12 cells (data not shown). LPA activated the transcription of the luciferase gene through activation of the Elk1 in EDG4-expressing PC12 cells but not in
EDG2- or EDG7-expressing cells (Fig. 8), suggesting that only EDG4 is coupled to MAP kinase activation among the cloned LPA receptors.

**Tissue and Cellular Distributions of EDG7**—The tissue distribution of EDG7 was examined by subjecting several human tissues and cancer cell lines to Northern blot analysis. EDG7 transcripts of about 4.3 kilobases were detected in the heart, pancreas, prostate, and testis and, to a lesser extent, in the lung and ovary (Fig. 9). EDG7 transcripts were also found weakly in the human cancer cell lines HeLa, K562, SW480, A549, and G361, the last of which, a melanoma cell line, contained the highest level (data not shown).

**DISCUSSION**

In this study, we isolated a new member of the EDG family of G-protein-coupled receptors, EDG7, and showed that it functions as a cellular receptor for LPA. Interestingly, EDG7 transduce increases in both [Ca^{2+}]_{i} and the cAMP level upon stimulating only with LPA containing an unsaturated fatty-acyl chain at the sn-1 or sn-2 position but not with LPA containing any of saturated fatty acids. EDG7 also reacted with cPA with unsaturated fatty acid (Fig. 5). In contrast, EDG4 can be stimulated by LPAs with both an unsaturated and saturated fatty acid. Moreover, Hecht et al. (9) have previously demonstrated that both oleoyl- and stearoyl-LPA induce EDG2-dependent cell rounding in EDG2-transfected cells. Erickson et al. (29) also reported that LPAs with both saturated and unsaturated fatty acid activate EDG2 in the yeast pheromone response pathway. Thus, EDG2 seems to recognize LPAs with both saturated and unsaturated fatty acids like EDG4. It has been previously demonstrated that LPAs with unsaturated fatty acids evoked greater Ca^{2+} responses of the human epidermoid carcinoma cell line A431 than LPAs with saturated fatty acids (30). In addition, Tokumura et al. (31) reported that degree of unsaturation in the acyl moiety of LPA affects proliferation of cultured vascular smooth muscle cells from rat aorta by LPA. A newly identified LPA receptor, EDG7, may account for these responses.

Several molecular species of LPA with saturated (stearoyl-
or EDG4 to demonstrate that PTX completely blocks LPA-TAg-Jurkat T cells transiently transfected with human EDG2 expressing Chinese hamster ovary cells was only observed examined EDG7-transduced Ca\textsuperscript{2+} mobilization of [Ca\textsuperscript{2+}]\textsubscript{i} was significantly in Sf9 cells, indicating that EDG7 and EDG4 are coupled to a PTX-sensitive G\textsubscript{i} protein(s), possibly G\textsubscript{i}. Although the human EDG2 was expressed significantly in Sf9 cells, Ca\textsuperscript{2+} mobilization was not observed in the cells, indicating that none of G-proteins in Sf9 cells can be coupled to human EDG2 (Ref. 17 and present study). It is interesting to note that no increase in the specific binding of LPA in EDG2-expressing Sf9 cells was observed either. Consistently with this observation, Figler et al. (37) demonstrated that specific binding site for ligand in GPCR was available only when appropriate G-protein(s) are coupled to the receptor.

The effect of LPA on the cell growth is either proliferative or antiproliferative. In most fibroblastic cell lines, LPA induces mitogenic responses and inhibits adenyl cyclase via a G\textsubscript{i} mediated signaling event (33). In fact, LPA inhibited adenyl cyclase in EDG2-transfected mammalian cells (9). On the other hand, in nonfibroblastic cell line Sp2/0-Ag14 myeloma, which does not express either EDG2 or EDG4 (22), LPA induced an increase in cAMP and inhibited cell proliferation in a PTX-sensitive manner, which is accompanied by an increase in cytoplasmic Ca\textsuperscript{2+} (4). They also indicated that LPA with a higher degree of unsaturation and longer acyl chains increased the antiproliferative effects (4). A newly identified EDG7, which enhanced adenyl cyclase in a PTX-sensitive manner, may account for those responses. The idea is further supported by the observation that EDG7 is not coupled to MAP kinase activation, which leads to cell proliferation in various types of cells. In EDG7- and EDG4-expressing Sf9 cells increases in cAMP by LPA were observed only after adenyl cyclase was first stimulated with forskolin (Fig. 7). Felder et al. (38) reported that accumulation of cAMP by CB1 antagonists in CB1-expressing Chinese hamster ovary cells was only observed when cells were pretreated with forskolin. Some isoforms of adenyl cyclase(s) that are coupled to EDG7 and EDG4 in Sf9 insect cells may require an initial priming for activation like type II or IV adenyl cyclases in mammalian cells as reported by Tang et al. (39). LPA is known to exert an effect on the cytoskeleton that can lead to changes in cell shapes and motility, which includes inducing the production of stress fiber through Rhod-GTPase activation. We examined the effect of LPA stimulation on the formation of actin stress fibers in EDG7-expressing CHO-K1 and PC12 cells by staining an actin filament with FITC-phalloidin but did not observe any obvious differences between the EDG7-expressing and control cells (data not shown).

In conclusion, EDG7 is a specific LPA receptor that shows distinct properties from known cloned LPA receptors in ligand specificity, Ca\textsuperscript{2+} response, and modulation of adenyl cyclase and MAP kinase. Further studies are definitely needed in understanding a physiological role of this receptor.

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