A Novel Phosphatidic Acid-selective Phospholipase A1 That Produces Lysophosphatidic Acid*

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Lysophosphatidic acid (LPA) is a lipid mediator with diverse biological properties, although its synthetic pathways have not been completely solved. We report the cloning and characterization of a novel phosphatidic acid (PA)-selective phospholipase A1 (PLA1) that produces 2-acyl-LPA. The PLA1 was identified in the GenBank™ data base as a close homologue of phosphatidylserine (PS)-specific PLA1 (PS-PLA1). When expressed in insect Sf9 cells, this enzyme was recovered from the Triton X-100-insoluble fraction and did not show any catalytic activity toward exogenously added phospholipid substrates. However, culture medium obtained from Sf9 cells expressing the enzyme was found to activate EDG7/LPA3, a cellular receptor for 2-acyl-LPA. The activation of EDG7 was further enhanced when the cells were treated with phorbol ester or a bacterial phospholipase D, suggesting involvement of phospholipase D in the process. In the latter condition, an increased level of LPA, but not other lysophospholipids, was confirmed by mass spectrometry analyses. Expression of the enzyme is observed in several human tissues such as prostate, testis, ovary, pancreas, and especially platelets. These data show that the enzyme is a membrane-associated PA-selective PLA1 and suggest that it has a role in LPA production.

Lysophosphatidic acid (1- or 2-acyl-lysophosphatidic acid; LPA) is a lipid mediator with multiple biological functions (1–3). These include induction of platelet aggregation, smooth muscle contraction, and stimulation of cell proliferation. LPA also promotes specific responses of the cytoskeleton such as generation of actin stress fibers in fibroblasts or inhibition of neurite outgrowth in neuronal cells. LPA evokes its multiple effects through G-protein-coupled receptors that are specific to LPA (see below), with consequent activation of phospholipase C (PLC) and phospholipase D (PLD), Ca2+ mobilization, inhibition of adenyl cyclase, activation of mitogen-activated protein kinase, and transcription of serum-response-element transcriptional reporter genes, such as c-fos. Recent studies (4, 5) have identified a new family of receptor genes for LPA. Members of this family include three G-protein-coupled receptors belonging to the endothelial differentiation gene (EDG) family, EDG2/LPA1 (4), EDG4/LPA2 (5), and EDG7/LPA3 (6). These proteins may explain various cellular responses to LPA (6–8).

In contrast to the signal transduction mediated by LPA receptors, the molecular mechanisms for LPA production are poorly understood. LPA is produced both in biological fluids such as serum (9) and in various cells such as platelets (10) and ovarian cancer cells (12, 13). In these latter studies, it was speculated that LPA is produced by phospholipase A2 (PLA2) from phosphatidic acid (PA) that is generated as a result of PLD activation (12, 13). Tokumura et al. (14) demonstrated that LPA is also produced in plasma from lysophosphatidylcholine (LPC) by the action of lysophospholipase D, which may account for the accumulation of LPA in aged plasma.

LPA, with various fatty acid species, has been detected in several biological systems. For example, human serum contains LPA with both saturated (16:0 and 18:0) and unsaturated (16:1, 18:1, 18:2, and 20:4) fatty acids (15). A similar LPA species was detected in human platelets (10). The activity of LPA has been shown to be modulated by the length, degree of unsaturation, and linkage to the glycerol backbone of the fatty acyl chain (16–21). Of particular interest is the detection of 2-linoleoyl-LPA in ascites from ovarian cancer patients, which may account for the increased ability of the ascites to activate the growth of ovarian cancer cell lines (22). We recently identified a novel LPA receptor, EDG7/LPA3, which shows a relatively high affinity for 2-acyl-LPA with unsaturated fatty acid (8, 23). It is generally accepted that the sn-1 position of glycerophospholipids is occupied by saturated fatty acids, whereas the sn-2 position is occupied by unsaturated fatty acids. This suggests that phospholipase A1 (PLA1) as well as PLA2 are involved in LPA production.

PLA1 enzymes hydrolyze the sn-1 fatty acids from phosphatidate; MES, 4-morpholinoesanesulfonic acid; MS, mass spectrometry; PMA, phorbol 12-myristate 13-acetate; cPA, cyclic PA; nPLA1, novel PLA1.
lipids. Although PLA1 activities are detected in many tissues and cell lines, a limited number of PLA2s have been purified and cloned. We have purified and cloned a cDNA for phosphatidylinserine-specific PLA2 (PS-PLA2), a member of the lipase family, from the culture medium of activated rat platelets. PS-PLA2 specifically hydrolyzes PS (24) and produces 2-acyl-lyso-phosphatidylserine (LPS), which is a lipid mediator for mast cells (25), T cells (26), and neural cells (27). We recently showed that PS-PLA2 stimulates histamine release from rat peritoneal mast cells by hydrolyzing PS exposed on the surface of some cell types such as apototic cells and activated platelets (25). Accordingly we searched GenBank™ for sequences similar to PS-PLA2, and found one PS-PLA2 homologue. Here we demonstrate that the PS-PLA2 homologue is a membrane-associated PA-selective PLA2 (mPA-PLA2) that can produce a bioactive lysophospholipid, 2-acyl-LPA, by hydrolyzing PA generated by PLD.

EXPERIMENTAL PROCEDURES

Materials—Phospholipase D from Actinomadura (28) was kindly donated by Meito Sangyo (Tokyo, Japan). 1-Oleoyl-LPA (18:1) and 1-[3H]oleoyl-LPA (18:1) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL) and Amersham Biosciences, respectively. 2-Oleoyl-LPA (18:1) was prepared as described previously (23). Other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Clone Identification—The EST clone 789124 (GenBank™ accession number AA149791) was identified by searching the GenBank™ EST database using the amino acid sequence of rat PS-PLA2. The cDNA clone was purchased from American Type Culture Collection (ATCC). The nucleotide sequence of the clone was determined by DNA sequencing using an ABI PRISM 377 DNA sequencer. We also amplified the cDNA of mPA-PLA2 (nPLA2) by reverse transcription (RT)-PCR using human colon-derived total RNA (see below). The nucleotide sequence data reported in this paper have been submitted to the GenBank™ database under the accession number AF536912 for human mPA-PLA2.

Expression of mPA-PLA2 in Sf9 Cells—The DNA fragment covering the coding region of mPA-PLA2 (EcoRI-HindIII fragment) was subcloned into the EcoRI and HindIII sites of pFASTBAC1 expression vector (Invitrogen) to generate a donor plasmid. Recombinant viruses were prepared using the Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer’s protocol. The resulting recombinant baculovirus was used to infect Sf9 cells. Sf9 cells in insect cell grown in serum-free EX-CELL 420 insect cell medium (Nichirei, Tokyo, Japan) at 27 °C. For infection, Sf9 cells were mixed with recombinant or wild-type Autographa californica nuclear polyhedrosis virus to produce a multiplicity of infection of 10, and the infected cells were further cultured for 48 or 72 h at 27 °C.

PLA2 Activity Assay—Sf9 cells were harvested 72 h after baculovirus infection. 100,000 cells were suspended in phosphate-buffered saline (137 mm NaCl, 2.7 mm KCl, 8.1 mm Na2HPO4, 1.5 mm KHPO4) and homogenized using a Potter-Elvehjem homogenizer. The supernatant obtained by centrifugation of the homogenate at 190 × g for 10 min was centrifuged at 100,000 × g for 60 min, and the resulting pellet was used as the “membrane fraction.” Dioloyl-PA, dioloyl-PS, dioleyl-phosphatidylethanolamine (dioleyl-PE), and dioleyl-phosphatidylinositol (dioleyl-PI), containing a 14C-labeled fatty acid at sn-1 position, were prepared as described previously (24). The PA, PS, PE, or PC (40 μl each) were incubated at 37 °C for 30 min with membrane fraction prepared from mPA-PLA2-expressing cells (100 μg of protein) in 100 μl Tris-HCl (pH 7.5) with 4 mm CaCl2. The fatty acid liberated was extracted by the modified Dole’s method, and radioactivity was measured with a scintillation counter as described previously (24).

Evaluation of EDG7 Activation—Activation of EDG7 wasactivation was done by Ca2+ assays using EDG7-expressing Sf9 cells (Sf9-EDG7 cells) as described (23). 48 h after EDG7-baculovirus infection, the cells were harvested and suspended in 2 ml 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 MBS (10 mm NaCl, 60 mm KCl, 17 mm MgCl2, 10 mm CaCl2, 110 mm sucrose, 4 mm HEPES, 0.1% fatty acid-free BSA (Sigma), and 10 mM MES (pH 6.2)) to produce a concentration of 106–107/ml. To examine the activity of LPA, the measurement of the ratio of emission wavelength of 500 nm by excitation wavelengths at 340 and 380 nm was performed in quarto cuvettes (total volume 100 μl) using a CAF-110 spectrofluorometer (Japan Spectroscopy, Inc., Tokyo, Japan) or in 96-well (total volume 200 μl) using an ARGUS-50/CA image analysis system (Hamamatsu Photonics K.K., Hamamatsu, Japan).

Exogenous PLD Treatment—Sf9 cells were harvested 72 h after baculovirus infection and suspended in MBS. Then PLD from Actinomadura was added exogenously to the suspension to a final concentration of 0.25 units/ml, and the mixture was incubated for 30 min at 27 °C. After removing the cells by centrifugation, the supernatant was used as “conditioned medium.”

Lipid Preparation—Phospholipids were extracted by the method of Bligh and Dyer in acidic condition (lower the pH to 3.0 with 1 N HCl). Lipids in the aqueous phase were re-extracted and pooled with the previous organic phase. The extracted lipids were dried, dissolved in chloroform/methanol (2:1) and used for functional bioassays and mass spectrometry (MS) analysis. The recovery of lipids was monitored by the addition of trace amounts of 1-[3H]oleoyl-LPA to the samples. Under the above conditions, recovery of 1-[3H]oleoyl-LPA was always >95%. For MS analysis the lipids were concentrated 20-fold.

MS Analysis—MS analysis was performed essentially as described previously (29). Lipid extracts from cells and conditioned media were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Antibodies—A peptide consisting of the C-terminal 18 amino acids of mPA-PLA2 (TGCGAAGTAAATCATTCTTGAA) was conjugated with keyhole limpet hemocyanin. The conjugate was injected into the hind foot pads of WKY/Izm rats using Freund’s complete adjuvant. The enriched medial iliac lymph nodes from the rats were used for cell culture with mouse myeloma cells, PAI. The antibody-secreting hybridoma cells were selected by screening with enzyme-linked immunosorbent assay (ELISA), immunofluorescence, and Western blotting.

Western Blotting—Protein samples were separated by SDS-PAGE and transferred to nitrocellulose membranes using the Bio-Rad protein transfer system. The membranes were blocked with Tris-buffered saline containing 5% (w/v) skimmed milk and 0.05% (v/v) Tween 20, and incubated with mouse monoclonal anti-mPA-PLA2 antibody (clone 11H3), and then treated with anti-rat IgG conjugated with horseradish peroxidase. Proteins bound to the antibodies were visualized with an enhanced chemiluminescence kit (ECL, Amersham Biosciences).

Immunofluorescent Staining—Sf9 cells infected with baculoviruses grown on cover glasses were fixed with ice-cold methanol and blocked with 10% goat serum. After incubation with anti-mPA-PLA2 monoclonal antibody (culture supernatant prepared from clone 11H3), and then treated with anti-rat IgG conjugated with Alexa Fluor 488 (Molecular Probes Inc.), the bound antibody was detected with a fluorescence microscope (Axio phot 2, Zeiss, Germany) and a confocal laser scanning microscope (Fluoview, Olympus, Tokyo, Japan).

Northern Blotting—Human multiple tissue Northern Blots were purchased from CLONTECH (Palo Alto, CA). The membrane was hybridized with a random-primed 32P-labeled EcoRI-XhoI 2.5-kb DNA probe generated by PCR (see below) using as the template a cDNA clone prepared using RT-PCR with the primers described in “Experimental Procedures.” The blot was rinsed in 2× SSC at room temperature for 5 min, washed twice in 0.1% SSC at 65 °C for 20 min, and used to expose Kodak X-Omat AR film (Eastman Kodak Co.). The blots were re-hybridized with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe (CLONTECH) as an internal standard.

RT-PCR—Human platelets were collected from healthy volunteers using EDTA as described previously (30). Total RNA was prepared using isogen (Wako, Osaka, Japan). RT-PCR was performed using Superscript reverse transcriptase (Invitrogen), Ex-Taq polymerase (Takara, Kyoto, Japan). The sequences of the two oligonucleotides used in RT-PCR were TGGAGAATTAAATACATCTTGTTGAA (nucleotide position 39–62) and TGGTACATCCATTAGGCCTACTG (nucleotide position 1589 to 1566). Nucleotide sequences of the PCR products were determined by direct sequencing.
RESULTS

Identification of a Novel PLA1 (nPLA1)—Our initial efforts to identify new phospholipases homologous to PS-PLA1 failed using low stringency cross-hybridization techniques with PS-PLA1 sequences. A precise search of the human EST database was successful, and one PS-PLA1-related gene fragment was identified (GenBank accession number AA149791). DNA sequence analysis of the clone revealed that the sequence was highly homologous with the entire open reading frames (ORFs) of rat and human PS-PLA1. This cDNA clone contained a 1353-bp ORF, starting with the initiation codon (ATG) at nucleotide 91, numbered as 1, and ending with a stop codon (TAA) at position 1444–1446 (Fig. 1A). This ORF was flanked by 5′- and 3′-untranslated sequences of 90 and 1,001 bp, respectively, and encoded 451 amino acids with a predicted molecular mass of 50,859 Da. Four possible N-linked glycosylation sites and a hydrophobic sequence composed of 18 amino acid residues at the N terminus were detected in the deduced amino acid sequence. This hydrophobic sequence was probably a short signal sequence. By RT-PCR we detected a cDNA that was identical to the DNA sequences in several human tissues (data not shown), indicating the EST clone is not an artificial clone. The deduced amino acid sequence had 34.0% identity with that of human PS-PLA1 (Fig. 1B), and the first half of the molecule, which corresponded to the N-terminal catalytic domain of PS-PLA1, had an identity of about 40%. Three of the amino acid residues in the ORF, Ser-154, Asp-178, and His-248, were completely conserved in the lipase family and are thought to make up a catalytic triad.

Like PS-PLA1, the molecule has a short lid composed of 12 amino acid residues, and a part of the loop that is found in other lipases is deleted (Fig. 1B). Interestingly, the same molecular features were also observed in all the hornet PLA1s that have been reported so far (30–32). The lids and loop domains are shaded boxes. C, phylogenetic relationship of the lipase family and nPLA1. A phylogenetic tree was generated from ClustalW alignment data using the GENETYX-MAC version 10.1.6 (Software Development Co. Ltd., Tokyo, Japan). This analysis found that nPLA1 and PS-PLA1 form a subfamily in the lipase family.

PLRP1, pancreatic lipase-related protein 1; PLRP2, pancreatic lipase-related protein 2.

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Like PS-PLA1, the molecule has a short lid composed of 12 amino acid residues, and a part of the β9 loop that is found in other lipase families is deleted (Fig. 1B). Interestingly, the same molecular features were also observed in all the hornet PLA1s that have been reported so far (30–32). The lids and β9 loops in lipases are implicated in the substrate recognition. The phylogenetic tree in Fig. 1C and a BLAST search (data not shown)
showed that the protein is the closest homologue of PS-PLA₁ not only in the lipase family but also in the data base. Thus we tentatively designated this as a novel PLA₁ (nPLA₁).

nPLA₁ Is a Membrane-associated Protein

In order to detect enzyme activity of the recombinant protein, we first tried to express the protein in Sf9 insect cells by a baculovirus system, and we detected it as a protein band with an apparent molecular mass of 55 kDa on a Western blotting using a monoclonal antibody raised against the C-terminal 18 amino acids (see “Experimental Procedures”) (Fig. 2A). Unlike PS-PLA₁, which was mostly secreted into the culture medium when expressed in Sf9 cells (24), the recombinant protein was detected only in the cell fraction (Fig. 2A). After high speed centrifugation of the cell homogenate, the recombinant protein was detected exclusively in the pellet (data not shown). In addition, it was found that the protein was resistant to solubilization by Triton X-100 (Fig. 2A). Immunofluorescence images using the anti-nPLA₁ antibody and a confocal laser microscope are shown in Fig. 2B. This analysis confirmed that the protein is localized exclusively to the plasma membrane.

Conditioned Medium from nPLA₁-expressing Sf9 Cells Activates EDG7—We hypothesized that the newly identified protein would exhibit PLA₁ and/or lipase activity based on the similarity of its sequence to sequences of proteins in the lipase family. We first determined whether the enzyme has PLA₁ activity by an in vitro conventional assay using various phospholipids as substrate and the membrane fraction from Sf9 cells expressing nPLA₁ (Sf9-nPLA₁ cells) as an enzyme source. However, the protein in the membrane fraction did not show any PLA activity toward exogenously added phospholipids (data not shown).

Previously, we used Sf9 cells to characterize a novel LPA receptor that we isolated, EDG7, which is a member of LPA receptor family. Cells expressing EDG7 (Sf9-EDG7 cells) were found to be strongly stimulated by exogenously added LPA (8). When we accidentally mixed the cultures of Sf9-nPLA₁ and Sf9-EDG7 cells, we detected a small but significant [Ca²⁺] increase in Sf9-EDG7 cells (data not shown). These preliminary data suggested that Sf9 cells expressing the newly identified protein may produce LPA, which then stimulates EDG7.
Therefore, we decided to analyze this phenomenon in more detail. When we incubated SF9-nPLA1 cells with medium containing 0.1% BSA, we found that the conditioned medium did induce a transient Ca\(^{2+}\) response in SF9-EDG7 cells (Fig. 3A). The response was not induced at all by a conditioned medium prepared from SF9 cells infected with wild-type baculovirus (SF9-WT cells) (Fig. 3A). It was also shown that the conditioned medium from the SF9-nPLA1 cells desensitized the Ca\(^{2+}\) response induced by 100 nM LPA (Fig. 3A) and that it did not induce any Ca\(^{2+}\) response in Fura-2-loaded SF9-WT cells (data not shown), confirming that the Ca\(^{2+}\) response is mediated by EDG7.

We next determined whether catalytic activity of nPLA1 is required for the induction of a Ca\(^{2+}\) response. To do this we prepared a baculovirus to express mutant PLA1, in which the putative active serine residue (Ser-154), which is conserved among members of the lipase/PLA1 family (Fig. 1B), was replaced with an alanine residue. SF9 cells infected with the mutant baculovirus (SF9-mutPLA1 cells) expressed mutPLA1 protein at almost the same level as SF9-nPLA1 cells (Fig. 3A). However, the conditioned medium from SF9-mutPLA1 cells did not induce any Ca\(^{2+}\) response in SF9-EDG7 cells (Fig. 3B). This result indicated that Ser-154 was actually the active serine residue and that catalytic activity of nPLA1 was required for the activation of EDG7. These results, taken together, indicated that LPA was continuously produced and released from SF9-nPLA1 cells.

**Synthetic Pathway(s) for LPA in SF9-nPLA1 Cells**—From the data shown above, two pathways for LPA production were postulated in which nPLA1 is involved. In the first pathway, nPLA1 hydrolyzes phospholipids which results in an accumulation of lysophospholipids and a consequent degradation of the lysophospholipids to LPA by the action of phospholipase D (PLD). In the second pathway, nPLA1 hydrolyzes PA and produces LPA. To test the first pathway, we determined whether lysophospholipids accumulated in SF9-nPLA1 cells. For this we extracted phospholipids from both SF9-nPLA1 and SF9-WT cells and performed lipid analysis by electrospray ionization mass spectrometry (ESI-MS). As shown in Fig. 4, several compounds were detected in the lipid fractions from both cell types. These included LPC (m/z 538 (16:1-LPC ion paired with HCOOH) and 566 (18:1-LPC ion paired with HCOOH) in negative ion scan mode), lysophosphatidylethanolamine (LPE) (m/z 450 (16:1-LPE) and 478 (18:1-LPE) in negative ion scan mode), lysophosphatidylinositol (LPI) (m/z 569 (16:1-LPI), 597 (18:1-LPI), 599 (18:0-LPI) in negative ion scan mode), and LPS (m/z 494 (16:1-LPS) and 522 (18:1-LPS), not shown). However, we did not observe any significant differences in the expression profiles of lysophospholipids between SF9-nPLA1 and SF9-WT cells. We also examined the lysophospholipid profiles in the conditioned media and did not observe any difference in the expression of LPC, LPE, LPS, and LPI (data not shown). Although a significant difference in the activation of EDG7 was observed in the conditioned media prepared from SF9-nPLA1.
and Sf9-WT cells (Fig. 3), signals corresponding to LPA were not detected either in the conditioned medium or in the cells under the present conditions, possibly due to a low sensitivity of the ESI-MS compared with the bioassay. Indeed, the lower limit of LPA detection was only 10 \( \text{nM} \) for ESI-MS, whereas the bioassay could detect 1-oleoyl-LPA concentrations as low as 100 \( \text{nM} \) and 2-oleoyl-LPA concentrations as low as 10 \( \text{nM} \).

To confirm the second pathway, we next examined whether activation of endogenous PLD (Fig. 5) or exogenously added PLD (Fig. 6) affected the ability of the conditioned medium from Sf9-nPLA1 cells to activate EDG7. First we used the ability of phorbol 12-myristate 13-acetate (PMA) to activate PLD via protein kinase C and that of short chain alcohol to inhibit PLD activity. As shown in Fig. 5, treatment of the cells with 100 nM PMA for 30 min significantly enhanced the \( \text{Ca}^{2+} \) response in Sf9-EDG7 cells initiated by the addition of conditioned medium from Sf9-nPLA1 cells. The enhancement was not induced by addition of conditioned medium from Sf9-WT cells treated with PMA (Fig. 5). We further examined the effect of 1-butanol or 2-butanol on PMA-enhanced \( \text{Ca}^{2+} \) response. Incubation of Sf9-nPLA1 cells with 100 nM PMA in the presence of 1-butanol at 0.5%, a concentration that completely inhibits PLD from the Sf9-nPLA1 cells to activate EDG7. First we used the ability of phorbol 12-myristate 13-acetate (PMA) to activate PLD via protein kinase C and that of short chain alcohol to inhibit PLD activity. As shown in Fig. 5, treatment of the cells with 100 nM PMA for 30 min significantly enhanced the \( \text{Ca}^{2+} \) response in Sf9-EDG7 cells initiated by the addition of conditioned medium from Sf9-nPLA1 cells. The enhancement was not induced by addition of conditioned medium from Sf9-WT cells treated with PMA (Fig. 5). We further examined the effect of 1-butanol or 2-butanol on PMA-enhanced \( \text{Ca}^{2+} \) response. Incubation of Sf9-nPLA1 cells with 100 nM PMA in the presence of 1-butanol at 0.5%, a concentration that completely inhibits PLD...
activity, completely blocked the Ca\(^{2+}\) response enhanced by PMA treatment (Fig. 5), whereas 2-butanol, a positional isomer of 1-butanol that does not have the ability to block PLD, at 0.5% did not show such an effect (Fig. 5). PMA, 1-butanol, or 2-butanol alone did not affect the Ca\(^{2+}\) responses (data not shown).

As shown in Fig. 6, the treatment of Sf9-nPLA\(_1\) cells with exogenously added PLD significantly enhanced the ability of the conditioned medium to activate EDG7. The ability of the conditioned medium from Sf9-nPLA\(_1\) cells to activate EDG7 was increased at least 100 times by the PLD treatment (Fig. 6). The conditioned medium from Sf9-WT and Sf9-mutPLA\(_1\) cells induced a small Ca\(^{2+}\) response in Sf9-EDG7 cells after the PLD treatment, but they were at least 10 times less potent than the conditioned medium from Sf9-nPLA\(_1\) cells (Fig. 6), showing that a small amount of LPA is produced after the PLD treatment. These two lines of evidence indicate that nPLA\(_1\) produced 2-acyl-LPA by hydrolyzing PA generated on membranes by either endogenously expressed or exogenously added PLD.

**nPLA\(_1\) is PA-selective PLA\(_1\)**—To elucidate further the substrate specificity of nPLA\(_1\), we next performed lipid analysis of the conditioned medium prepared from Sf9-nPLA\(_1\) cells after the PLD treatment using ESI-MS. As shown in Fig. 7, two major ion peaks (m/z 407 and 435) were detected by ESI-MS (negative ion scan mode) in the lipid fraction extracted from the conditioned medium from Sf9-nPLA\(_1\) cells after PLD treatment. These peaks were estimated from their molecular weights to be 16:1-LPA and 18:1-LPA, respectively (29). They were also weakly detected in the lipid fractions from Sf9-WT and Sf9-mutPLA\(_1\) cells even after the PLD treatment (Fig. 7). In the positive ion scan mode, four minor peaks, m/z 409, 426, 437, and 454, were detected that were not detected in the lipid fractions from Sf9-WT and Sf9-mutPLA\(_1\) cells (Fig. 7). 16:1-LPA and 18:1-LPA had m/z values of 407 and 435, respectively, in the negative ion scan mode, 409 and 437, respectively, in the positive ion scan mode, and 426 and 454, respectively, complexed with ammonium ion observed only in the positive ion scan mode. The identities of the peaks were further confirmed by MS/MS analysis of the daughter ions. The detected major fragment peaks from the precursor ion, m/z 435, were m/z 78.7, 152.7, and 280.9. They correspond to PO\(_3\), cyclic glycerophosphate, and oleic acid (18:1), respectively (data not shown). A similar result was obtained from peak m/z 407 (data not shown). Thus we concluded that LPA with 16:1 and 18:1 was produced in Sf9-nPLA\(_1\) cells after the PLD treatment. Other than LPA, we detected four ion peaks in the negative ion scan mode with m/z values 389 (16:1-cPA), 407 (16:1-LPA), 417 (18:1-cPA), 435 (18:1-LPA), 450 (16:1-LPE), and 478 (18:1-LPE) in negative ion scan mode, and 409 (16:1-LPA), 426 (16:1-LPA ion paired with NH\(_3\)), 437 (18:1-LPA), 454 (18:1-LPA ion paired with NH\(_3\)), 494 (16:1-LPC), and 522 (18:1-LPC) in positive ion scan mode.

**Expression of mPA-PLA\(_1\)**—We finally examined the tissue distribution of mPA-PLA\(_1\) by Northern blotting using the full-length cDNA as a probe. The Northern blotting indicated that most of the human tissues examined had a transcript of 3.3 kb and that some had transcripts of 4.4, 2.2, and 1.5 kb. mPA-PLA\(_1\) is most abundantly expressed in prostate, testis, ovary, colon, pancreas, kidney, and lung and is expressed at lower levels in spleen, brain, and heart (Fig. 8A). Interestingly, the expression pattern is similar, but not completely identical, to

![Fig. 7. Detection of LPA in the conditioned medium from Sf9-nPLA\(_1\) cells after PLD treatment by MS analysis. The ESI-MS spectra of phospholipids from the conditioned media from Sf9-nPLA\(_1\), Sf9-WT, and Sf9-mutPLA\(_1\), cells after they were treated with PLD from Actinomadura. Results from both negative and positive ion scan mode are shown. The values representing 100% of the y axis of negative and positive ion scan modes are 7.7 and 8.0 \(\times 10^{-4}\) eV, respectively. The major ions and their identities are 389 (16:1-cPA), 407 (16:1-LPA), 417 (18:1-cPA), 435 (18:1-LPA), 450 (16:1-LPE), and 478 (18:1-LPE) in negative ion scan mode, and 409 (16:1-LPA), 426 (16:1-LPA ion paired with NH\(_3\)), 437 (18:1-LPA), 454 (18:1-LPA ion paired with NH\(_3\)), 494 (16:1-LPC), and 522 (18:1-LPC) in positive ion scan mode.](http://www.jbc.org/)
that of EDG7, which is highly expressed in prostate, pancreas, ovary, testis, lung, and heart (8). We also examined mPA-PLA1 expression in human platelets because the cells have been well characterized as LPA-producing cells (10, 11). As shown in Fig. 8B, expression of mPA-PLA1 in human platelets was confirmed by both mRNA and protein levels. A high level of protein expression was observed in the cells and was almost the same level observed in Sf9 overexpressing mPA-PLA1 (Fig. 8B).

DISCUSSION

The metabolic pathways for LPA synthesis are currently poorly understood, and at least three pathways have been postulated. In the first pathway, LPA is converted from PA by PLA1 or PLA2, which has been observed to occur in erythrocytes and ovarian cancer cells (12, 13, 33). In the second pathway, which may occur in platelets, diacylglycerol produced by PLC could be deacylated by diacylglycerol lipase, with the resulting monoacylglycerol being further phosphorylated into LPA (34, 35). The third pathway involves lysophospholipase D acting on LPC in plasma and may explain the large accumulation of LPA in aged plasma (14). A similar reaction may occur on the cell surface, in which LPC was converted to LPA by bacterial PLD (36). Enzymes involved in these processes of LPA synthesis have not been characterized fully. However, several PLA2 isoforms identified and characterized biochemically have been implicated in LPA production. For example, studies using inhibitors of PLA2 isoforms have suggested that sPLA2-IB, Ca2+-independent PLA2, and cytosolic PLA2 were partially involved in the LPA production of ovarian cancer cells (12, 13). It was also proposed that sPLA2-IIA was able to produce LPA by hydrolyzing PA exposed on the cell surface after phospholipid scrambling (37) or by hydrolyzing PA on membrane microvesicles shed from erythrocytes (33).

The present investigation led to several interesting observations, allowing us to propose a role of a novel PLA1 molecule, mPA-PLA1, in LPA production. Our results from this study are as follows. (i) A low level of LPA that could activate EDG7 was continuously produced and released into the medium in Sf9-mPA-PLA1 cells. (ii) The production of LPA in Sf9-mPA-PLA1 cells was significantly increased after PLD administration (Figs. 6 and 7). (iii) The expression of mPA-PLA1 did not promote accumulation of any lysophospholipids including LPC, LPE, LPI, and LPS in the cells (Fig. 4). (iv) We also observed that cPA was equally detected in the media from Sf9-mPA-PLA1, Sf9-WT, and Sf9-mutPLA1 cells only after the PLD treatment (Fig. 7). The bacterial PLD (from Actinomadura) used in this study converts lysophospholipids (LPC, LPE, LPS, and LPI) to cPA but not to LPA.2 All these results clearly indicate that mPA-PLA1 produces LPA by hydrolyzing PA. We could not detect PLA1 activity of mPA-PLA1 toward exogenously added PA liposome using a conventional assay for PLA1 or A2. It can be speculated that the availability of exogenous substrate to the enzyme is limited, as mPA-PLA1 is tightly associated with membrane phospholipids. mPA-PLA1 may hydrolyze such phospholipids, which surround the enzyme on the plasma membrane, after the phospholipids are converted to PA.

2 T. Kobayashi, Ochanomizu University, personal communication.
PA is a very minor component of phospholipids in mammalian cells and also in SF9 cells (38). This is consistent with the result that the LPA level was very low under normal conditions (Figs. 5 and 6). It is thus reasonable to assume that the rate-limiting step for LPA production in this pathway is generation of PA. PA could be generated by PLD or sequentially by PLC and diacylglycerol kinase. We observed that exogenously added PLD strongly promoted the production of LPA (Figs. 6 and 7) and that PMA-stimulated production of LPA was suppressed by a PLD inhibitor, 1-butanol (Fig. 5). Thus, it is likely that PLD is involved in the production of LPA mediated by mPA-PLA1. In mammalian cells, the molecular identities of the two isozymes of PLD, PLD1 and PLD2, have been elucidated. Among these two isozymes, PLD2 is activated by PMA both in vivo and in vitro through an activation of protein kinase Ca (39). Although information about PLD isozyme(s) in SF9 insect cells is limited (40), the observation that PMA stimulated LPA formation in SF9-mPA-PLA1 cells (Fig. 5) suggests an involvement of a PLD-like molecule in the insect cells. Consistent with this, it is reported by Shen et al. (12) that LPA is produced and secreted from ovarian cancer cells after they were treated with PMA.

LPA produced by mPA-PLA1 in SF9 cells was rich in oleic acid (18:1) and palmitoleic acid (16:1) (Fig. 7). Marheineke et al. (38) reported that the major fatty acids in the phospholipids from SF9 cells were oleic acid, palmitoleic acid, and stearic acid (18:0), with a small amount of palmitic acid (16:0). This explains why LPA with linoleic acid (18:2) and arachidonic acid (20:4), which are the major fatty acids at the sn-2 position of phospholipids of mammalian cells, was not detected. We observed that mPA-PLA1 is abundantly expressed in human platelets that have been characterized well as LPA-producing cells (10, 11). In activated platelet, LPA with both saturated (16:0, 18:0) and unsaturated 16:1, 18:1, 18:2, and 20:4 has been detected. This suggests that both PLA1 and PLA2 isozymes are involved in the LPA production in the cells.

Although it is possible that EDG7 is activated by an entity other than LPA, this seems unlikely for two reasons. First, the amount of LPA in the conditioned medium of Sf9-mPA-PLA1 cells is limited (40), the observation that PMA stimulated LPA production in the cells is consistent with this, it is reported by Shen et al. (12) that LPA is produced and secreted from ovarian cancer cells after they were treated with PMA.
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A Novel Phosphatidic Acid-selective Phospholipase A_1 That Produces Lysophosphatidic Acid
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