J. Biochem. 2009;146(2)283–293
doi:10.1093/jb/mvp068

Lysophosphatidylmethanol is a pan lysophosphatidic acid receptor agonist and is produced by autotaxin in blood

Tomoko Endo[1,2,*], Kuniyuki Kano[2,*], Rie Motoki[1,*], Kotaro Hama[1], Shinichi Okudaira[1,2], Mayuko Ishida[3], Hideo Ogiso[3], Masayuki Tanaka[1,2], Norio Matsuki[1], Ryo Taguchi[3], Motomu Kanai[1], Masakatsu Shibasaki[1], Hiroyuki Arai[1,4] and Junken Aoki[2,5,†]

[1]Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033; [2]Graduate School of Pharmaceutical Sciences, Tohoku University, Aobayama, Aoba-ku, Sendai, 980-8578; [3]Graduate School of Medicine, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-0033; [4]CREST; and [5]PRESTO, Japan Science and Technology Corporation, Japan

Received February 23, 2009; accepted April 16, 2009; published online May 4, 2009

Lysophosphatidic acid (LPA) is a simple phospholipid but has numerous biological effects through a series of G-protein-coupled receptors specific to LPA. In general, LPA is short-lived when applied *in vivo*, which hinders most pharmacological experiments. In our continuing study to identify stable LPA analogues capable of *in vivo* applications, we identified here lysophosphatidylmethanol (LPM) as a stable and pan-LPA receptor agonist. A synthetic LPM activated all five LPA receptors ($LPA_{1–5}$), and stimulates both cell proliferation and LPA-receptor-dependent cell motility. In addition, LPM showed a hypertensive effect in rodent when applied *in vivo*. We found that, when fetal calf serum was incubated in the presence of methanol, formation of LPM occurred rapidly, whereas it was completely blocked by depletion of autotaxin (ATX), a plasma enzyme that converts lysophosphatidylcholine (LPC) to LPA. When recombinant ATX was incubated with LPC in the presence of methanol, both LPM and LPA were produced with a ratio of 1:10, showing that ATX has transphosphatidylation activity in addition to its lysophospholipase D activity. Administration of methanol in mice resulted in the formation of several micromoles of LPM in plasma, which is much higher than that of LPA. The present study identified LPM as a novel and stable lysophospholipid mediator with LPA-like activities and ATX as a potential synthetic enzyme for LPM.

Key words: lysophosphatidic acid, lysophosphatidylmethanol, lysophosphatidylcholine, autotaxin, transphosphatidylation.

Abbreviations: LPA, lysophosphatidic acid; LPM, lysophosphatidylmethanol; LPC, lysophosphatidylcholine; ATX, autotaxin; LPEt, lysophosphatidylethanol; LPBt, lysophosphatidylbutanol; MS, mass spectrometry.

Lysophosphatidic acid (1- or 2-acyl-lysophosphatidic acid; LPA) is a bioactive lipid that mediates multiple cellular processes (*1–3*), including platelet aggregation, smooth muscle contraction, cell proliferation and cytoskeletal reorganization (*e.g.* generation of actin stress fibres and inhibition of neurite outgrowth). LPA is also a vasoactive lipid that induces either hypertension or hypotension, depending on animal species (*2*, *4*). Most of these LPA actions are thought to be mediated by G-protein-coupled receptors (GPCRs) that are specific to LPA (*5*, *6*). Recent studies have identified new families of receptor genes for LPA (*6*). Members of this family include three GPCRs belonging to the EDG (endothelial cell differentiation gene) family, LPA_1/EDG2 (*7*), LPA_2/EDG4 (*8*), LPA_3/EDG7 (*9*) and two GPCRs, GPR23/LPA_4 (*10*) and GPR92/LPA_5 (*11*, *12*), which are structurally distant from the EDG family. These LPA receptors, by coupling with different G-proteins, may explain various cellular responses to LPA. Indeed, genetic manipulation of these GPCRs revealed that LPA has critical and specific roles in brain development (*13*, *14*), preventing apoptosis at the intestinal epithelium (*15*, *16*) and embryo implantation in the uterus (*17*).

LPA can be produced by many types of cells and also in blood through diverse pathways in which multiple phospholipases are involved (*18–20*). The most abundant source of LPA is serum, where LPA is produced by the action of a plasma enzyme called autotaxin (ATX). ATX converts lysophosphatidylcholine (LPC) to LPA by its lysophospholipase D activity (*21*, *22*). ATX acts on various lysophospholipids such as LPC, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lyso platelet-activating factor, sphingosylphosphorylcholine, but not on phospholipids with two long methylene chains (*19*, *21*). Thus, ATX is a phospholipase D that is specific to the 'lyso' form of phospholipids as physiological substrates. Transphosphatidylation is a reaction that introduces a primary alcohol to the polar head of phospholipids and is catalysed by phospholipase D (*23*). Tsuda *et al*. (*24*) recently showed

*These authors contribute equally to this work.
†To whom correspondence should be addressed.
Tel: +81-3-5841-4723, Fax: +81-3-3818-3173,
E-mail: jaoki@mail.pharm.tohoku.ac.jp

that ATX catalyses intramolecular tansphosphatidylation reaction, producing cyclic PA (cPA) from LPC. It is not clear whether ATX catalyses a reaction to introduce a primary alcohol to the polar head of lysophospholipids.

LPA in general is a short-lived compound *in vivo*. For example, when applied *in vivo* the hypertensive activity of LPA lasts only for a few minutes (*4*). In addition, in culture cell system, LPA is degraded quickly. The major degrading route is dephosphorylation, which is catalysed by lipid phosphate phosphatases (LPPs) (*25*). Stable and, thus, long-lived LPA analogues are desired to examine the biological activity of LPA. To search for such stable LPA analogues we have developed a series of LPA analogues with a modified phosphate group. T13 (*26*) and OMPT (*27*, *28*), which has a thiophosphate at the polar head, and XY-17 (*28*), in which the bridging oxygen of the monophosphate was replaced by an a-monofluoromethylene (-CHF-), were such stable LPA analogues and showed specificity to LPA_3. In this study, as part of our continuing study to search for such stable LPA analogues, we identified lysophosphatidylmethanol (LPM). In addition, we describe several lines of evidence showing that LPM is produced by ATX in blood by its transphosphatidylation activity.

MATERIALS AND METHODS

Reagents—1-oleoyl LPA was purchased from Avanti polar lipids Inc. Synthetic phosphatidylethanol (di-oleoyl) and phosphatidylbutanol (di-oleoyl) were purchased from Biomol.

Synthesis of LPM—Synthesis ofLPM with oleic acid (18:0) started from commercially available enantiomerically pure isopropylidene-glycerol **30S** or **30R** as shown in Supplementary Data. Diol **32** was synthesized via two steps following the procedure of Prestwitch et al. (*29*, *30*). The primary hydroxyl group of **32** was selectively acylated with oleoyl chloride in the presence of triethylamine, giving **33** as a single regio-isomer. Mono methyl ester cleavage was conducted with $^{t}BuNH_2$ with excellent yield (*31*). Acyl migration took place during the reaction, and the obtained LPM was a mixture of regio-isomers. Myristate LPM (14:0) was prepared from myristate LPC as follows. Myristate LPC (2 mM) was mixed with recombinant ATX in 100 mM Tris–HCl pH 9.0, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 500 mM NaCl, 0.05% Triton X-100 in the presence of methanol (25% v/v). After extracting the lipids, LPM was purified using preparative thin-layered chromatography. The resulting myristate LPM was quantified and used in the mass spectrometry (MS) analyses as an internal standard. The MS spectra indicated that any minor contamination other lysophospholipids including LPA was detected in the LPM preparation. In addition, we also checked the contamination of LPA in the LPA preparation using colorimetric assay (*19*).

Preparation of Lysophosphatidylethanol (LPEt) and Lysophosphatidylbutanol (LPBt)—Phosphatidylethanol and phosphatidylbutanol were digested by phospholipase A_2 from Naja Naja (Sigma) and the resulting lysophospholipids were extracted by the Bligh and Dyer method (*32*).

Measurement of Intracellular Calcium—cDNA fragments encoding human $LPA_{1–5}$ were inserted into the pCAGGS expression vector. The resulting plasmid DNAs were transfected to HeLa cells, which do not express detectable levels of LPA_1, LPA_2, LPA_3, LPA_4 or LPA_5 at the mRNA level, using lipofectamine2000 transfection reagent (Invitrogen). Twenty-four hours after transfection, cells were washed with PBS and loaded with 2 mM Fura-2 AM for 30 min at 37°C in Calcium Ringer solution (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 5 mM HEPES-NaOH) containing 0.1% BSA. After washing with PBS, the cells were re-suspended in Calcium Ringer solution at a concentration of 1.0×10^6 cells/well. Following stimulation with LPA or other ligands, the intracellular calcium level ($[Ca^{2+}]_i$) was measured by monitoring the fluorescence intensity at an emission wavelength of 500 nm and at excitation wavelengths of 340 and 380 nm using a CAF-110 (JASCO). We could not detect LPA_5-dependent $[Ca^{2+}]_i$ mobilization in our system. We have performed the assay at least three times for each compound and typical data were shown.

Reporter Gene Assay—The ability of each LPA analogue to activate each LPA receptor was evaluated using a reporter gene assay. PC12h cells were transfected with 500 ng of pCAGGS expression vector encoding mouse $LPA_{1–5}$, 455 g of Zif 268-firefly luciferase-pGL2, 25 ng of cytomegalovirus (CMV) promoter-driven Renilla luciferase-pRL, 20 ng of G16-pME18S using Superfect transfection reagent (Qiagen). The transfected cells (2×10^5 cells/ml) in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.5% horse serum and 0.25% fetal calf serum (FCS) were cultured for 48 h in a collagen-coated 24-well plate. Cells were starved for 90 min at 37 C by changing the medium to serum-free and phenol red-free DMEM. Cells were then stimulated with ligands dissolved in serum-free and phenol red-free DMEM for 6 h. Firefly and Renilla luciferase activities in the cell lysates were measured using PICAGENE Dual Seapansy (TOYO INK MFG) and Luminescencer AB-2200 luminometer (ATTO). The relative luciferase activity was calculated by dividing the firefly luciferase value by the Renilla luciferase value.

Evaluation of Cell Proliferation and Cell Migration—Rat hepatoma cells, McA-Rh7777, which stably express LPA_1 (*21*), were plated in 96-well plates at a density of 1×10^4 cells/well in 100 μl of media, and cultured for 48 h. After cells were starved for 24 h, cells were stimulated with LPA or LPA analogues and cultured for 48 h as described previously (*21*). The cell numbers were determined using a cell-counting kit (Dojin) based on the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assays. Cell migration was evaluated with the Boyden-chamber assay as described previously (*33*) using the PC-3 human prostate cancer cell line, which shows an LPA_1-dependent migratory response to LPA (*33*).

Measurement of Blood Pressure—Male C57 BL/6J mice (25–30 g) anaesthetized with urethane (1.5 mg/kg) were fixed on their back. Under a stereoscopic microscope, the trachea was exposed and cannulated. A polyethylene-tipped cannula filled with heparinized saline was inserted into the carotid artery and the

femoral vein for direct measurements of arterial pressure and for drug administration, respectively. The arterial cannula was connected to a transducer (Nihon Kohden) and blood pressure signals were recorded using PowerLab (ADinstruments). Compounds were dissolved at various concentrations in saline containing 0.01% BSA, and injected using a syringe (1 ml) through the femoral vein cannula.

Formation of LPM In Vitro and In Vivo—To form LPM *in vitro*, FCS or mouse serum was incubated at 37°C in the presence or absence of methanol. Alternatively 18:1-LPC (2 mM) was mixed with recombinant ATX in 100 mM Tris–HCl pH 9.0, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 500 mM NaCl, 0.05% Triton X-100 in the presence or absence of methanol. To form LPM *in vivo*, C57BL/6J mice were injected intravenously with methanol. As high dose of methanol was found to be toxic, we injected 50 μl of methanol intravenously three times with 8 h interval. Twenty-four hours after the first methanol injection, blood was drawn from the mice and EDTA plasma was prepared.

Preparation of ATX-Depleted FCS—ATX-depleted serum was prepared as described previously (*34*). Briefly, the monoclonal antibody against ATX (5E5) was coupled to Sepharose 4B beads (GE Healthcare). FCS was incubated with 5E5-Sepharose 4B and resulting supernatant was used.

Extraction of Lipids from Biological Samples—Extract cartridges (Oasis HLB, Hydrophilic-Lipophilic Balance, 30 mg, Waters) were used to extract lipids for MS analyses. The cartridges were first conditioned by rinsing with methanol (1 ml), followed by water (1 ml). Then, the lipids were loaded onto the cartridge and were absorbed to the cartridges by centrifuging at 1,0000 r.p.m. for 6 min. After washing the cartridges with water (1 ml) and then with chloroform (300 μl), the lipids were eluted with methanol (200 μl). To quantify LPM, a known amount of 14:0-LPM, which was not detected *in vivo*, was added to the samples before extracting lipids as an internal standard. As standard LPM was only available for LPM-18:1, we could only quantify LPM-18:1 in this study.

ESI-MS/MS Analysis—LPM was detected as $[M - H]^-$ in the negative ion mode at m/z 449. The only major fragment obtained from 18:1 was fatty acid anions, $RCOO^-$ (m/z 281 for 18:1-LPM) (shown in Supplementary Fig. 2), thus common neutral loss of 168 mass units were used for detecting all acyl LPM species. Neutral loss scanning was performed on a programmable pump model 305 (Gilson; Middleton, WI) equipped with a 20 μl sample loop and a hybrid quadruple and linear ion trap mass spectrometer, Quantum Ultra (Thermo). A total of 20 μl of sample was subjected to each analysis. The solvent system was used at a flow rate of 100 μl/min. Collision energy was 20–30 eV. The mobile phase consisted of methanol-20% aqueous ammonium-water (1000:1:1, pH8.0) containing 0.1% acetic acid. We could only quantify 18:1-LPM because standard LPM is only available for the LPM species. For quantification of LPM (1-oleoyl), standard curve was drawn from the ion intensity of LPM (1-oleoyl), which is normalized, by the ion intensity of LPM (14:0) used as an internal standard. For ionization, air was used as nebulizar gas and electro spray ionization (ESI) methods were applied.

Evaluation of LPM Stability—To evaluate the stability of LPM in comparison to LPA, LPM (1-oleoyl) was incubated with cultured McAh7777 cells. We prepared 20 μM of LPM and LPA in DMEM culture medium containing 0.1% BSA. After the indicated time, aliquots of the cultured medium were examined for LPA or LPM levels. LPA levels were determined by an enzyme-linked fluorometric assay previously established. LPM was quantified as described above.

RESULTS

LPM is a Pan-LPA receptor Agonist—We previously designed and synthesized 2-acyl-LPA analogue T10 (**2**) (Fig. 1), which was found to be a potent LPA_3-selective agonist (*26*). T10 (**2**) has restricted conformational flexibility due to the introduction of a ring structure derived from carbohydrates used as a scaffold (*26*). We further introduced methyl (**3**), dimethyl (**4**), benzyl (**5**) and ethyl (**6**) groups to the phosphate group of T10 (**2**) (Fig. 1), and examined their potency to activate LPA_3. In the intracellular calcium ($[Ca^{2+}]_i$) mobilization assay, only methyl-T10 (**3**) was found to have an activity for LPA_3 (Supplementary Fig. 1). Methyl-T10 (**3**) was found to be a poor agonist for LPA_1 and LPA_2 (data not shown). From these experiments, we attempted to synthesize LPM (**7**, racemic, and examine its potency to activate LPA receptors using $[Ca^{2+}]_i$ mobilization and luciferase assays. As a result, LPM (**7**) was found to activate all LPA receptors (LPA_{1-5}) (Fig. 2). However, LPM (**7**) was about 5–20 times less potent than LPA depending on LPA receptor subtypes and assay systems employed (Fig. 2). LPM (*2R*) (**8**), which has a natural R-configuration, activated LPA_1, LPA_2 and LPA_3 more efficiently than LPM (*2S*) (**9**), indicating the *R* configuration at the *sn*-2 position of LPM is preferred by LPA receptors (Fig. 3). Both LPEt and LPBt were found to be poor agonists against the four LPA receptors tested (LPA_{1-4}) (Fig. 2A–D). It is stressed here that LPA was never detected in our LPM preparation using both colorimetric (*19*) and MS assays (data not shown) indicating that the biological response of LPM is not explained by the presence of trace amount of LPA as an impurity.

Biological Activities of LPM—Stimulation of cell proliferation and cell migration is the most feature of LPA. As shown in Fig. 4, LPM (**7**) stimulated both cell proliferation (McA-Rh7777 rat hepatoma) and cell migration (PC-3 human prostate cancer cells). Consistent with the results of the LPA receptor activation (Fig. 3), LPM (*2S*, **9**) showed a weaker migratory response than LPM (**7**) and LPM (*2R*, **8**) (data not shown). In addition, the cell migration-stimulating effect of LPM was blocked by Ki16425, an LPA_1 antagonist (Fig. 4B). LPEt and LPBt did not show such effects (data not shown).

Similar to LPA (**1**), LPM (**7**, racemic) induced transient hypertension in mice when it is intravenously injected (Fig. 5A). LPM (**7**, racemic) showed similar hypertension-inducing activity (Fig. 5A, B and F). We found that LPM (*2R*, **8**) and LPM (*2S*, **9**) showed a similar vasopressive effect to LPM (*7*), while LPM induced

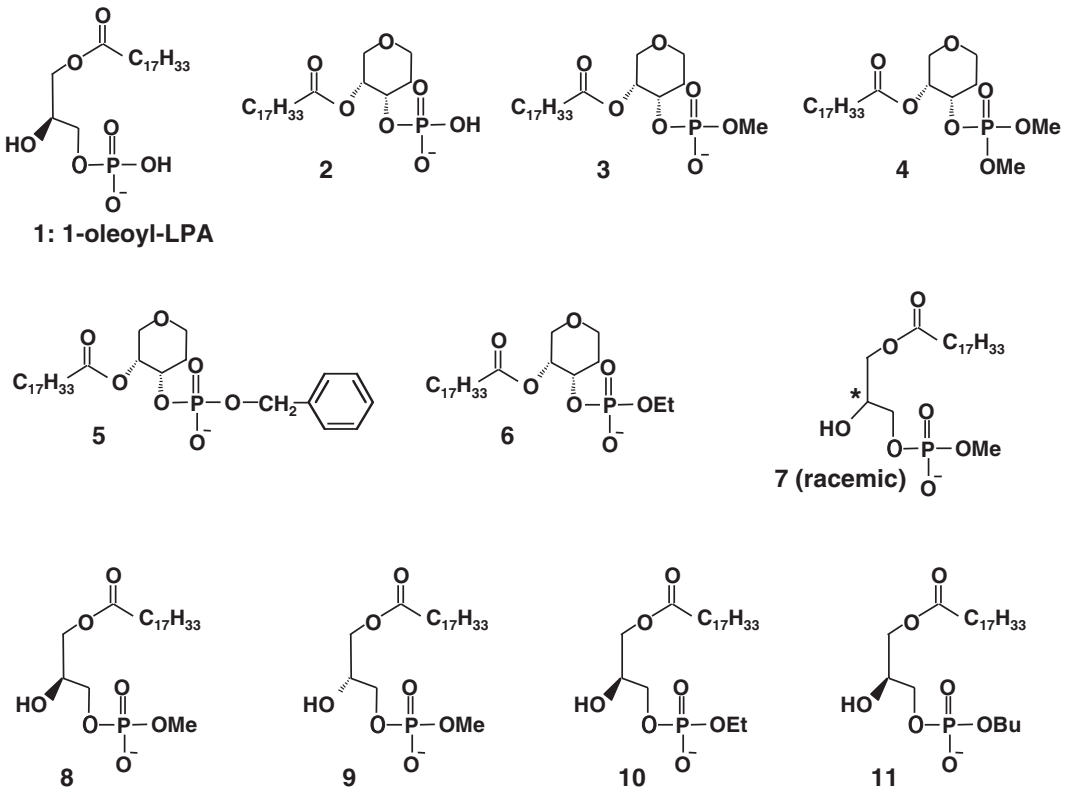

Fig. 1. **Chemical structure of LPA analogues synthesized and characterized in this study.** Structures of various compounds synthesized based on the structure of LPA or LPA_3-selective agonist T10 as a lead compound. Note that LPA analogues synthesized in this study have modified phosphate groups (**3–11**).

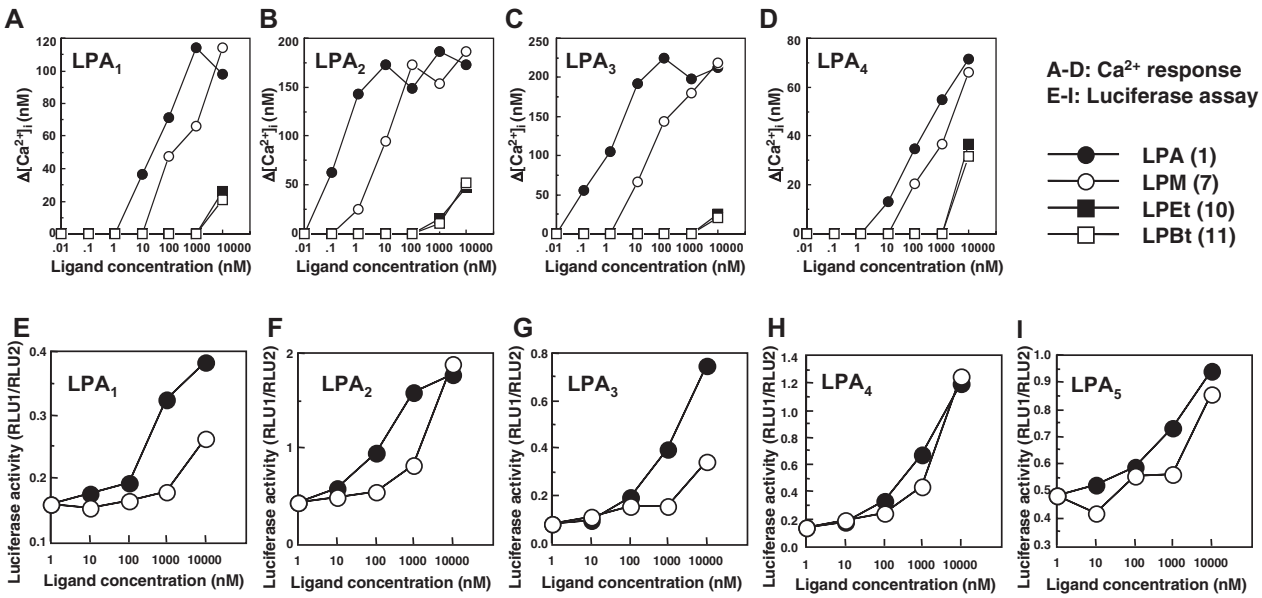

Fig. 2. **LPM is a pan LPA receptor agonist.** (A–D). Ca^{2+} response of HeLa cells expressing LPA_1 (A), LPA_2 (B), LPA_3 (C) or LPA_4 (D) to lysophosphatidyl methanol [LPM (**7**)], lysophosphatidylethanol [LPEt (**10**)], lysophosphatidylbutanol [LPBt (**11**)] and LPA (**1**). HeLa cells were transfected with $LPA_{1–4}$-expressing cDNA, loaded with the fluorescent Ca^{2+} indicator Fura-2 AM and stimulated with each ligand. (E–I) Effect of LPM (**7**), LPEt (**10**), LPBt (**11**) and LPA (**1**) on zif268-directed luciferase activity in PC-12 cells expressing LPA_1 (E), LPA_2 (F), LPA_3 (G), LPA_4 (H) or LPA_5 (I). Filled circles, LPA (**1**); open circles [LPM (**7**)], filled squares [LPEt (**10**)] and open squares [LPBt (**11**)]. Note that we could not detect LPA-dependent Ca^{2+} response in LPA_5-expressing HeLa cells.

a significantly lower response than LPA (Fig. 5A, C, D and F). Both LPEt and LPBt were found to be poor agonists in inducing hypertension in mice (Fig. 5E)

LPM is Produced in Serum—As LPM is a candidate of novel lysophospholipid mediators with LPA-like activities, we tried to detect it in biological samples. We could detect the LPM in mouse serum by MS. Molecular related ion of the standard 18:1-LPM was detected at m/z 499 and only major fragment ion detected from this precursor ion by MS/MS analysis was m/z 281 as indicated in Supplementary Fig. 2. MS/MS spectrum obtained from the peak at m/z 449 detected in the sample was exactly same as that of standard 18:1-LPM. We, thus, employed neutral loss scanning to detect and quantify LPM in biological samples. Indeed, we could detect the LPM in mouse serum by MS. The level, however, was estimated to be <20 nM (data not shown). We found, by chance, that a significant amount of LPM is produced in FCS when it is incubated in the presence of methanol. When FCS was mixed with methanol (25%, v/v) and incubated for 12 or 24 h, LPM was produced in the FCS. An MS analysis detected LPM species with

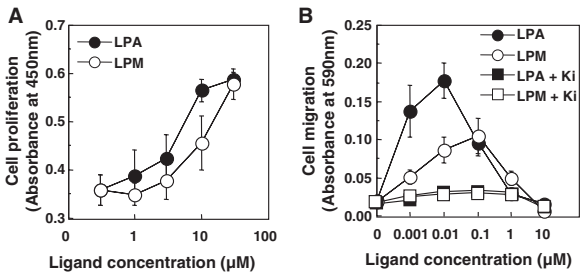

Fig. 4. **LPM stimulates cell proliferation and cell migration.** (A) Serum-starved rat hepatoma McA Rh7777 cells stably expressing LPA_1 were stimulated with indicated concentrations of LPM (open circles) and LPA (filled circles) and cell number was evaluated using an MTT assay. Data represented as mean ± SD. The number of each group is three. (B) Human prostate cancer cells which show LPA_1-dependent LPA-induced cell migration were stimulated with indicated concentrations of LPM (open circles) and LPA (filled circles) in a Boyden-chamber assay. Cells were also stimulated with LPA (filled squares) or LPM (open squares) in the presence of LPA_1 antagonist, Ki16425 (5 μM). Data represented as mean ± SD. The number of each group is three.

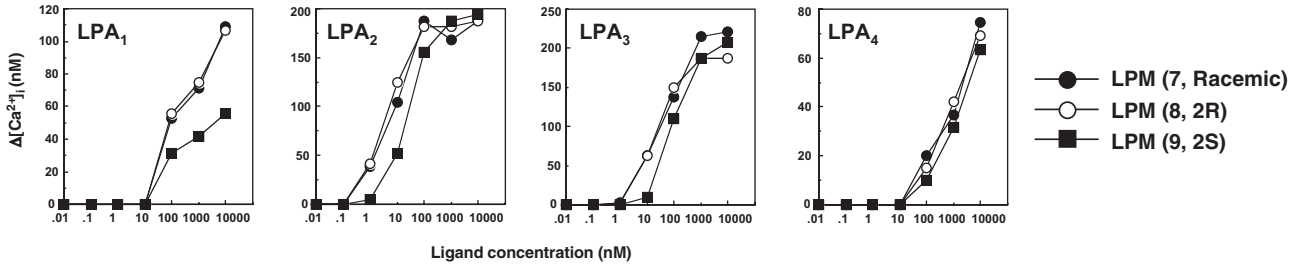

Fig. 3. **LPA receptors prefer LPM (*2R*) to LPM (*2S*).** Ca^{2+} response of HeLa cells expressing LPA_1 (A), LPA_2 (B), LPA_3 (C) or LPA_4 (D) to LPM [racemic, **7**, filled circles)], LPM (*2R*, **8**, open circles) and LPM (*2S*, **9**, filled squares). HeLa cells were transfected with LPA_{1-4}-expressing cDNA, loaded with the fluorescent Ca^{2+} indicator Fura-2 AM and stimulated with each ligand. We have repeated the assay at leased three times for each compound and typical data were shown.

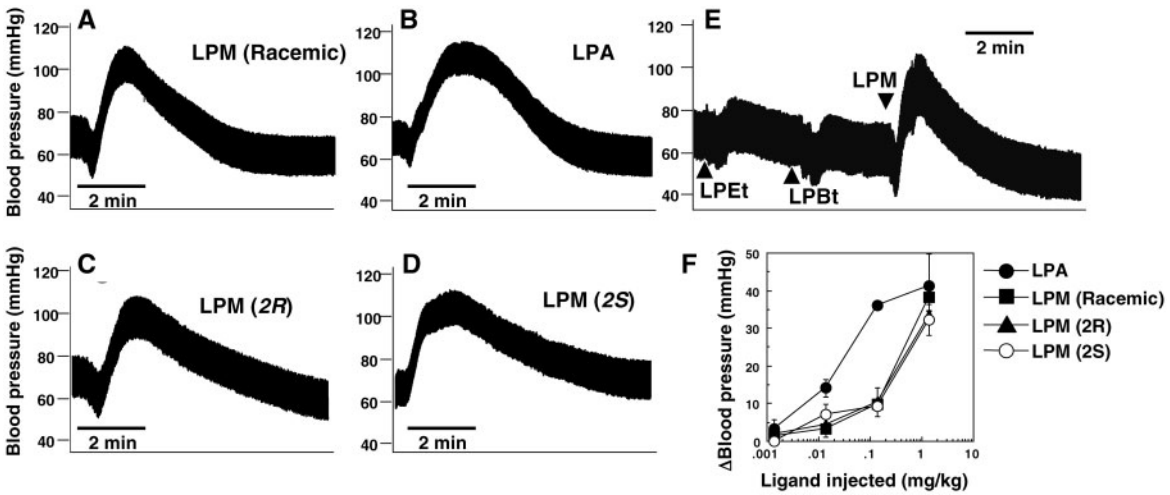

Fig. 5. **LPM induces transient hypertension in mice.** LPM and its related compounds [(A) LPM racemic (7), (B) LPA (**1**), (C) LPM (*2R*) (**8**), (D) LPM (*2S*) (**9**), (E) LPEt (**10**) and LPBt (**11**), each 1.4 mg/kg] were intravenously injected into mice through a polyethylene-tipped cannula. The cannula was also connected to blood pressure measuring equipment. Dose–response curves of each compound are shown in (F). Filled circle, LPA (**1**); filled square, LPM racemic (7); filled triangle, LPM (*2R*) (**8**); and open circle, LPM (*2S*) (**9**). Data represented as mean ± SD. The number of each group is three.

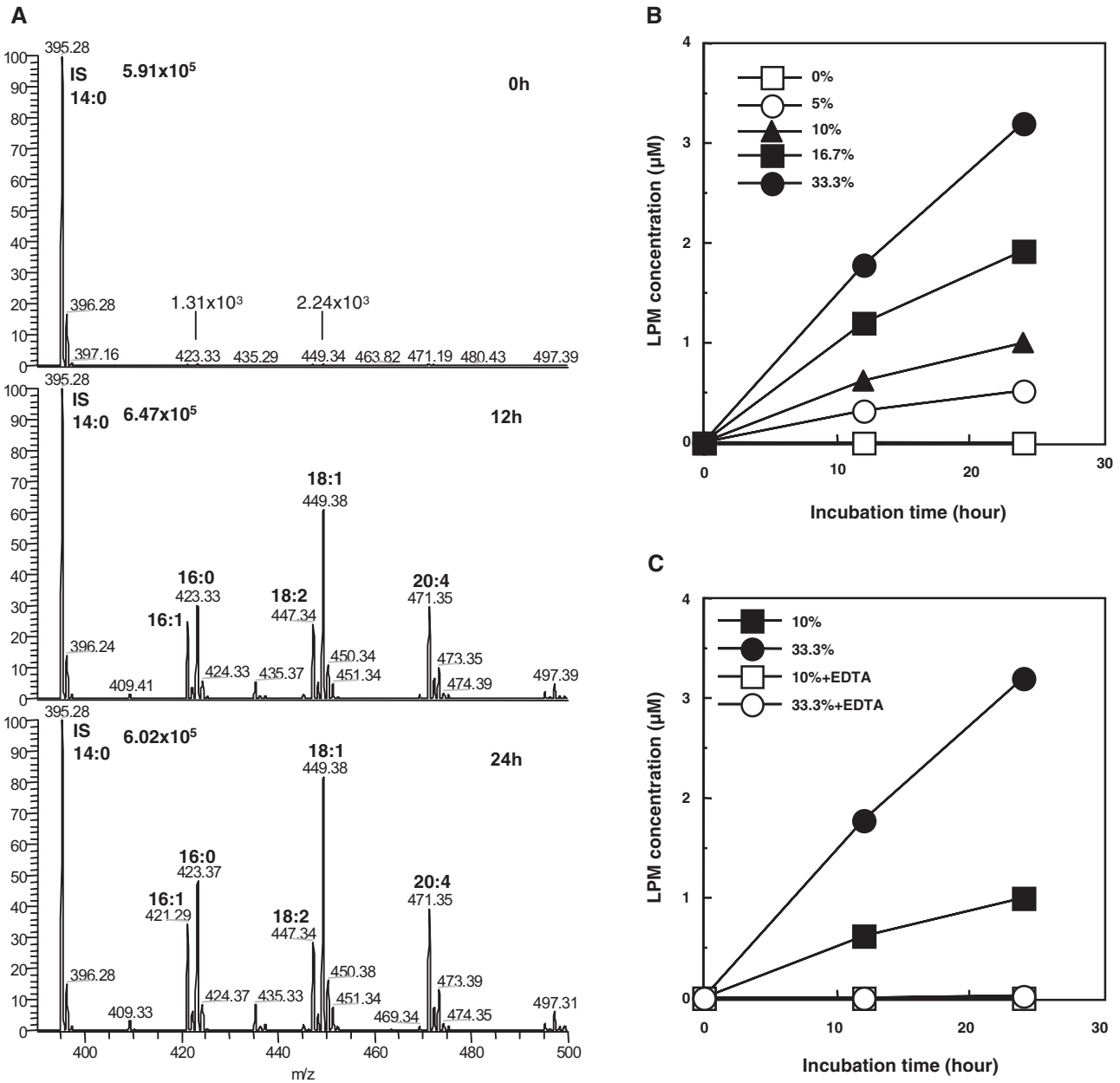

Fig. 6. **Production of LPM in the presence of methanol in incubated fetal calf serum.** (A) FCS was incubated at 37°C in the presence of 33.3% (v/v) of methanol. After 12 and 24 h lipids were extracted and LPM production was examined using mass spectrometry (MS). LPM was detected by neutral loss scanning of 168 Da in the negative ion mode. We added 3 μM of 14:0-LPM to the samples as an internal standard. (B) FCS was incubated at 37°C for 24 h in the presence of indicated concentrations of methanol [5–33.3% (v/v)] and LPM production was examined using MS. Among the LPM species, the amount of 18:1-LPM was quantified using 14:0-LPM as an internal standard and 18:1-LPM as a standard LPM. We have repeated the assay at leased three times for each point and typical data were shown. (C) FCS was incubated with 10% (v/v) or 33.3% (v/v) of methanol at 37°C both in the presence and absence of 10 mM EDTA. After 24 h, lipids were extracted and the production of 18:1-LPM was examined as in (B). We have repeated the assay at leased three times for each point and typical data were shown.

16:0, 16:1, 18:1, 18:2 and 20:4, with an order of abundance of 18:1 > 16:0 = 20:4 > 16:1 > 18:2 (Fig. 6A). Molecular species similar to LPM were also detected when mouse serum was incubated with methanol (data not shown). Using standard LPM (18:1) and LPM (14:0) as an internal standard (IS), we quantified the formation of 18:1-LPM in FCS (Fig. 6B). The production of LPM was dependent on incubation time and concentration of methanol, and nearly 3 μM of 18:1-LPM was produced in 24 h in the presence of 33.3% methanol (Fig. 6B). As standard for each LPM species (16:0, 16:1, 18:2 or 20:4) was not available, and because ionization efficiencies of each LPM species differ, we could not precisely quantify the other species of LPM except for 18:1-LPM. However, we can estimate from the MS profiles that about 10 μM of LPM is produced during 24-h incubation at 37°C. As ATX

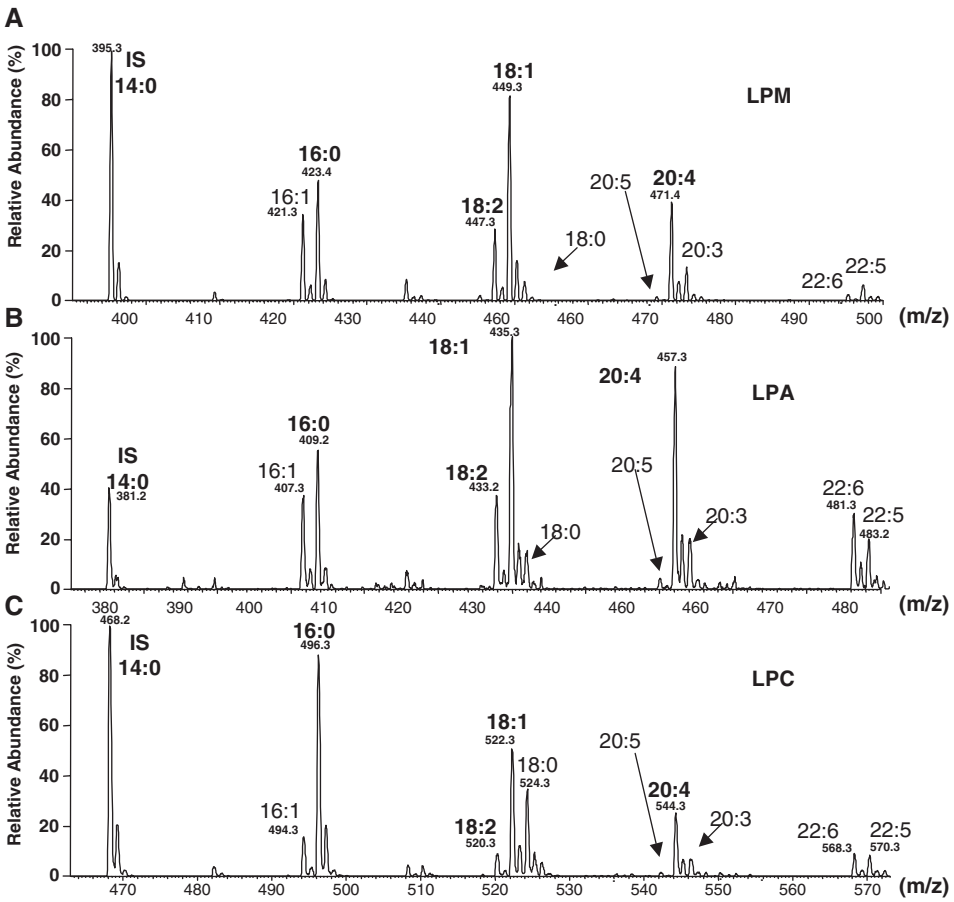

Fig. 7. **Molecular species of LPM, LPA and LPC in incubated FCS.** FCS was incubated at 37°C in the presence of 25% (v/v) methanol. After 24 h, lipids were extracted and molecular species of (A) LPM, (B) LPA and (C) LPC were determined by MS. We added 3 μM of 14:0-LPM, 15 μM of 14:0-LPA and 50 μM of 14:0-LPC to the samples as internal standards.

requires divalent cations for its lysoPLD activity, we tested the effect of EDTA on the production of LPM. As shown in Fig. 6C, the production of LPM was completely inhibited in the presence of EDTA and was not observed when FCS was heat treated (80°C × 30 min, data not shown), implying the involvement of divalent cation-dependent enzymes. We found that the abundance of each molecular species of LPA is quite similar, but not identical, to those of LPM (Fig. 7A and B). For example, the MS profiles of the molecular species of LPM and LPA possessing 16:1, 16:0, 18:1 or 18:2 seem to be quite similar with slight differences in the ratio of polyunsaturated (20:4 and 22:6) species. By contrast, the MS profiles of molecular species of LPC were quite different from those of LPM and LPA (Fig. 7C). Together, these data suggest that LPM is produced by similar mechanism to LPA. We, thus, hypothesized that LPM is generated from LPC by the action of autotaxin (ATX)/lysoPLD, because the enzyme is responsible for almost all LPA production in serum (*35*, *34*).

Autotaxin is Responsible for LPM Production—To test this hypothesis, we incubated ATX-depleted FCS in the presence of methanol. It was found that, in ATX-depleted FCS, the LPM production was completely absent (Fig. 8A and B). Quantification of the formation of 18:1-LPM is shown in Fig. 8C. When recombinant ATX was incubated with LPC in the presence of methanol, a significant amount of LPM was produced (Fig. 8D). ATX seems to utilize a similar catalytic mechanism for LPM production to that for LPA, because an ATX^{T209A} mutant, which lacks the activity to convert LPC to LPA, also failed to produce LPM from LPC (Fig. 8D). In the presence of 25% methanol the formation of LPA decreased by 40% (Fig. 8E). It was also found that the ratio of LPM and LPA produced was 1:10 (Fig. 8D and E). These experiments clearly show that LPM is produced by ATX from LPC in the presence of methanol.

Formation of LPM In Vivo—When methanol (50 μl) was injected intravenously in mice, LPM with 18:2, 18:1, 20:4 and 16:0 was detected in plasma even 8 h after the methanol administration (Fig. 9A). The level of these LPM species further increased when methanol was additionally injected (Fig. 9B). The amount of 18:1-LPM was calculated to be 0.37 μM (8 h) and 1.67 μM (24 h) after methanol administration. From the MS profiles, the total LPM concentration was estimated to be at least ~1 μM at 8 h and ~5 μM at 24 h. At the same time, the LPA level as judged by colorimetric assay were 87 nM (0 h), 91 nM (8 h) and 82 nM (24 h). These analyses clearly show that LPM is present and is

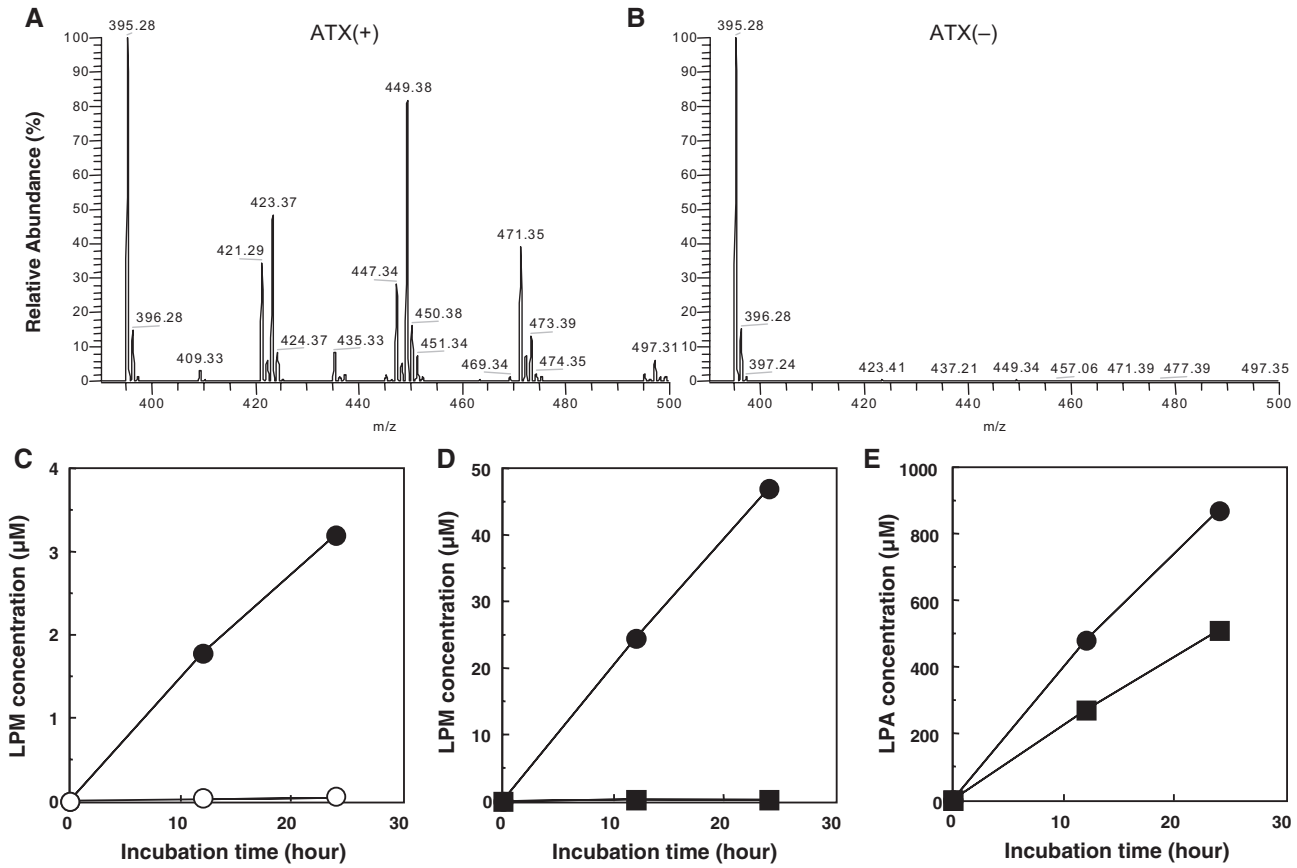

Fig. 8. **ATX is responsible for LPM production in incubated FCS.** (A and B) FCS (A) or ATX-depleted FCS (B) was incubated at 37°C in the presence of 33.3% (v/v) methanol. After 24 h, lipids were extracted and LPM was quantified using was by neutral loss scanning of 168 mass units. (C) FCS (filled circles) or ATX-depleted FCS (open circles) was incubated at 37°C in the presence of 33.3% (v/v) methanol. After 24 h lipids were extracted, amount of 18:1-LPM was quantified using 14:0-LPM as an internal standard and 18:0-LPM as a standard LPM. (D) 18:1-LPC (2 mM) was incubated with recombinant wild-type ATX (filled circles) or catalytically inactive mutant ATX (T209A) (filled squares) in the presence of 33.3% (v/v) methanol. After 24 h, lipids were extracted, and the amount of 18:1-LPM was quantified as in (C). (E) 18:1-LPC (2 mM) was incubated with recombinant wild-type ATX in the presence (filled squares) or absence (filled circles) of 33.3% (v/v) methanol. After 12 and 24 h, production of LPA was determined with a colorimetric assay.

generated in blood when methanol is present. In addition, this demonstrates that LPM is stable *in vivo*.

LPM is More Stable than LPA—To know if LPM is really a stable LPA analogue, we examined the stability of LPM in culture cell system. Figure 10 shows that, following incubation with Rat hepatoma Rh7777 cells for 6 h, <10% of LPA (18:1) remained, while 40% of LPM (18:1) was still present. Thus, LPM is more stable than LPA, possibly by escaping degradation by lipid phosphate phsophatases due to the modification of phosphate group.

DISCUSSION

In this study we showed that LPM, which has an additional methyl group on LPA, retains most of the biological activities of LPA. LPM stimulated cell proliferation (Fig. 4A) and cell migration (Fig. 4B). In addition, LPM induced a hypertension in rodents (Fig. 5). It appears that LPM exerts its LPA-like activities through LPA receptors because LPM behaves as an LPA receptor agonist (LPA_{1-5}) (Fig. 2). Modification of the phosphate group of LPA by an ethyl or butyl residue (*i.e*. LPEt or LPBt), however, did not show such effects (Fig. 2A–D), indicating that introduction of these residues causes steric hindrance. Interestingly, LPA acyltransferase β (LPAATβ) utilizes both LPA and LPM as its donors, although LPAATα discriminates LPA from LPM (*36*). Thus, LPA-targeting molecules recognize LPA in different manners.

In this study we also found that, like classical Phospholipase D (PLD) isozymes (*23*), ATX has transphosphatidyl activity in addition to its lysophospholipase D activity. Classical PLD isozymes prefer ethanol and butanol as their donor. Thus, it is interesting to test which alcohol-like compounds ATX utilizes as a donor in the transphosphatidyl reaction. In addition, it is interesting to know how these two enzymes, classical PLD and lysophospholipase D, exert their transphosphatidyl reaction because the structure and catalytic mechanisms of ATX are quite distinct from those of PLD isozymes (*37*, *38*). We showed clearly that LPM was generated

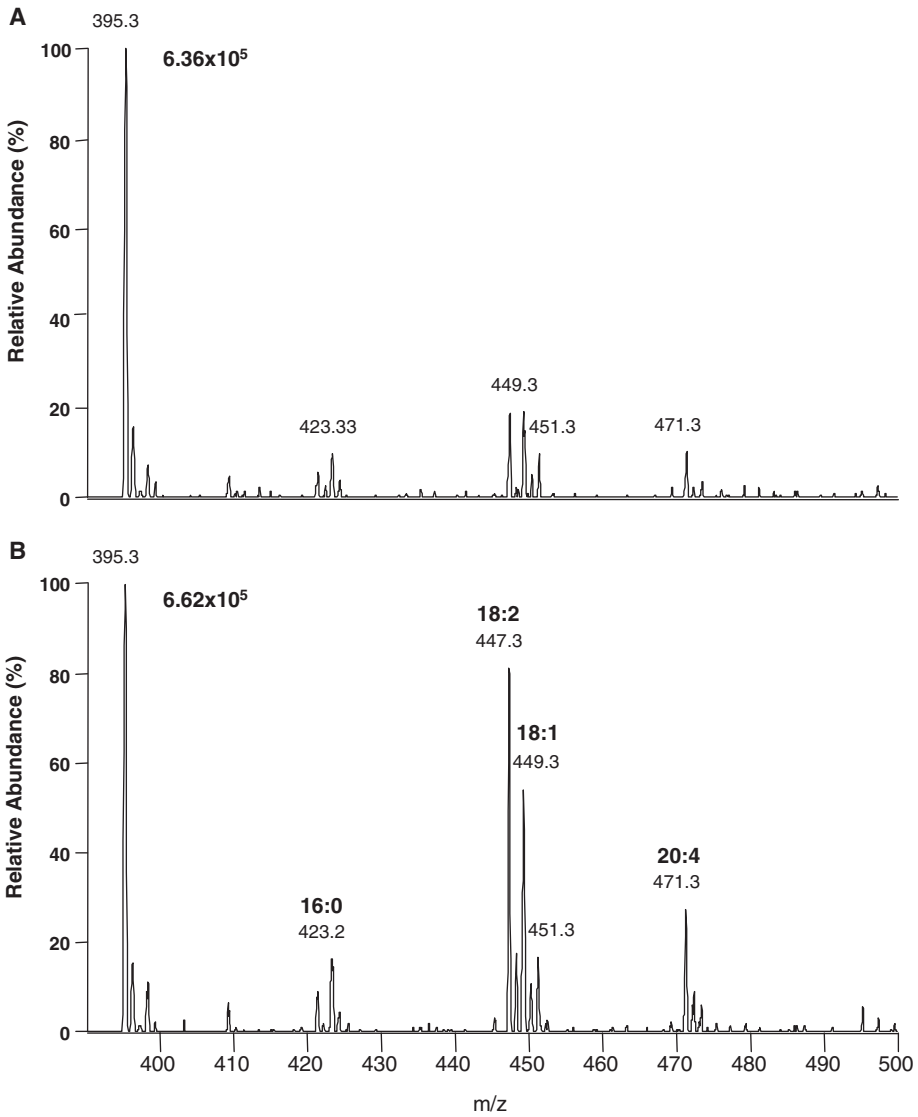

Fig. 9. **LPM production *in vivo*.** Mice were intravenously injected with 50 μl of methanol three times at intervals of 8 h. Eight hours after the first (A) and the third (B) injections, lipids were extracted from the plasma of the mice and LPM was detected by neutral loss scanning of 168 mass units using MS. 14:0-LPM (final 3 μM) was added to the plasma just before the lipid extraction. LPM species with 18:2, 18:1, 20:4 and 16:0 were detected both in the presence and absence of methanol.

in vivo by the action of ATX, which is known to be responsible for LPA production in blood. After administration of methanol *in vivo*, we detected LPM in mouse plasma (Fig. 9). It appears that approximately several micromoles of LPM are present in the plasma of methanol-administered mice. It is now well accepted that the LPA concentration in plasma is kept at a low level (*24*, *35*). By suppressing spontaneous production of LPA due to the ATX activity during the preparation of plasma, Recently, Nakamura *et al*. (*39*) demonstrated clearly that the basal LPA concentration in plasma is <100 nM. Thus, it is likely that the LPM level in plasma is much higher than the LPA level (Fig. 9), because LPM is resistant to degradation by lipid phosphate phosphatases due to its modification of the phosphate group and, thus, is more stable than LPA (Fig. 10).

The physiological significance of LPM is currently unknown. However, considering the high metabolic stability of LPM, it is reasonable to assume that LPM exerts its LPA-like actions *in vivo* when methanol intake occurs. Methanol intake may sometimes occur because denatured ethanol is known to contain methanol (*40*). Methanol is highly toxic. Although the main mechanisms of methanol toxicity seems to be its CNS depressant properties as well as formation of formic acid in liver, the conversion to LPM and the consequent activation of LPA receptors may partly contribute the expression of methanol toxicity *in vivo*.

LPA was first reported to be a vasoactive compound. However, the mechanism how LPA modulates blood pressure remains to be solved. Tokumura *et al*. showed that LPA with unsaturated fatty acid, such as linoleic

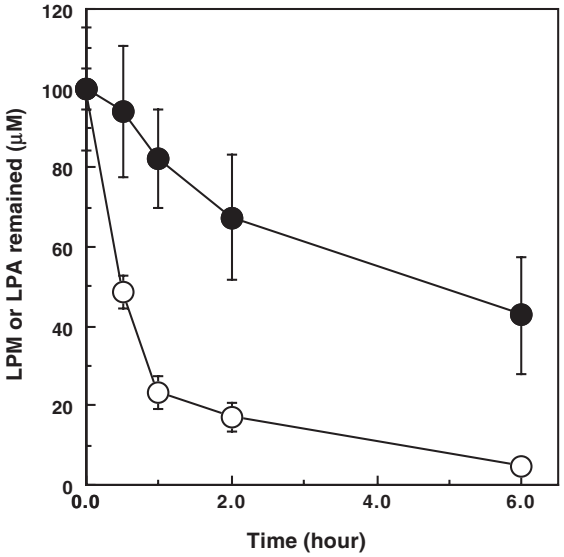

Fig. 10. **Stability of LPM and LPA in culture cells.** Solutions of 20 μM 1-oleoyl-LPM (filled circle) and 20 μM 1-oleoyl-LPA (open circle) were added to rat hepatoma Rh7777 cells. After incubation for the indicated times, aliquots were taken and the concentrations of LPM and LPA were determined either by mass spectrometry (for LPM) or by an enzymatic colorimetric method (for LPA). Each point represents the mean ± SD of three independent experiments.

acid (18:2) and linolenic acid (18:3), was more potent in inducing hypertension in rodent than LPA with saturated fatty acid (*2*, *4*). We showed that LPM (*2R*) and LPM (*2S*) showed almost equal activity in inducing hypertension (Fig. 5), although these enantiomers showed quite different patterns in activating each LPA receptor (LPA_{1-5}) (Fig. 3). Thus, it is strongly suggested that the classical LPA receptors are not involved in the LPA-induced hypertension.

It was recently shown by several investigators that ATX is responsible for bulk LPA production in blood (*34*, *35*), indicating that most of the biological action of ATX *in vivo* is mediated by LPA. However, ATX gives rise to other enzymatic products. *In vitro*, ATX is capable of producing cyclic PA (cPA) from lysophospholipids (*34*) and sphingosine 1-phosphate (S1P) from sphingosylphosphorylcholine (SPC) (*41*). We showed in this study that under certain circumstances, ATX produces LPM. It is possible that, like PLD isozymes, ATX incorporates ethanol and butanol, producing LPEt and LPBt. It is interesting to test if these products have some roles *in vivo* in further studies.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

FUNDING

The Japan Science and Technology Agency (to J.A.); the Ministry of Education, Science and Culture (MEXT) of Japan (to J.A., M.S. and H.A.); the Target Protein Project of MEXT (to J.A.); ONO Science Foundation (to J.A.); and Takeda Science Foundation (to J.A.).

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Moolenaar, W.H., van Meeteren, L. A., and Giepmans, B.N. (2004) The ins and outs of lysophosphatidic acid signaling. *Bioessays* **26**, 870–881
2. Tokumura, A. (1995) A family of phospholipid autacoids: occurrence, metabolism and bioactions. *Prog. Lipid Res.* **34**, 151–184
3. van Meeteren, L. A. and Moolenaar, W.H. (2007) Regulation and biological activities of the autotaxin-LPA axis. *Prog. Lipid Res.* **46**, 145–160
4. Tokumura, A., Fukuzawa, K., and Tsukatani, H. (1978) Effects of synthetic and natural lysophosphatidic acids on the arterial blood pressure of different animal species. *Lipids* **13**, 572–574
5. Anliker, B. and Chun, J. (2004) Lysophospholipid G protein-coupled receptors. *J. Biol. Chem.* **279**, 20555–20558
6. Ishii, I., Fukushima, N., Ye, X., and Chun, J. (2004) Lysophospholipid receptors: signaling and biology. *Annu. Rev. Biochem.* **73**, 321–354
7. Hecht, J.H., Weiner, J.A., Post, S.R., and Chun, J. (1996) Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J. Cell Biol.* **135**, 1071–1083
8. An, S., Bleu, T., Zheng, Y., and Goetzl, E.J. (1998) Recombinant human G protein-coupled lysophosphatidic acid receptors mediate intracellular calcium mobilization. *Mol. Pharmacol.* **54**, 881–888
9. Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami, M.K., Tsujimoto, M., Arai, H., and Inoue, K. (1999) Molecular cloning and characterization of a novel human G-protein- coupled receptor, EDG7, for lysophosphatidic acid. *J. Biol. Chem.* **274**, 27776–27785
10. Noguchi, K., Ishii, S., and Shimizu, T. (2003) Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J. Biol. Chem.* **278**, 25600–25606
11. Lee, C.W., Rivera, R., Gardell, S., Dubin, A.E., and Chun, J. (2006) GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J. Biol. Chem.* **281**, 23589–23597
12. Kotarsky, K., Boketoft, A., Bristulf, J., Nilsson, N.E., Norberg, A., Hansson, S., Owman, C., Sillard, R., Leeb-Lundberg, L.M., and Olde, B. (2006) Lysophosphatidic acid binds to and activates GPR92, a G protein-coupled receptor highly expressed in gastrointestinal lymphocytes. *J. Pharmacol. Exp. Therap.* **318**, 619–628
13. Contos, J.J., Fukushima, N., Weiner, J.A., Kaushal, D., and Chun, J. (2000) Requirement for the lpA1 lysophosphatidic acid receptor gene in normal suckling behavior. *Proc. Natl Acad. Sci USA* **97**, 13384–13389
14. Contos, J.J., Ishii, I., Fukushima, N., Kingsbury, M.A., Ye, X., Kawamura, S., Brown, J.H., and Chun, J. (2002) Characterization of lpa(2) (Edg4) and lpa(1)/lpa(2) (Edg2/Edg4) lysophosphatidic acid receptor knockout mice: signaling deficits without obvious phenotypic abnormality attributable to lpa(2). *Mol. Cell Biol.* **22**, 6921–6929
15. Deng, W., Balazs, L., Wang, D.A., Van Middlesworth, L., Tigyi, G., and Johnson, L.R. (2002) Lysophosphatidic acid protects and rescues intestinal epithelial cells from radiation- and chemotherapy-induced apoptosis. *Gastroenterology* **123**, 206–216
16. Deng, W., Shuyu, E., Tsukahara, R., Valentine, W.J., Durgam, G., Gududuru, V., Balazs, L., Manickam, V., Arsura, M., Van Middlesworth, L., Johnson, L.R., Parrill, A.L., Miller, D.D., and Tigyi, G. (2007)

The lysophosphatidic acid type 2 receptor is required for protection against radiation-induced intestinal injury. *Gastroenterology* **132**, 1834–1851
17. Ye, X., Hama, K., Contos, J.J., Anliker, B., Inoue, A., Skinner, M.K., Suzuki, H., Amano, T., Kennedy, G., Arai, H., Aoki, J., and Chun, J. (2005) LPA3-mediated lysophosphatidic acid signalling in embryo implantation and spacing. *Nature* **435**, 104–108
18. Aoki, J. (2004) Mechanisms of lysophosphatidic acid production. *Semin. Cell Dev. Biol.* **15**, 477–489
19. Aoki, J., Taira, A., Takanezawa, Y., Kishi, Y., Hama, K., Kishimoto, T., Mizuno, K., Saku, K., Taguchi, R., and Arai, H. (2002) Serum lysophosphatidic acid is produced through diverse phospholipase pathways. *J. Biol. Chem.* **277**, 48737–48744
20. Sano, T., Baker, D.L., Virag, T., Wada, A., Yatomi, Y., Kobayashi, T., Igarashi, Y., and Tigyi, G. (2002) Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine-1-phosphate generation in blood. *J. Biol. Chem.* **277**, 21197–21206
21. Umezu-Goto, M., Kishi, Y., Taira, A., Hama, K., Dohmae, N., Takio, K., Yamori, T., Mills, G.B., Inoue, K., Aoki, J., and Arai, H. (2002) Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. *J. Cell Biol.* **158**, 227–233
22. Tokumura, A., Majima, E., Kariya, Y., Tominaga, K., Kogure, K., Yasuda, K., and Fukuzawa, K. (2002) Identification of human plasma lysophospholipase D, a lysophosphatidic acid-producing enzyme, as autotaxin, a multifunctional phosphodiesterase. *J. Biol. Chem.* **277**, 39436–39442
23. Yu, C.H., Liu, S.Y., and Panagia, V. (1996) The transphosphatidylation activity of phospholipase D. *Mol. Cell Biochem.* **157**, 101–105
24. Tsuda, S., Okudaira, S., Moriya-Ito, K., Shimamoto, C., Tanaka, M., Aoki, J., Arai, H., Murakami-Murofushi, K., and Kobayashi, T. (2006) Cyclic phosphatidic acid is produced by autotaxin in blood. *J. Biol. Chem.* **281**, 26081–26088
25. Pyne, S., Long, J.S., Ktistakis, N.T., and Pyne, N.J. (2005) Lipid phosphate phosphatases and lipid phosphate signalling. *Biochem. Soc. Trans.* **33**, 1370–1374
26. Tamaruya, Y., Suzuki, M., Kamura, G., Kanai, M., Hama, K., Shimizu, K., Aoki, J., Arai, H., and Shibasaki, M. (2004) Identifying specific conformations by using a carbohydrate scaffold: discovery of subtype-selective LPA-receptor agonists and an antagonist. *Angew. Chem.* **43**, 2834–2837
27. Hasegawa, Y., Erickson, J.R., Goddard, G.J., Yu, S., Liu, S., Cheng, K.W., Eder, A., Bandoh, K., Aoki, J., Jarosz, R., Schrier, A.D., Lynch, K.R., Mills, G.B., and Fang, X. (2003) Identification of a phosphothionate analogue of lysophosphatidic acid (LPA) as a selective agonist of the LPA3 receptor. *J. Biol. Chem.* **278**, 11962–11969
28. Xu, Y., Aoki, J., Shimizu, K., Umezu-Goto, M., Hama, K., Takanezawa, Y., Yu, S., Mills, G.B., Arai, H., Qian, L., and Prestwich, G.D. (2005) Structure-activity relationships of fluorinated lysophosphatidic acid analogues. *J. Med. Chem.* **48**, 3319–3327
29. Xu, Y., Qian, L., and Prestwich, G.D. (2003) Synthesis of monofluorinated analogues of lysophosphatidic acid. *J. Org. Chem.* **68**, 5320–5330
30. Allende, M.L., Sasaki, T., Kawai, H., Olivera, A., Mi, Y., Van, E., Deckert, G., Hajdu, R., Rosenbach, M., Keohane, C.A., Mandala, S., Spiegel, S., and Proia, R.L. (2004) Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. *J. Biol. Chem.* **279**, 52487–52492
31. Smith, D.J.H., Ogilvie, K.K., and Gillen, M.F. (1980) The methyl group as phosphate protecting group in nucleotide synthesis. *Tetrahedron Lett.* **21**, 861–864
32. Bligh, E.G. and Dyer, W.J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917
33. Hama, K., Aoki, J., Fukaya, M., Kishi, Y., Sakai, T., Suzuki, R., Ohta, H., Yamori, T., Watanabe, M., Chun, J., and Arai, H. (2004) Lysophosphatidic acid and autotaxin stimulate cell motility of neoplastic and non-neoplastic cells through LPA1. *J. Biol. Chem.* **279**, 17634–17639
34. Tanaka, M., Okudaira, S., Kishi, Y., Ohkawa, R., Iseki, S., Ota, M., Noji, S., Yatomi, Y., Aoki, J., and Arai, H. (2006) Autotaxin stabilizes blood vessels and is required for embryonic vasculature by producing lysophosphatidic acid. *J. Biol. Chem.* **281**, 25822–25830
35. Tsuda, S., Okudaira, S., Moriya-Ito, K., Shimamoto, C., Tanaka, M., Aoki, J., Arai, H., Murakami-Murofushi, K., and Kobayashi, T. (2006) Cyclic phosphatidic acid is produced by autotaxin in blood. *J. Biol. Chem.* **281**, 26081–26088
36. Hollenback, D., Bonham, L., Law, L., Rossnagle, E., Romero, L., Carew, H., Tompkins, C.K., Leung, D.W., Singer, J.W., and White, T. (2006) Substrate specificity of lysophosphatidic acid acyltransferase beta – evidence from membrane and whole cell assays. *J. Lipid Res.* **47**, 593–604
37. Murata, J., Lee, H.Y., Clair, T., Krutzsch, H.C., Arestad, A.A., Sobel, M.E., Liotta, L.A., and Stracke, M.L. (1994) cDNA cloning of the human tumor motility-stimulating protein, autotaxin, reveals a homology with phosphodiesterases. *J. Biol. Chem.* **269**, 30479–30484
38. Morris, A.J., Hammond, S.M., Colley, C., Sung, T.C., Jenco, J.M., Sciorra, V.A., Rudge, S.A., and Frohman, M.A. (1997) Regulation and functions of phospholipase D. *Biochem. Soc. Trans.* **25**, 1151–1157
39. Nakamura, K., Kishimoto, T., Ohkawa, R., Okubo, S., Tozuka, M., Yokota, H., Ikeda, H., Ohshima, N., Mizuno, K., and Yatomi, Y. (2007) Suppression of lysophosphatidic acid and lysophosphatidylcholine formation in the plasma in vitro: proposal of a plasma sample preparation method for laboratory testing of these lipids. *Anal. Biochem.* **367**, 20–27
40. Lachenmeier, D.W., Rehm, J., and Gmel, G. (2007) Surrogate alcohol: what do we know and where do we go? *Alcohol. Clin. Exp. Res.* **31**, 1613–1624
41. Clair, T., Aoki, J., Koh, E., Bandle, R.W., Nam, S.W., Ptaszynska, M.M., Mills, G.B., Schiffmann, E., Liotta, L.A., and Stracke, M.L. (2003) Autotaxin hydrolyzes sphingosylphosphorylcholine to produce the regulator of migration, sphingosine-1-phosphate. *Cancer Res.* **63**, 5446–5453