Human platelets respond differentially to lysophosphatidic acids having a highly unsaturated fatty acyl group and alkyl ether-linked lysophosphatidic acids

Akira TOKUMURA*[1], Junya SINOMIYA*, Seishi KISHIMOTO†, Tamotsu TANAKA‡, Kentaro KOGURE*, Takayuki SUGIURA†, Kiyoshi SATOUCHI‡, Keizo WAKU† and Kenji FUKUZAWA*

*Faculty of Pharmaceutical Sciences, The University of Tokushima, 1-78 Shomachi, Tokushima 770-8505, Japan, †Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-0195, Japan, and ‡Department of Applied Biological Science, Fukuyama University, Fukuyama, Hiroshima 729-0292, Japan

Lysophosphatidic acid (LPA) is a physiological agonist that is produced by lysophospholipase D, phospholipase A_1 and phospholipase A_2 in the blood of animals. It exerts diverse biological actions on a broad range of animal cells. Specific receptors for this important agonist have been characterized. In this investigation, for the first time we prepared LPAs having a highly unsaturated fatty acyl group, such as the eicosapentaenoyl or docosahexaenoyl residue, and their acetylated derivatives. Human platelets aggregated more potently in response to the highly unsaturated acyl-LPAs than to LPAs with a C_{18} fatty acyl group, such as an oleoyl group, while alkyl ether-linked LPAs (alkyl-LPA) had much stronger aggregating activity. Two positional isomers of LPAs with an arachidonoyl, eicosapentaenoyl or docosahexaenoyl group had equipotent aggregatory activity as well as the positional isomers of their acetylated analogues, indicating that putative LPA receptors could not distinguish the difference between the positional isomers. We found that platelet preparations from two individuals showed no aggregatory response to alkyl-LPAs, although they contained mRNAs for known LPA receptors in the following order of expression level: endothelial differentiation gene (Edg)-4 > Edg-7 > Edg-2. We also obtained evidence that 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082), a phospholipase A_2 inhibitor, potentiated alkyl-LPA-induced platelet aggregation, but inhibited highly unsaturated acyl-LPA-induced platelet aggregation. These results indicated that human platelets express acyl-LPA-selective and alkyl-LPA-selective receptors on their plasma membrane.

Key words: bovine serum albumin, lysophosphatidylcholine, platelet aggregation, positional isomer.

INTRODUCTION

Much interest has recently become directed to a family of lysolipid intercellular mediators [1–5]. Lysophosphatidic acid (LPA; radyl-*sn*-glycerol-3-phosphate) is the prototype of the lysolipid mediator family, and functions as an agonist in a wide range of animal cells [1–3]. The molecular diversity of LPA is well recognized; it comprises three subclasses (acyl-, alkenyl- and alkyl-LPAs) containing a long hydrophobic chain with different chain lengths [1,2,6]. In addition, the acyl subclass includes two positional isomers having the same fatty acyl group: 1-acyl- and 2-acyl-LPAs.

Acyl-LPAs are widely distributed in rat tissues [7], whereas definitive evidence was given on the occurrence of alkyl-LPA only in rat brain [8] and human ascitic fluid [9], and on that of alkenyl-LPA in human ascitic fluid [9] and rabbit aqueous humour [10]. The potencies of their biological activities were found to depend on the binding mode (subclass), the binding position (positional isomers) and the chain length and the degree of unsaturation (molecular species) of its long hydrophobic chain. In accordance with the molecular heterogeneity of LPA, three different Edg (endothelial differentiation gene) genes, encoding LPA receptors, have been characterized; *Edg-2*, *Edg-4* and *Edg-7* [3,11,12]. They appear to distinguish structural differences among various types of LPA to variable extents. Edg-7 has a unique feature; it responds well to unsaturated acyl-LPAs, but not to saturated acyl-LPAs [12].

There are several studies reporting the occurrence of various molecular species of LPA in tissues [7,8], body fluids [13–18] and platelets [19]. Among the three predominant molecular species of LPA was included a polyunsaturated LPA: linoleoyl (18:2)-LPA in rat liver [7], 18:2-LPA in rat heart [7] and arachidonoyl (20:4)-LPA in thrombin-stimulated platelets [19]. (Esterified fatty acids are indicated by their total numbers of carbons and degrees of unsaturation, e.g. 16:0, 18:0, 18:1, 18:2, 18:3, 20:4, 20:5 and 22:6 for the palmitoyl, stearoyl, oleoyl, linoleoyl, α-linolenoyl, arachidonoyl, eicosapentaenoyl and docosahexaenoyl group, respectively. The fatty alcohol groups, hexadecyl and octadecyl, attached to the glycerol backbone of lysophosphatidic acid are represented as 16:0-alkyl and 18:0-alkyl, respectively.) Furthermore, LPA accumulated in rat plasma during its incubation at 37 °C for 24–48 h was enriched with polyunsaturated molecular species of LPA such as 18:2- and 20:4-LPA, since lysophospholipase D was found to have substrate specificity with preference for unsaturated lysophosphatidylcholines (LPCs) over saturated LPCs [13,15]. A noteworthy finding was the co-existence in human and rat plasma of 1-acyl isomers of LPCs having 18:2, 20:4 and docosahexaenoyl (22:6) groups with their 2-acyl isomers [16,20], both of which could be the substrates of plasma lysophospholipase D [16].

Abbreviations used: Edg, endothelial differentiation gene; FAB-MS, fast atom bombardment-MS; GP, *sn*-glycerol-3-phosphate; GPC, *sn*-glycero-3-phosphocholine; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; ONO-RS-082, 2-(*p*-amylcinnamoyl)amino-4-chlorobenzoic acid; PC, phosphatidylcholine; tBDMS, t-butyldimethylsilyl; RT, reverse transcriptase; TMS, trimethylsilyl; PAF, platelet-activating factor.

[1] To whom correspondence should be addressed (e-mail tokumura@ph.tokushima-u.ac.jp).

To our knowledge, there have been only three studies showing that some types of cell are able to respond to LPA having an arachidonoyl group (20:4-LPA). The first was our work on the *in vivo* hypertensive effect on rats of its intravenous injection [21]. Second, Jalink et al. [22] reported that 20:4-LPA increased intracellular Ca^{2+} mobilization in A231 human colonic cancer cells. Third, Bandoh et al. [23] reported interesting results concerning Ca^{2+} mobilization induced by 20:4-LPAs (1-acyl and 2-acyl isomers) in Sf9 insect cells expressing Edg-2, Edg-4 or Edg-7. However, we know of no report on the biological activity of LPA containing eicosapentaenoate (20:5) or 22:6. From the viewpoint of the physiological and pathophysiological significances of the balance of phospholipids having an $n-6$ or $n-3$ polyunsaturated fatty acyl group [24], comparison of the biological activities of LPAs having an $n-6$ or $n-3$ highly polyunsaturated fatty acyl group seems informative. In this study, therefore, we first attempted to prepare LPAs having the highly unsaturated fatty acyl group at either the *sn*-1 or *sn*-2 position. Next we examined aggregatory responses of human platelets to the highly unsaturated LPAs, because previous studies suggested that they might be exposed to the highly unsaturated LPAs generated in the blood circulation *in vivo* [13,15,16,19,25]. During the course of this study, we obtained results indicating that two different receptors for alkyl-LPA and acyl-LPA exist on the surface of human platelets, and that the putative receptor for alkyl-LPA is distinct from known Edg-type LPA receptors.

MATERIALS AND METHODS

Materials

1,2-Diarachidonoyl-*sn*-glycero-3-phosphocholine (GPC) and 1,2-didocosahexaenoyl-GPC were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.). LPAs such as 1-palmitoyl- (16:0-), stearoyl- (18:0-), 18:2- and α-linolenoyl- (18:3-) *sn*-glycerol-3-phosphates (GPs) were prepared as described previously [26]. LPAs with an *sn*-1-*O*-hexadecyl or octadecyl group (16:0- and 18:0-alkyl-LPAs) were also prepared in our laboratory as described earlier [27]. Docosahexaenoic acid was purchased from Doosan Serdary Research Laboratories (London, ON, Canada). 1-Palmitoyl-GPC (16:0-LPC), 1-oleoyl-GP (18:1-LPA), 2-(*p*-toluidino)naphthalenesulphonic acid, phospholipase A_2 from bee venom, phospholipase C from *Bacillus cereus*, phospholipase D from *Streptomyces chromofuscus*, fatty acid-free BSA, ADP and fibrinogen were products of Sigma (St. Louis, MO, U.S.A.). Trimethylsilyldiazomethane and t-butyldimethylchlorosilane were from Tokyo Chemical Industry (Tokyo, Japan). *N*-Palmitoyl-L-serine phosphate was prepared as described by Sugiura et al. [28]. 2-(*p*-Amylcinnamoyl)amino-4-chlorobenzoic acid (ONO-RS-082), a phospholipase A_2 inhibitor [29], was a product of Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.).

Preparation of 1-highly unsaturated acyl-2-lyso-GP and 1-highly unsaturated acyl-2-acetyl-GP

Phosphatidylcholines (PCs) having the same two highly unsaturated fatty acyl groups were prepared by reaction of the GPC–cadmium complex with arachidonic, eicosapentaenoic and docosahexaenoic anhydrides, as described previously [30]. The fatty acid anhydrides were synthesized using dicyclohexylcarbodi-imide as a catalyst [31]. The PCs with two polyunsaturated acyl groups were then hydrolysed with phospholipase A_2 from bee venom (10-fold higher activity) in Tris/HCl buffer (pH 8.9) containing 50 mM $CaCl_2$ in the presence of ethyl ether, essentially as described previously [32]. The reaction mixture was vigorously stirred at 37 °C overnight. Lipids were extracted by the method of Bligh and Dyer [33] after adjusting the pH of the aqueous phase to 2.5. The LPCs were purified by TLC on a Merck silica gel 60 plate developed with a solvent system of chloroform/methanol/water (13:7:1, by vol.). Portions of LPCs were immediately acetylated with acetic anhydride in the presence of pyridine, as described previously [16], and subjected to experiments for measurement of the ratio of their positional isomers, as described below. LPCs (mainly 1-acyl isomers) or their acetylated derivatives dispersed in Tris/HCl buffer (pH 7.4) were hydrolysed with phospholipase D from *S. chromofuscus* (10 equivalent activity) at 37 °C for 1 h under vigorous stirring, as described previously [26]. The resultant LPAs or their acetylated derivatives were extracted from the reaction mixture by the method of Bligh and Dyer [33] after acidification of the aqueous phase (pH 2.5), and purified by TLC on silica gel 60 plates in a solvent system of chloroform/methanol/water (13:7:1, by vol.).

Preparation of 1-lyso-2-highly unsaturated acyl-GP

PC from beef heart was treated with alkali, and the resultant LPCs having an *sn*-1-*O*-alkyl or alkenyl group were recovered and purified as described previously [30]. PCs having an *sn*-2-highly unsaturated fatty acyl group were prepared by treating the alkenyl- and alkyl-LPCs with arachidonic, eicosapentaenoic and docosahexaenoic anhydrides for 14 h at 80 °C. The PCs were then hydrolysed with phospholipase D from *S. chromofuscus* essentially as described previously [26]. The phosphatidic acids were purified by TLC on Merck silica gel 60 plates developed with a solvent system of chloroform/methanol/water (13:7:1, by vol.). The purified phosphatidic acids were treated with 2.5 M HCl, and LPAs with an *sn*-2-highly unsaturated fatty acyl group were immediately extracted from the reaction mixture by the method of Bligh and Dyer [33], and purified by TLC, as described above.

Preparation of 1-acetyl-2-highly unsaturated acyl-GP

1-Lyso-2-20:4-, 20:5- or 22:6-GPC was prepared from beef heart choline phosphoglycerides as described in [30]. In brief, PCs with an *sn*-1-alkenyl or alkyl and an *sn*-2-highly unsaturated acyl group were dispersed in a mixture of 0.8 ml of 2.5 M HCl, 2 ml of methanol and 1 ml of chloroform for 10 min at room temperature with vigorous stirring. Resultant LPCs having an *sn*-2-highly unsaturated fatty acyl group were extracted from the reaction mixtures by the method of Bligh and Dyer [33] after addition of 1 ml each of chloroform and distilled water. Immediately after their recovery, the 2-acyl-LPCs were acetylated by incubation with 0.2 ml each of acetic anhydride and pyridine at 35 °C for 12 h. The acetylated products were extracted by the method of Bligh and Dyer [33] after addition of 1 ml of distilled water, and purified by TLC on silica gel 60 plates developed with the chloroform/methanol/water solvent system (13:7:1, by vol.). Proportions of the acetylated LPCs were analysed for measurement of the ratio of their positional isomers, as described below. Finally, 1-acetyl-2-acyl-GPCs were hydrolysed to 1-acetyl-2-acyl-GPs with *S. chromofuscus* phospholipase D, as described for preparation of 1-acyl-2-acetyl-GPs.

Fast atom bombardment-MS (FAB-MS) of LPAs

Aliquots of 1 μl of solutions of LPAs or their related phospholipids in a mixture of chloroform/methanol (1:2, v/v) were mixed with 1 μl of magic bullet/thioglycerol (1:2, v/v) on the insertion probe of a JEOL JMS-SX 102A mass spectrometer. The magic bullet was comprised of dithiothreitol and dithio-

erythritol (3:1, v/v). Negative-ion mass spectra were measured with Xe gas under the following conditions: 3 kV of gun high voltage, 1 A filament current and 5 mA emission current.

GC-MS of volatile derivatives of LPAs

Highly unsaturated acyl-LPAs (50 μg) were dissolved in 0.5 ml of chloroform/methanol mixture (2:1, v/v), and the solutions were mixed with 10 μl of trimethylsilyldiazomethane for 5 min at room temperature. The solutions were immediately dried under a stream of N_2 gas. The major reaction product that migrated with an R_F value of 0.57 on a Merck silica gel 60 plate developed with chloroform/methanol (10:1, v/v) was recovered from the silica by the method of Bligh and Dyer [33]. The hydroxyl groups at the *sn*-2-position of the glycerol backbone of the dimethylated LPAs were then modified to trimethylsilyl (TMS) ether by heating at 60 °C in 0.1 ml of a solution of pyridine and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 0.2 % trimethylchlorosilane (1:1, v/v), essentially as described previously [34]. Alternatively, the hydroxyl group was masked with a t-butyldimethylsilyl (tBDMS) group by heating the LPAs in 0.2 ml of dimethylformamide containing 40 mg of imidazole and 143 mg of tBDMS chloride at 60 °C for 1 h. The volatile derivatives of LPAs were analysed by GC-MS in a JEOL JMS-AM150 mass spectrometer coupled with a gas chromatograph equipped with a DB-1 fused column (30 m × 0.25 mm inner diameter, 0.25 μm thickness; J & W Scientific, Folsom, CA, U.S.A.). The column temperature was increased from 200 to 320 °C at a rate of 10 °C/min. Electron-impact ionization mass spectra were measured under the following conditions: 70 eV ionizing voltage, 3 kV accelerating voltage, 300 μA ionizing current and 280 °C ion source temperature.

Analysis of positional isomers of LPC

Ratios of positional isomers of LPCs were determined by GC-MS after the acetylation of their hydroxyl group at the *sn*-1- or 2-position, hydrolysis with *B. cereus* phospholipase C to acylacetylglycerols and conversion into volatile tBDMS ether derivatives, as described previously [16].

Determination of lipid phosphate

Lipid phosphate was determined by a modified Malachite Green method, as described by Chalvardjian and Rudnicki [35].

Measurement of platelet aggregation

Blood (160–190 ml) was withdrawn into a plastic bag containing 20 ml of citrate/phosphate/dextrose solution (102 mM sodium citrate, 17 mM citrate, 129 mM dextrose and 16 mM NaH_2PO_4) from an antecubital vein of healthy volunteers of both sexes aged 21–33 years, who had not taken any drug for at least 2 weeks. Approx. 13 ml aliquots of anticoagulated blood were transferred to plastic tubes, and centrifuged at 830 ***g*** for 10 min at room temperature. Platelet-rich plasma was recovered, and platelets were isolated essentially as described in [36]. In brief, platelets were separated from plasma components by centrifugation at 1000 ***g*** for 15 min, and resultant pellets were washed twice with Tyrode-Hepes buffer with no Ca^{2+} (pH 6.5; 137 mM NaCl, 2.6 mM KCl, 12.0 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 1.0 mM $MgCl_2$, 5.0 mM Hepes and 0.56 mM glucose) containing 10 μM adenosine.

Aliquots (0.1 ml) of platelet suspension [$(1.2–1.3) \times 10^9$ platelets/ml] in Tyrode-Hepes buffer with no Ca^{2+} (pH 6.5) were diluted in 0.4 ml of Tyrode-Hepes buffer with 1.33 mM Ca^{2+} (pH 7.4). The mixtures were warmed to 37 °C for 2 min and then ADP and fibrinogen were added at final concentrations of 10 μM and 0.2 mg/ml, respectively, and platelet aggregation was monitored continuously for 5 min under 1100 rev./min in an automatic aggrecorder PA-3210 (Kyoto Daiichi Chemical, Kyoto, Japan). Samples of LPA and its related lipids were dissolved in saline containing 0.1 % BSA, and aliquots of 5 μl were added to 0.5 ml of platelet suspension. Changes in light transmission were recorded in platelet samples (0 %) and a blank sample without platelets (100 %).

Detection of mRNAs encoding Edg-2, Edg-4 and Edg-7 by reverse transcriptase (RT)-PCR

For detection of mRNAs encoding Edg-2, Edg-4 and Edg-7, platelets were prepared from samples of anticoagulated blood (about 200 ml each) from four volunteers including a person whose platelets were sensitive to acyl-LPA, but not to alkyl-LPA. To minimize contamination of erythrocytes and leucocytes, the isolation procedures for washed platelets were modified as follows. Half the platelet-rich plasma was withdrawn for the second centrifugation. Then, the platelet-rich plasma was centrifuged twice, and four-fifths of the supernatant was withdrawn for the next centrifugation to reduce the numbers of contaminating erythrocytes and leucocytes. Finally, one more washing of the isolated platelets was added to the procedure described previously [36].

The washed platelets were suspended in 2–2.5 ml of Tyrode-Hepes buffer without Ca^{2+} (pH 6.5) at a cell density of 5×10^8 cells/ml. The final platelet suspension (essentially no erythrocytes and leucocytes measured in a Coulter counter) was mixed with a 3-fold volume of ISOGEN-LS (Nippon Gene Co., Tokyo, Japan). Total RNA was isolated from the platelet lysate according to the instructions for ISOGEN-LS. Neutrophils were isolated from peripheral blood of a healthy volunteer using dextran sedimentation and Percoll density-gradient centrifugation [37]. Total RNA was isolated from mixtures of washed human neutrophils with ISOGEN-LS.

Syntheses of cDNA from the total RNA (0.5 μg each) were performed at 42 °C for 60 min in a 10 μl reaction mixture containing 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM $MgCl_2$, 0.75 μM dNTPs, 10 μg/ml oligo-dT_{18} primer and 100 units of Superscript II RT (Invitrogen, Carlsbad, CA, U.S.A.). After inactivation of the enzyme by heating at 98 °C for 5 min, 0.4 μl of the reaction mixture was used for PCR. The sequences of PCR primer sets were: 5′-CAATCGAGAGGCACATTACGGT-3′ (sense) and 5′-GATGTGAGCATAGAGAACCACC-3′ (antisense) for Edg-2 (257 bp); 5′-AGACTGTTGTCATCATCCTGGG-3′ (sense) and 5′-AAGGGTGGAGTCATCAGTGGGT-3′ (antisense) for Edg-4 (331 bp); 5′-CTGCTCATTTTGCTTGTCTGGG-3′ (sense) and 5′-CCACAACCATGATGAGGAAGGC-5′ (antisense) for Edg-7 (175 bp); 5′-ATCCTCTTCTGCAACCTGGTCA-3′ (sense) and 5′-GGTCTAAGACACAGTTGGTGCT-3′ (antisense) for platelet-activating factor (PAF) receptor (265 bp); and 5′-CAGAGCAAGAGAGGCATCCT-3′ (sense) and 5′-AGGATCTTCATGAGGTAGTC-3′ (antisense) for β-actin (404 bp). Amplification of each gene was conducted with 30, 35 and 40 cycles, consisting of 1 min denaturation at 94 °C, 1 min annealing (54, 58, 57, 55 and 55 °C for Edg-2, Edg-4, Edg-7, PAF receptor and β-actin, respectively) and 1 min elongation at 72 °C in a 20 μl reaction mixture [66.7 mM Tris/HCl (pH 8.8), 16.7 mM $(NH_4)_2SO_4$, 6.67 mM $MgCl_2$, 10 μM 2-mercaptoethanol, 6.67 μM EDTA, 167 μg/ml BSA, 1 μM sense and antisense primers, 15 mM dNTP, 1 unit of *Taq* polymerase (Takara Shuzo, Kyoto, Japan) and 0.4 μl of RT reaction mixture]. After amplification,

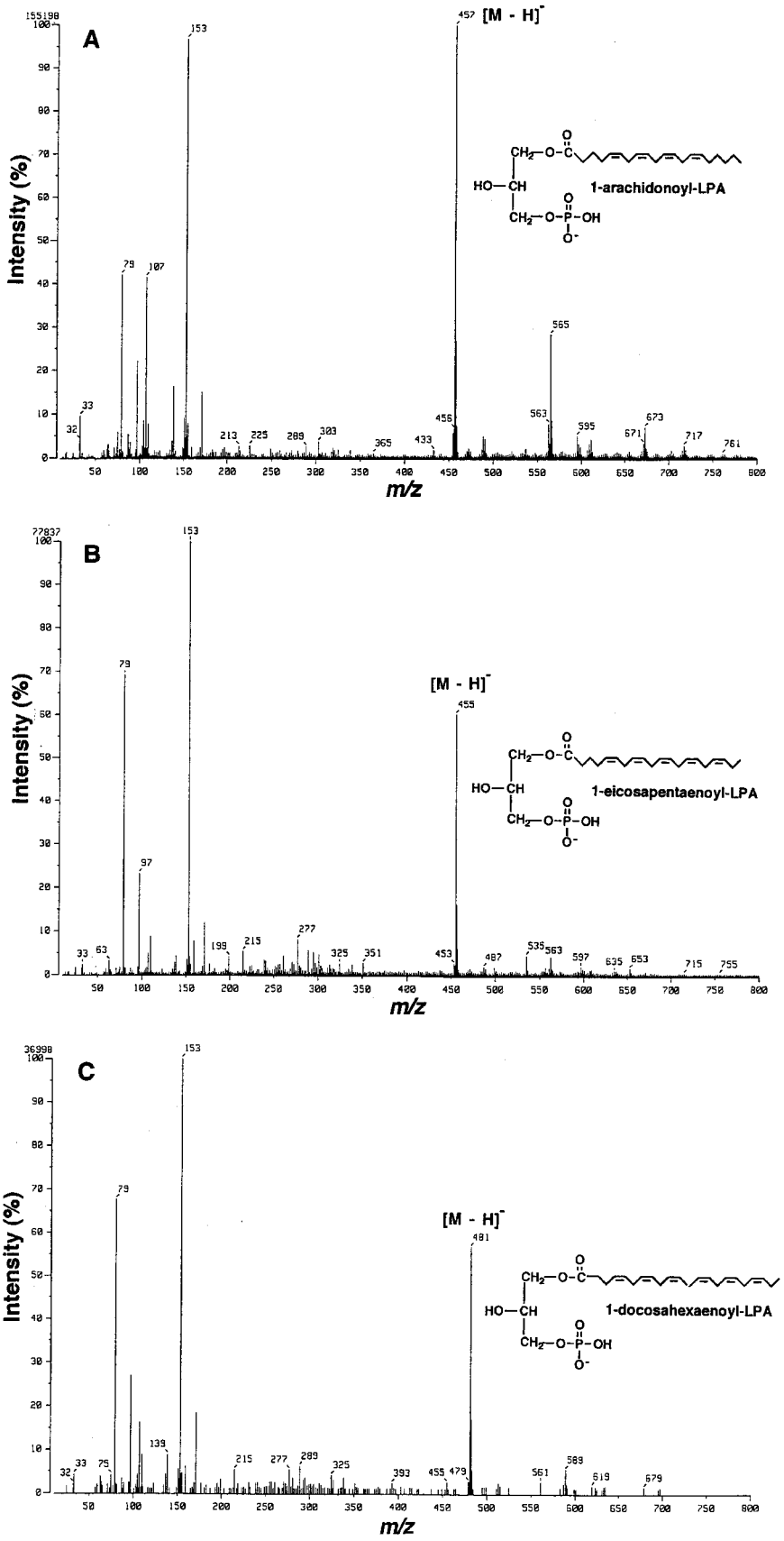

Figure 1 FAB-MS of LPAs with an *sn*-1-highly unsaturated acyl group

(**A**) 20:4-LPA, (**B**) 20:5-LPA and (**C**) 22:6-LPA.

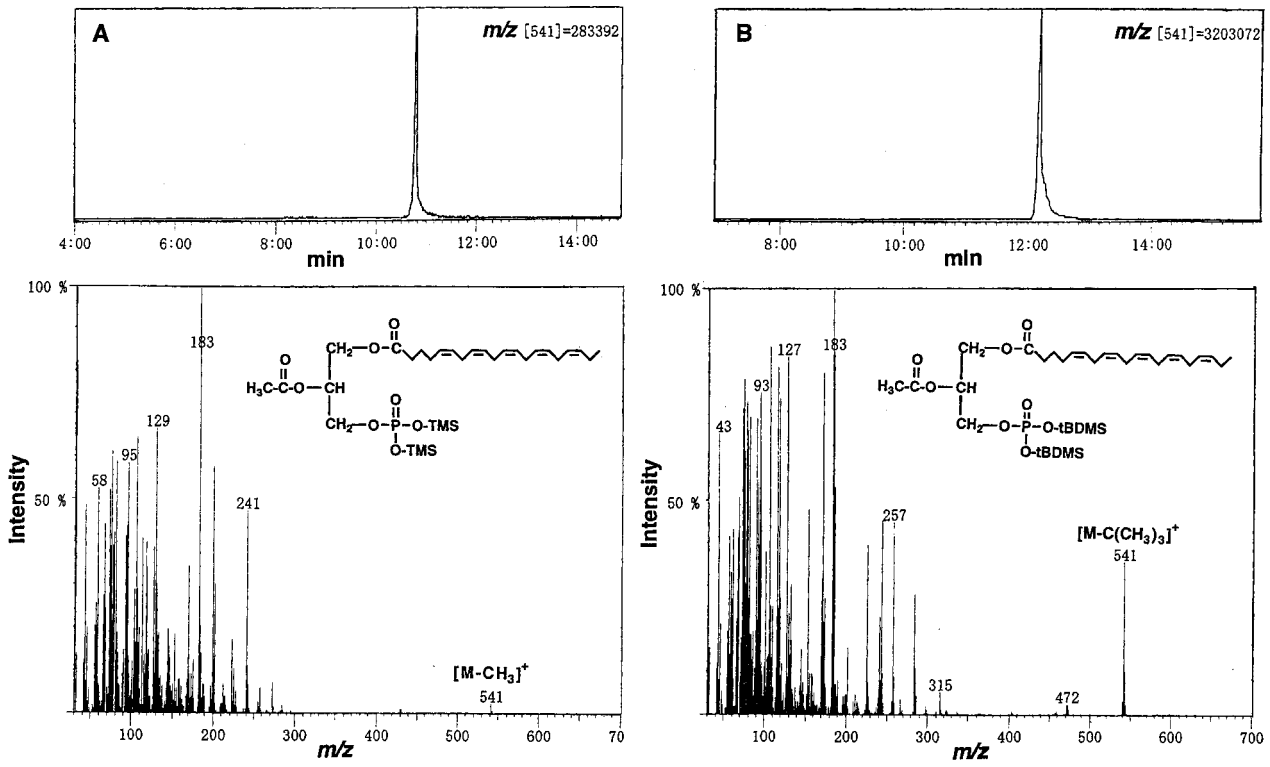

Figure 2 GC-MS of TMS (A) and tBDMS (B) ether derivatives of 1-20:5-LPA

Electron-impact ionization mass spectra were measured at tips of peaks in ion chromatograms of $[M-CH_3]^+$ (**A**) and $[M-C(CH_3)_3]^+$ (**B**) monitoring *m/z* 541.

10 μl aliquots of the reaction mixture were electrophoresed on a 2% agarose gel, and the gel was stained with ethidium bromide and photographed under a UV lamp.

Statistics

Values are means ± S.E.M. Differences between means were analysed by Student's *t* test, and $P < 0.05$ was considered significant.

RESULTS

Preparation of highly unsaturated LPAs and acetylated analogues

1-Acyl-LPAs having 20:4, 20:5 and 22:6 groups were prepared from PCs with the same highly unsaturated fatty acyl groups in yields of 36.3, 15.2 and 36.6% when treated by two hydrolytic procedures with bee venom phospholipase A_2 and *S. chromofuscus* phospholipase D, as described in the Materials and methods section. Their structural analyses were first carried out by FAB-MS in a negative-ion mode. The base ion was observed at *m/z* 153 [glycerol-3-phosphate $-H_2O-H]^-$ in the mass spectra of 20:4-, 20:5- and 22:6-LPAs, as shown in Figure 1. For 20:4-LPA, the diagnostic ions observed at *m/z* 457 and 565 were assigned to $[M-H]^-$ and [*M* + thioglycerol $-H]^-$, respectively, as well as fragment ions at *m/z* 97 ([orthophosphate-H]$^-$) and 79 ([orthophosphate-H_2O-H]$^-$). Similar mass spectrometric results were obtained for 20:5- and 22:6-LPAs; the deprotonated molecular ion and its adduct ion with thioglycerol were shifted to ion peaks at *m/z* 455 and 563 for 20:5-LPA (Figure 1B) and those at *m/z* 481 and 589 for 22:6 (Figure 1C).

Our second approach to the structural analysis of highly unsaturated LPAs was GC-MS of their volatile derivatives. The phosphate moiety of LPAs was methylated with trimethyldiazomethane, and the dimethyl esters of LPAs were then converted into TMS or tBDMS ether derivatives. Typical results on TMS and tBDMS ether derivatives of 20:5-LPA are shown in Figure 2. When the ion at *m/z* 541 was monitored, an intense ion peak was seen at 10.8 min. This peak would be due to $[M-CH_3]^+$ of TMS-20-5-LPA. On electron-impact ionization of the mass spectrum measured at the tip of the peak, the base peak at *m/z* 183 $[OP(OCH_3)_2+OSi(CH_3)_2]^+$ was seen besides $[M-CH_3]^+$ at *m/z* 541 (Figure 2A). Other diagnostic ions were $[M-CH_3-RCOO+H]^+$ (*m/z* 241) and $[M-CH_3-RCO+H]^+$ (*m/z* 257), where RCOOH was 20:5. The tBDMS ether derivative of 20:5-LPA eluted at a slower rate than that of the TMS derivative. The mass spectrum measured at the tip (12.2 min) of the peak monitored with *m/z* 541 $\{[M-C(CH_3)_3]^+\}$ is shown in Figure 2(B). The ion profile of the mass spectrum was analogous with that of the TMS ether derivative of 20:5-LPA, although the relative intensity of the ion at *m/z* 541 in the mass spectrum of the tBDMS ether derivative was higher than that observed for the TMS ether derivative.

Platelet-aggregating activities of 1-highly unsaturated acyl-LPAs

Responses of washed human platelets to LPAs were stable, when the platelets were incubated with a threshold concentration of ADP (10 μM) in the presence of fibrinogen and Ca^{2+}, as previously reported [28,36]. The potencies of aggregating activities of 1-20:4-, 1-20:5- and 1-22:6-LPAs towards washed human platelets were compared with those of other 1-acyl- and 1-*O*-alkyl-LPAs. All LPAs were found to induce platelet aggregation in a concentration-dependent manner at submicromolar concentrations in our assay medium including ADP and

Table 1 Platelet-aggregating activities of different molecular species of acyl- and alkyl-LPA

EC_{50} values were calculated from concentration-response curves constructed with at least six different concentrations of LPA, and are shown as means ± S.E.M. Maximal aggregation values are means ± S.E.M.

LPA	*n*	EC_{50} (nM)	Relative activity	Maximal aggregation (%)
Alkyl-LPA				
16:0	5	8.5 ± 2.3	100	67 ± 6.0
18:0	4	9.0 ± 1.7	94	67 ± 7.8
Acyl-LPA				
16:0	3	36 ± 6.2	24	68 ± 7.3
18:0	6	177 ± 27	4.8	65 ± 2.5
18:1	5	108 ± 25	7.9	70 ± 6.4
18:2	4	160 ± 82	5.3	68 ± 8.7
18:3	4	183 ± 24	4.6	71 ± 7.4
20:4	10	55 ± 23	15	65 ± 4.3
20:5	10	49 ± 7.0	17	63 ± 5.1
22:6	4	38 ± 4.4	22	64 ± 3.7

fibrinogen. The extent of the maximal aggregation varied in different platelet samples, but was only slightly altered among the molecular species of LPA tested (Table 1). Therefore, we also compared the EC_{50} values of various LPAs, as shown in Table 1. Three LPAs having a highly unsaturated fatty acid moiety were equipotent to 16:0-LPA in inducing platelet aggregation, but their activities were higher than those of LPAs with a C_{18} fatty acyl group. Consistent with previous results [28,38,39], the platelet-aggregating activities of alkyl-LPAs were much higher than those of acyl-LPAs.

In human blood, albumin exists at a high concentration of more than 3% and binds to various kinds of endogenous substance, including fatty acid and lysolipids. A large portion of LPA in mammalian plasma and serum is bound to albumin [16,40,41]. Indeed, LPA has been reported to bind to three high-affinity binding sites for free fatty acids, while the affinity of LPA to these sites was less than that of oleic acid [41]. Thus the platelet-aggregating activity of LPA was predicted to be reduced by its binding to albumin in a competitive manner with its own receptors on platelets under *in vivo* conditions [38,39]. In preliminary experiments, we examined the inhibitory effects of various concentrations of fatty acid-free BSA on platelet aggregation induced by different LPAs. Figure 3(A) shows a typical result of such experiments with 18:3-LPA, showing concentration-dependent inhibition by BSA of the LPA-induced platelet aggregation. Similar concentration-dependency of the inhibitory effect of BSA was observed for platelet aggregation induced by several LPAs other than 18:3-LPA (results not shown). Based on previous reports and our preliminary results, we selected a final concentration of fatty acid-free BSA of 0.05% (w/v) for comparison of efficiencies of BSA to inhibit platelet aggregation induced by various LPAs. Pre-addition of BSA to the assay solution (final concentration, 0.05%) prevented aggregation of human platelets in response to different LPAs to various extents, as shown in Figure 3(B). The inhibitory effect of 0.05% BSA was assessed by calculating the ratio of the EC_{50} value in the presence of BSA to that in its absence. The ratios for 20:4-, 20:5- and 22:6-LPAs were 19–22, being higher than those of 16:0-LPA, 16:0-alkyl-LPA and 18:0-alkyl-LPA (10–14-fold), but less than those of LPAs with a C_{18} fatty acyl group (45–62-fold). These results suggest that BSA has a higher affinity for LPAs with a C_{18} fatty acid that have less potent platelet-aggregating activity, than to alkyl-LPAs and other acyl-LPAs with more potent platelet-aggregating activity compared with

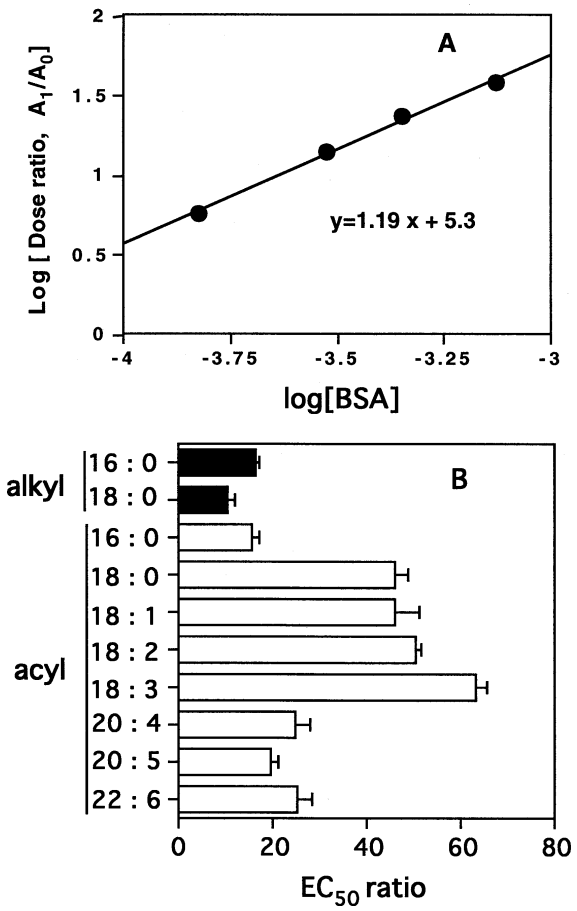

Figure 3 Inhibitory effects of BSA on platelet-aggregating activities of various LPAs

(**A**) Concentration-dependent inhibition by BSA of platelet aggregation induced by 18:3-LPA. Dose ratios of EC_{50} values obtained in the presence of 0.01, 0.02, 0.03 and 0.05% BSA to EC_{50} value in the absence of BSA were calculated. (**B**) Ratios of EC_{50} values obtained in the presence of 0.05% BSA in the assay medium to EC_{50} value in the absence of BSA were calculated. Values are means ± S.E.M. from 3–10 separate experiments.

those of the highly unsaturated LPAs. So, under *in vivo* conditions, the rank order of the platelet-aggregating activity among different LPAs would be extended beyond than that obtained by our *in vitro* experiments.

Comparison of platelet-aggregating activities of positional isomers of LPA and acetylated analogues

First, we prepared 2-20:4-LPA by hydrolysis of 1-alkenyl-2-20:4-GPC with HCl. The product was immediately extracted and purified by TLC with a neutral solvent system to minimize the migration of the *sn*-2-acyl group, as described in the Materials and methods section. The purified 2-20:4-LPA was dispersed in saline containing 0.01% BSA just before the assay of its platelet-aggregating activity and immediately added to the assay solution. Figure 4 shows typical results on the platelet-aggregating activities of both isomers that were equipotent and concentration-dependent. These results indicated that the LPA receptor on human platelets could interact with 20:4-LPA with a similar affinity even if the position of its long-chain acyl group attached to the glycerol backbone was different. However, we could not exclude the possibility that 2-acyl-LPA rapidly changed to

Table 2 GC-MS analysis of positional isomers of acetylated LPCs as tBDMS ether derivatives

Acetylated LPCs (1-acyl- or 2-acyl-) were hydrolysed with *B. cereus* phospholipase C, and resultant acyl-acetyl-glycerols were converted into tBDMS derivatives and analysed by GC-MS as described in the Materials and methods section. The positional isomer composition (%) was determined based on peak areas of $[M-57]^+$ derived from the tBDMS derivatives.

		Positional isomer composition (%)	
Acetylated LPC	tBDMS derivative ...	1-Acyl-2-acetyl-3-tBDMS	1-Acetyl-2-acyl-3-tBDMS
1-20:4-2-Acetyl		86	14
1-20:5-2-Acetyl		98	2
1-22:6-2-Acetyl		85	15
1-Acetyl-2-20:4		6	94
1-Acetyl-2-20:5		10	90
1-Acetyl-2-22:6		14	86

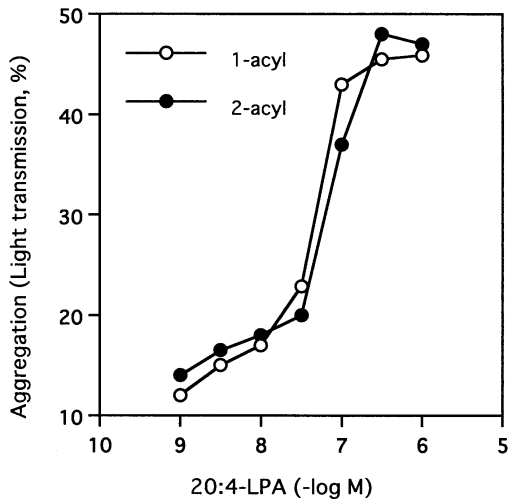

Figure 4 Concentration-response curves of platelet-aggregating activities of 1-20:4-LPA and 2-20:4-LPA

Results are typical of four separate experiments.

1-acyl-LPA during its preparation, storage and dispersion in aqueous solution for measurement of its platelet-aggregating activity. To resolve this issue, the purity of the 2-acyl-LPA as a positional isomer should be confirmed. Unfortunately, no method for determination of the positional isomer composition of LPA has been established, and our attempts to analyse the positional isomers as acetylated analogues with or without hydrolytic pretreatment were unsuccessful. Therefore, we decided to prepare 1-highly unsaturated acyl-2-acetyl-GP (acetylated 1-acyl-LPA) and 1-acetyl-2-highly unsaturated acyl-GP (acetylated 2-acyl-LPA) from the corresponding acetylated LPCs, and to compare their platelet-aggregating activities. Using this approach, we were able to predict the positional isomer purity of the acetylated LPAs by analysing tBDMS ether derivatives of 1-acyl-2-acetyl-glycerol and 1-acetyl-2-acyl-glycerol derived from the acetylated LPCs, the precursors of the acetylated LPAs. This method was based on the observation that the enzymic hydrolysis of acetylated LPC to acetylated LPAs with bacterial phospholipase D was quantitative under our experimental conditions.

The yields of 1-acyl-2-acetyl-GPs having 20:4, 20:5 and 22:6 through a three-step procedure from PCs having two highly unsaturated acyl groups were 37.7, 13.8 and 18.3 %, respectively. 1-Acetyl-2-acyl-GPs were prepared in yields of 50.3, 29.2 and 33.1 % through the three steps of procedure from 1-alkenyl-2-highly unsaturated acyl-GPC. Their structures were confirmed by negative-ion FAB-MS. Intense ions assigned to $[M-H]^-$ were seen at m/z 499 (1- and 2-20:4-LPAs), 497 (1- and 2-20:5-LPAs) and 523 (1- and 2-22:6-LPAs). Table 2 shows the compositions of 1-acyl-2-acetyl and 1-acetyl-2-acyl isomers in three preparations of LPA. Their purities with respect to the positional isomer were somewhat variable, but high enough to compare their biological activities. Figures 5(A), 5(B) and 5(C) show concentration-response curves for positional isomers of acetylated 20:4-, 20:5- and 22:6-LPAs, respectively. Although their biological activities were slightly less than those of the parent LPAs, the two positional isomers were equipotent in inducing aggregation of human platelets in all cases. The EC_{50} values for 1-20:4-2-acetyl-, 1-20:5-2-acetyl- and 1-22:6-2-acetyl-GPs (acetylated LPAs) were 22.7 ± 5.6, 23.7 ± 7.3 and 36.7 ± 1.6 nM, whereas the values for 1-acetyl-2-20:4-, 1-acetyl-2-20:5- and 1-acetyl-2-22:6-GPs were 29.5 ± 6.9, 21.5 ± 3.8 and 39.3 ± 0.3 nM, respectively.

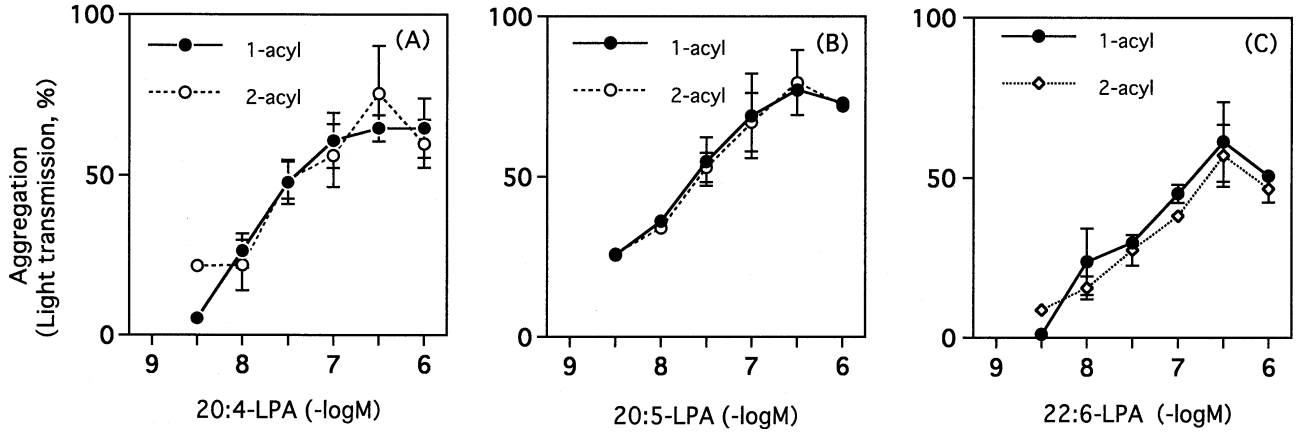

Figure 5 Concentration-response curves of platelet-aggregating activities of 1-acyl-2-acetyl-GPs and 1-acetyl-2-acyl-GPs

Values are means $\pm$ S.E.M. from three separate experiments. (**A**) 20:4, (**B**) 20:5 and (**C**) 22:6.

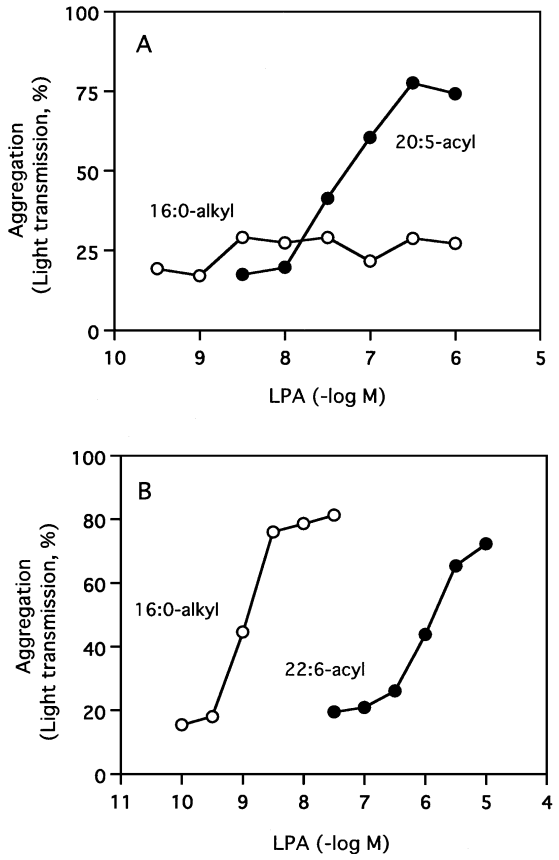

Figure 6 Unusual responses of human platelets to 16:0-alkyl-LPA and highly unsaturated acyl-LPA

Results are typical of at least three separate experiments with platelets prepared from the same person. (**A**) Concentration-response curves for aggregatory responses of human platelets to 16:0-alkyl-LPA and 1-20:5-LPA. (**B**) Concentration-response curves for aggregatory responses of human platelets to 1-22:6-LPA.

Unusual aggregatory responses of human platelets to alkyl-LPAs or highly polyunsaturated acyl-LPAs

During experiments on the platelet-aggregating activities of various LPAs, we realized that platelets from two of more than 30 subjects failed to respond to alkyl-LPAs, but aggregated normally in response to acyl-LPAs, including 20:4-, 20:5- and 22:6-LPAs. A typical result of this abnormal response is shown in Figure 6(A). This observation was confirmed from similar results in three independent experiments with platelet preparations prepared from the same volunteers. These results indicate that the platelets are deficient in a specific receptor for alkyl-LPA. Furthermore, a platelet preparation from a different individual was found to show a much lower aggregatory response to highly unsaturated acyl-LPAs, but responded normally to alkyl-LPAs. Typical results of these experiments with 16:0-alkyl-LPA and 22:6-acyl-LPA are shown in Figure 6(B), suggesting a reduced expression of a specific receptor for acyl-LPA. Taken altogether, we speculate that human platelets possess at least two distinct LPA receptors that are specific for acyl- and alkyl-LPAs. Both LPA receptors can couple to intracellular signalling pathways leading to aggregation, although the signalling pathways triggered by the LPA receptors may not be identical, as shown below.

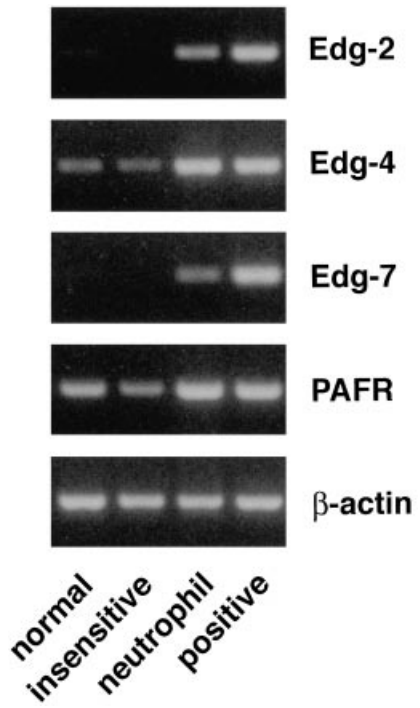

Figure 7 RT-PCR analysis of mRNAs encoding Edg-2, Edg-4 and Edg-7 in normal and alkyl-LPA-insensitive human platelets

RT-PCR was performed with primer sets specific for *Edg-2*, *Edg-4*, *Edg-7*, PAF receptor and β-actin genes using cDNA prepared from human normal platelets, alkyl-LPA-insensitive platelets and human neutrophils as templates. Typical results from three separate experiments are shown in photographs of agarose-gel electrophoresis with ethidium bromide staining. PCR for the positive control was carried out using cloned cDNA corresponding to each gene.

Identification of mRNAs encoding LPA receptors in normal and alkyl-LPA-insensitive platelets

To determine whether known Edg-type LPA receptors could be responsible for the abnormal platelet-aggregatory response, RT-PCR was performed with extracts of isolated human platelets. Predominant expression of the *Edg-4* gene, but not the *Edg-2* and *Edg-7* genes, was observed in both normal and alkyl-LPA-insensitive platelets in the RT-PCRs with 35 cycles (Figure 7). On 40-cycle amplification, an obvious band corresponding to *Edg-7* and a faint band corresponding to *Edg-4* were detected in these platelet preparations (results not shown). Expression of PAF receptor mRNA seems to be higher than that of Edg-4 mRNA in the 35-cycle amplifications (Figure 7), since a band corresponding to the PAF receptor gene, but not that corresponding to the *Edg-4* gene, was detected in 30-cycle amplification. It seems unlikely that expression of these mRNAs in the platelet preparations was mainly due to leucocytes such as neutrophils, since we confirmed essentially no contamination of leucocytes in isolated human platelets for mRNA.

N-Palmitoyl-L-serine phosphate inhibits platelet aggregation by alkyl-LPAs and acyl-LPAs

N-Palmitoyl-L-serine phosphate and *N*-palmitoyl-L-tyrosine phosphate are the only LPA receptor antagonists thus far recognized [28,42,43]. These are structural analogues of alkyl-LPAs, and were originally reported to inhibit alkyl-LPA-induced aggregation of human platelets, but not to prevent ADP-, arachidonate- and PAF-induced platelet aggregation [28].

Table 3 Opposing effects of ONO-RS-082 on platelet aggregation induced by acyl-LPA and alkyl-LPA

Human platelets were pretreated with or without 0.01 vol. of a solution of ONO-RS-082 dissolved in a mixture of DMSO and distilled water (1:1, v/v) at the final concentrations indicated. The platelets were then stimulated with different concentrations of 1-22:6-acyl-LPA or 16:0-alkyl-LPA in the presence of ADP and fibrinogen. Values are percentages of control values obtained without ONO-RS-082, and are shown as means ± S.E.M. from six experiments. N.D., not determined.

		Platelet aggregation (% of control)					
		Alkyl-LPA			Acyl-LPA		
ONO-RS-082 (μM)	Concentration (nM)...	3	10	30	30	100	300
0.3		N.D.	147 ± 11	122 ± 13	N.D.	73 ± 10	96 ± 15
1		140 ± 18	229 ± 19	183 ± 19	61 ± 6.6	63 ± 10	73 ± 11
3		63 ± 13	67 ± 8.5	59 ± 9.6	48 ± 13	56 ± 7.9	46 ± 12
10		59 ± 11	47 ± 12	48 ± 11	53 ± 12	41 ± 11	44 ± 10

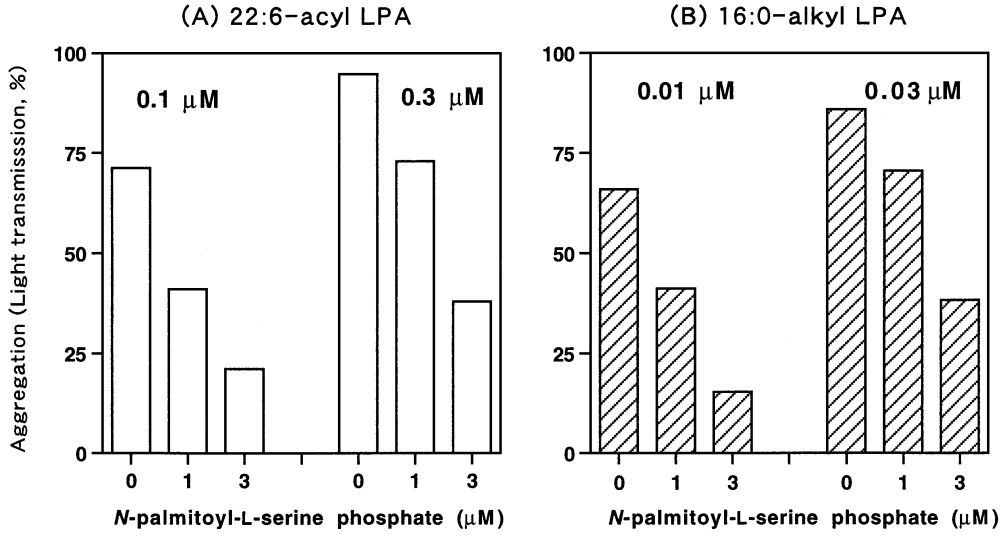

Figure 8 Inhibitory effects of *N*-palmitoyl-L-serine phosphate on (A) 1-22:6-LPA- and (B) 16:0-alkyl-LPA-induced platelet aggregation

Results are typical of four separate experiments. Platelets were pretreated with *N*-palmitoyl-L-serine phosphate (1 or 3 μM) for 2 min and then exposed to 1-22:6-LPA (0.1 or 0.3 μM) or 16:0-alkyl-LPA (0.01 or 0.03 μM) in the presence of ADP and fibrinogen, as described in the Materials and methods section.

However, there is no report of their ability to inhibit platelet aggregation induced by acyl-LPAs, although *N*-palmitoyl-L-serine phosphate was found to act as a weak agonist that induces calcium mobilization and to inhibit adenylate cyclase activity in a human breast cancer cell line [43]. In this study, therefore, we examined whether *N*-palmitoyl-L-serine phosphate inhibits the aggregation of human platelets induced by highly unsaturated acyl-LPAs. As shown in Figure 8, it inhibited 1-20:5-LPA-induced platelet aggregation in a concentration-dependent manner to a similar extent to that induced by 16:0-alkyl-LPA used for comparison.

Highly unsaturated acyl-LPA-induced signalling for platelet aggregation may be distinct from that induced by alkyl-LPA

During examinations of the signalling pathways triggered by different LPAs, we found that ONO-RS-082 affected the platelet aggregation induced by alkyl-LPAs and acyl-LPAs differently. The ONO-RS-082 compound at 0.3 or 1 μM inhibited 1-22:6-LPA-induced platelet aggregation to a significant extent, but potentiated platelet aggregation induced by alkyl-LPA (Table 3). However, on increasing the concentration of ONO-RS-082 to 3 and 10 μM, it inhibited the platelet aggregation induced by both 1-22:6-LPA and 16:0-alkyl-LPA, as shown in Table 3.

DISCUSSION

Determination of the position of the acyl group in lysophospholipid molecules has been helpful in assessing what pathways would be operative for the generation of the cellular lysophospholipids examined. However, early attempts to analyse the positional isomers of lysophospholipids were problematic, owing to the fast isomerization of the 2-acyl isomer at basic or even neutral pH in aqueous solution to generate the more stable 1-acyl isomer [44]. For example, standing of 2-22:6-LPC dissolved in neutral medium at room temperature overnight was shown to result in its 85% conversion into 1-22:6-LPC, but only 4% isomerization was observed during its standing in acidic medium (pH 4) at 4 °C [20]. Previously, we found that 83–87% of the polyunsaturated fatty acid residues of LPC in rat plasma were linked to carbon in the *sn*-1 position of the glycerol backbone [16]. Later, using rat and human plasma prepared by established centrifugation procedures, Croset et al. [20] found about 90% of the unsaturated fatty acids were in the *sn*-1

position, confirming our previous finding [16]. They reported, however, that acyl migration of the 2-acyl isomer of LPC still occurred during even a short period of centrifugation of blood for preparing plasma. This quick isomerization of LPC during the preparation of the plasma LPC was overcome by its direct extraction from quickly acidified blood, revealing that about half the polyunsaturated LPCs were 2-acyl isomers. This result indicated that about half of the 2-acyl isomers of LPCs secreted into the blood circulation of human subjects and rats could be converted into the corresponding 1-acyl isomers in the circulation before its re-utilization by tissues, including liver. It is very possible from this pioneering work of Croset et al. [20] that polyunsaturated LPAs in heparinized rat plasma after its incubation for 6 h or more would be mainly 1-acyl isomers under such *ex vivo* conditions, as noted in our previous work [16]. However, the demonstration of the occurrence of both 1-acyl and 2-acyl isomers of polyunsaturated LPCs in the blood circulation *in vivo* suggested that both isomers of LPA would be generated by lysophospholipase D in the circulation, and that the isomers of LPA could interact with vascular cells including platelets. Thus it is meaningful to compare the aggregatory responses of human platelets to both positional isomers of polyunsaturated LPAs.

The platelet-aggregating activities of acyl-LPAs were first reported by two independent groups [45,46]. In these early investigations, only limited numbers of acyl-LPAs were examined, and their potencies in inducing platelet aggregation were compared on human and feline platelet-rich plasma. A more systematic study was later conducted on platelet-rich plasma, and revealed that the rank order of the aggregating activities of 1-acyl-LPAs was as follows: $16:0 > 18:2 > 18:3 > 18:1 > 18:0 > 14:0 > 12:0$, based on their threshold concentrations [47]. The current work concerning the structure-activity relationship of 1-acyl-LPA-induced aggregation of washed human platelets was essentially compatible with our previous results on human and feline platelet-rich plasma [47]. The structure-activity relationship of LPA-induced platelet aggregation was further extended in the present investigation, leading to the novel finding that highly unsaturated LPAs with a C_{20} or C_{22} fatty acid moiety were more potent than those of LPAs having a C_{18} fatty acid residue.

Acetylated LPAs (acyl-acetyl-GPs) were also found to be active in inducing platelet aggregation in human and feline platelet-rich plasma, although their EC_{50} concentrations were higher than those of the corresponding LPAs [47]. From this finding we compared the platelet-aggregating activities of 1-highly unsaturated acyl-LPAs and 2-highly unsaturated acyl-LPAs acetylated analogues in order to overcome the possibility of their positional purity noted above. Our results clearly showed that acetylated 2-acyl-LPAs were equipotent with acetylated 1-acyl-LPAs in inducing aggregation of washed human platelets. This indicated that the putative LPA receptor for acyl-LPAs could not differentiate between the conformational structures of the positional isomers due to different positions of the acyl chain attachment to the glycerol backbone, as in the cases of known LPA receptors (Edg-2 and Edg-4) [23]. A recent report has shown that mRNAs of Edg-2, Edg-4 and Edg-7 are expressed in human platelets [48]. In this study, predominant expression of Edg-4 mRNA in alkyl-LPA-insensitive platelets as well as normal platelets was shown, indicating that putative receptor specific for alkyl-LPA is distinct from the Edg type receptors, and that acyl-LPA aggregates platelets through its binding to Edg-4. The latter may be supported by our results on the structure-activity relationship of acyl-LPA-induced platelet aggregation resembling those on the Ca^{2+}-mobilizing activities of various acyl-LPAs in LPA-non-responding Sf9 insect cells expressing cloned Edg-4. However, our results obtained by RT-PCR might not precisely reflect the expression of Edg-2, Edg-4 and Edg-7 on human platelets, and therefore the possibility that the aggregatory effect of acyl-LPA was mediated by an uncharacterized LPA receptor other than Edg-type receptors cannot be excluded.

The first study on the biological activities of alkyl-LPA was reported by Simon et al. [38], who found about a 30 times higher platelet-aggregating activity of alkyl-LPA than of LPA having an acyl group with the same chain length as that of the alkyl-LPAs. This interesting finding was later confirmed by additional evidence from more extensive studies [28,39,49]. Considering the presence of multiple LPA receptors in the animal body and the structure-activity relationships of LPA-induced platelet aggregation, human platelets are likely to express more than two distinct subclasses of LPA receptor, including an alkyl-LPA-specific receptor. In this study, we obtained results suggesting the occurrence of two distinct types of LPA receptor on human platelets. Our observations were as follows: two platelet preparations showed essentially no response to alkyl-LPA, but responded to acyl-LPAs, and vice versa for the cases of another platelet preparation. We expected that *N*-palmitoyl-L-serine phosphate, which was reported to inhibit selectively alkyl-LPA-induced aggregation of human platelets, but not those by other typical platelet agonists [28], may fail to inhibit acyl-LPA-induced platelet aggregation, since *N*-palmitoyl-L-serine phosphate was found to act as a weak agonist in assay systems using cells expressing Edg-type LPA receptors [43]. However, this was not the case; the compound prevented both highly unsaturated acyl-LPA- and alkyl-LPA-induced platelet aggregation to similar extents. Gueguen et al. [49] reported that *N*-palmitoyl-L-serine phosphate exerted a weak agonistic action on washed human platelets. The inconsistencies between these works could be due to different experimental conditions, especially the concentration of external calcium ions in the assay medium for measurement of platelet aggregation, as suggested in [49].

In an attempt to obtain further evidence, we found that ONO-RS-082, a phospholipase A_2 inhibitor [29], potentiated alkyl-LPA-induced platelet aggregation, but inhibited acyl-LPA-induced aggregation in a narrow concentration range. This may be explained by considering that the two LPA receptors are coupled to distinct intracellular signalling pathways. We found that other inhibitors of phospholipase A_2, such as aristrochic acid, 12-episcalaradial and arachidonyl trifluoromethyl ketone, did not show such opposing effects observed for ONO-RS-082 on platelet aggregation induced by alkyl-LPAs and acyl-LPAs (results not shown). Thus the ONO-RS-082 compound might affect other key enzymes besides phospholipase A_2. In this context, it should be mentioned that ONO-RS-082 has recently been shown to inhibit phospholipase D [50]. However, the exact mechanism of the potentiation by ONO-RS-082 of alkyl-LPA-induced platelet aggregation is still unknown at present.

Physiological significance of circulating LPC is also largely unknown, but LPCs associated with albumin were reported to be rapidly incorporated into various organs [51–53]. The preferable incorporation of highly unsaturated LPCs into brain tissues has been documented [54]. Our recent studies indicated that circulating LPCs play a novel role as precursors of chronically generated LPAs with diverse biological activities that could be supplied to a wide range of organs, tissues and cells [15,16]. The potencies of the physiological activities of LPA would depend on both its local concentration and the distribution of its receptors, the former of which may be affected by the activities of LPA-producing enzymes, levels of its precursors, activities of

LPA-degrading enzymes and capacities of LPA-binding proteins. Elevated production of LPAs in the blood circulation could disturb homoeostasis of blood/vascular functions, leading to thrombosis and coagulation. In fact, LPA was shown to enhance the adhesion of monocytes to vascular endothelial cells [55] and to increase vascular permeability [56]. A recent report that platelet aggregation induced by oxidized low-density lipoprotein, a key induction factor of atherosclerosis [57], was prevented by an LPA receptor antagonist, suggests a potential role for LPA in increased formation of thrombosis under conditions of increased oxidative stress [58]. Collectively, excess production of LPA in the blood circulation may have pathophysiological significance by altering the balance between aggregatory and anti-aggregatory potentials.

In summary, our results suggest that two distinct LPA receptors on human platelets may be responsible for acyl-LPA- and alkyl-LPA-induced aggregations of human platelets. Further studies are necessary to clarify whether the observed higher platelet-aggregating activities of LPAs having a highly unsaturated fatty acyl group (C_{20}, C_{22}) compared with those having a C_{18} fatty acyl group is explained by the ligand selectivity of known LPA receptors. An alternative possibility is the existence of an uncharacterized LPA receptor for acyl-LPA in human platelets. Furthermore, the present investigation suggests that human platelets also express a novel LPA receptor selective for alkyl-LPA, and thus its characterization and distribution in the body are important subjects that should be studied extensively. Evaluation of possible sources and the mechanism of generation of alkyl-LPA is also necessary to understand the physiological significance of alkyl-LPA.

We thank Yasuko Yoshioka, Rituko Kawakami, Noriko Tsuji, Tomoka Okayama and Hiroyuki Hirahata for technical assistance, and Dr Yan Xu for reading the manuscript. This study was supported in part by a grant from the Japan Health Sciences Foundation.

REFERENCES

1 Jalink, K., Hordijk, P. L. and Moolenaar, W. H. (1994) Growth factor-like effects of lysophosphatidic acid, a novel lipid mediator. Biochim. Biophys. Acta **1198**, 185–196

2 Tokumura, A. (1995) A family of phospholipid autacoids: occurrence, metabolism and bioactions. Progr. Lipid Res. **34**, 151–184

3 Goetzl, E. J. and An, S. (1998) Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. FASEB J. **12**, 1589–1598

4 Spiegel, S. and Milstien, S. (2000) Functions of a new family of sphingosine-1-phosphate receptors. Biochim. Biophys. Acta **1484**, 107–116

5 Sugiura, T., Kodaka, T., Nakane, S., Miyashita, T., Kondo, S., Suhara, Y., Takayama, H., Waku, K., Seki, C., Baba, N. and Ishima, Y. (1999) Evidence that the cannabinoid CB1 receptor is a 2-arachidonoylglycerol receptor. Structure-activity relationship of 2-arachidonoylglycerol, ether-linked analogues, and related compounds. J. Biol. Chem. **274**, 2794–2801

6 Gaits, F., Fourcade, O., Le Balle, F., Gueguen, G., Gaige, B., Gassama-Diagne, A., Fauvel, J., Salles, J. P., Mauco, G., Simon, M. and Chap, H. (1997) Lysophosphatidic acid as a phospholipid mediator: pathways of synthesis. FEBS Lett. **410**, 54–58

7 Das, A. K. and Hajra, A. K. (1989) Quantification, characterization and fatty acid composition of lysophosphatidic acid in different rat tissues. Lipids **24**, 329–333

8 Sugiura, T., Nakane, S., Kishimoto, S., Waku, K., Yoshioka, Y., Tokumura, A. and Hanahan, D. J. (1999) Occurrence of lysophosphatidic acid and its alkyl ether-linked analog in rat brain and composition of their biological activities toward cultured neuronal cells. Biochim. Biophys. Acta **1440**, 194–204

9 Xiao, Y., Schwartz, B., Washington, M., Kennedy, A., Webster, K., Belinson, J. and Xu, Y. (2001) Electrospray ionization mass spectrometry analysis of lysophospholipids in human ascitic fluids: comparison of the lysophospholipid contents in malignant vs nonmalignant ascitic fluids. Anal. Biochem. **290**, 302–313

10 Liliom, K., Guan, Z., Tseng, J. L., Desiderio, D. M., Tigyi, G. and Watsky, M. A. (1998) Growth factor-like phospholipid generated after corneal injury. Am. J. Physiol. **274**, C1065–C1074

11 Chun, J. (1999) Lysophospholipid receptors: implications for neural signaling. Crit. Rev. Neurobiol. **13**, 151–168

12 Bandoh, K., Aoki, J., Hosono, H., Kobayashi, S., Kobayashi, T., Murakami-Murofushi, 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

13 Tokumura, A., Harada, K., Fukuzawa, K. and Tsukatani, H. (1986) Involvement of lysophospholipase D in the production of lysophosphatidic acid in rat plasma. Biochim. Biophys. Acta **875**, 31–38

14 Tokumura, A., Miyake, M., Nishioka, Y., Yamano, S., Aono, T. and Fukuzawa, K. (1999) Production of lysophosphatidic acids by lysophospholipase D in human follicular fluids *in vitro* fertilization patients. Biol. Reprod. **61**, 195–199

15 Tokumura, A., Fujimoto, H., Yoshimoto, O., Nishioka, Y., Miyake, M. and Fukuzawa, K. (1999) Production of lysophosphatidic acid by lysophospholipase D in incubated plasma of spontaneously hypertensive rats and Wistar Kyoto rats. Life Sci. **65**, 245–253

16 Tokumura, A., Nishioka, Y., Yoshimoto, O., Shinomiya, J. and Fukuzawa, K. (1999) Substrate specificity of lysophospholipase D which produces bioactive lysophosphatidic acids in rat plasma. Biochim. Biophys. Acta **1437**, 235–245

17 Baker, D. L., Desiderio, D. M., Miller, D. D., Tolley, B. and Tigyi, G. (2001) Direct quantitative analysis of lysophosphatidic acid molecular species by stable isotope dilution electrospray ionization liquid chromatography-mass spectrometry. Anal. Biochem. **292**, 287–295

18 Shen, Z., Wu, M., Elson, P., Kennedy, A. W., Belinson, J., Casey, G. and Xu, Y. (2001) Fatty acid composition of lysophosphatidic acid and lysophosphatidylinositol in plasma from patients with ovarian cancer and other gynecological diseases. Gynecol. Oncol. **83**, 25–30

19 Gerrard, J. M. and Robinson, P. (1989) Identification of the molecular species of lysophosphatidic acid produced when platelets are stimulated by thrombin. Biochim. Biophys. Acta **1001**, 282–285

20 Croset, M., Brossard, N., Polette, A. and Lagarde, M. (2000) Characterization of plasma unsaturated lysophosphatidylcholines in human and rat. Biochem. J. **345**, 61–67

21 Tokumura, A., Fukuzawa, K. and Tsukatani, H. (1979) Effects of synthetic and natural lysophosphatidic acids on the arterial blood pressure of different animal species. Lipids **33**, 572–574

22 Jalink, K., Hengeveld, T., Mulder, S., Postoma, F. R., Simon, M. F., Chap, H., van der Marel, G. A., van Boom, J. H., van Blitterswijk, W. J. and Moolenaar, W. H. (1995) Lysophosphatidic acid-induced Ca^{2+} mobilization in human A431 cells: structure–activity analysis. Biochem. J. **307**, 609–616

23 Bandoh, K., Aoki, J., Taira, A., Tsujimoto, M., Arai, H. and Inoue, K. (2000) Lysophosphatidic acid (LPA) receptors of the EDG family are differentially activated by LPA species. Structure-activity relationship of cloned LPA receptors. FEBS Lett. **478**, 159–165

24 Okuyama, H., Kobayashi, T. and Watanabe, S. (1997) Dietary fatty acids – the n−6/n−3 balance and chronic elderly diseases. Excess linoleic acid and relative n−3 deficiency syndrome seen in Japan. Progr. Lipid Res. **35**, 409–457

25 Eichholtz, T., Jalink, K., Fahrenfort, I. and Moolenaar, W. H. (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. Biochem. J. **291**, 677–680

26 Tokumura, A., Iimori, M., Nishioka, Y., Kitahara, M., Sakashita, M. and Tanaka, S. (1994) Lysophosphatidic acids induces proliferation of cultured vascular smooth muscle cells from rat aorta. Am. J. Physiol. **267**, C204–C210

27 Tokumura, A., Maruyama, T., Fukuzawa, K. and Tsukatani, H. (1985) Effects of lysophosphatidic acids and their structural analogs on arterial blood pressure of cats. Arzneim. Forsch./Drug. Res. **35**, 587–592

28 Sugiura, T., Tokumura, A., Gregory, L., Nouchi, T., Weintraub, S. T. and Hanahan, D. J. (1994) Biochemical characterization of the interaction of lipid phosphoric acids with human platelets: comparison with platelet activating factor. Arch. Biochem. Biophys. **311**, 358–368

29 Banga, H. S., Simons, E. R., Brass, L. F. and Rittenhouse, S. E. (1986) Activation of phospholipase A and C in human platelets exposed to epinephrine: role of glycoproteins IIb/IIIa and dual role of epinephrine. Proc. Natl. Acad. Sci. U.S.A. **83**, 9197–9201

30 Satouchi, K., Sakaguchi, M., Shirakawa, M., Hirano, K. and Tanaka, T. (1994) Lysophosphatidylcholine from white muscle of bonito *Euthynnus pelamis* (Linnaeus): involvement of phospholipase A_1 activity for its production. Biochim. Biophys. Acta **1214**, 303–308

31 Selinger, Z. and Lapidot, Y. (1966) Synthesis of fatty acid anhydrides by reaction with dicyclohexylcarbodiimide. J. Lipid Res. **7**, 174–175

32 Satouchi, K., Oda, M., Yasunaga, K. and Saito, K. (1985) Evidence for production of 1-acyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine concomitantly with platelet-activating factor. Biochem. Biophys. Res. Commun. **128**, 1409–1417

33 Bligh, E. G. and Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**, 911–917

34 Tokumura, A., Harada, K., Yoshioka, Y., Tsukatani, H. and Handa, Y. (1984) Analyses of lysophosphatidic acids by gas chromatography mass spectrometry without hydrolytic pretreatment. Biomed. Mass Spectrom. **11**, 167–171
35 Chalvardjian, A. and Rudnicki, E. (1970) Determination of lipid phosphorus in the nanomolar range. Anal. Biochem. **36**, 225–226
36 Ekholm, J., Tatsumi, Y., Nouchi, T. and Hanahan, D. J. (1992) The human platelet as a reproducible and sensitive cell for the detection and assay of platelet-activating factor. Anal. Biochem. **204**, 79–84
37 Kishimoto, S., Shimadzu, W., Izumi, T., Shimizu, T., Fukuda, T., Makino, S., Sugiura, T. and Waku, K. (1990) Regulation by IL-5 of expression of functional platelet-activating factor receptors on human eosinophils. J. Immunol. **157**, 4126–4132
38 Simon, F., Chap, H. and Douste-Blazy, L. (1982) Human platelet aggregation induced by 1-alkyl-lysophosphatidic acid and its analogs: a new group of phospholipid mediator? Biochem. Biophys. Res. Commun. **108**, 1743–1750
39 Tokumura, A., Yoshida, J., Maruyama, T., Fukuzawa, K. and Tsukatani, H. (1987) Platelet aggregation induced by ether-linked phospholipids. 1. Inhibitory actions of bovine serum albumin and structural analogues of platelet activating factor. Thromb. Res. **46**, 51–63
40 Tigyi, G. and Miledi, R. (1992) Lysophosphatidates bound to serum albumin activate membrane currents in *Xenopus* oocytes and neurite retraction in PC12 pheochromocytoma cells. J. Biol. Chem. **267**, 21360–21367
41 Thumser, A. E. R., Voysey, J. E. and Wilton, D. C. (1994) The binding of lysophospholipids to rat liver fatty acid-binding protein and albumin. Biochem. J. **301**, 801–806
42 Liliom, K., Bittman, R., Swords, B. and Tigyi, G. (1996) *N*-Palmitoyl-serine and *N*-palmitoyl-tyrosine phosphoric acids are selective competitive antagonists of the lysophosphatidic acid receptors. Mol. Pharmacol. **50**, 616–623
43 Hooks, S. B., Ragan, S. P., Hopper, D. W., Honemann, M. E., Durieux, M. E., MacDonald, T. L. and Lynch, K. R. (1998) Characterization of a receptor subtype-selective lysophosphatidic acid mimetic. Mol. Pharmacol. **53**, 188–194
44 Plückthun, A. and Dennis, E. A. (1982) Acyl and phosphoryl migration in lysophospholipids: importance in phospholipid synthesis and phospholipase specificity. Biochemistry **21**, 1743–1750
45 Schumacher, K. A., Classen, H. G. and Spath, M. (1979) Platelet aggregation evoked *in vitro* and *in vivo* by phosphatidic acids and lysoderivatives: identity with substances in aged serum (DAS). Thromb. Haemost. **42**, 631–640
46 Gerrard, J. M., Kindom, S. E., Peterson, D. A., Peller, J., Krantz, K. E. and White, J. G. (1979) Lysophosphatidic acids. Influence on platelet aggregation and intracellular calcium flux. Am. J. Pathol. **96**, 423–436
47 Tokumura, A., Fukuzawa, K., Isobe, J. and Tsukatani, H. (1981) Lysophosphatidic acid-induced aggregation of human and feline platelets: structure-activity relationship. Biochem. Biophys. Res. Commun. **99**, 391–398
48 Motohashi, K., Shibata, S., Ozaki, Y., Yatomi, Y. and Igarashi, Y. (2000) Identification of lysophospholipid receptors in human platelets: the relation of two agonists, lysophosphatidic acid and sphingosine 1-phosphate. FEBS Lett. **468**, 189–193
49 Gueguen, G., Gaige, B., Grevy, J. M., Rogalle, P., Bellan, J., Wilson, M., Klaebe, A., Pont, F., Simon, M. F. and Chap, H. (1999) Structure-activity analysis of the effects of lysophosphatidic acid on platelet aggregation. Biochemistry **38**, 8440–8450
50 Li, Y., Shiels, A. J., Maszak, G. and Byron, K. L. (2001) Vasopressin-stimulated Ca^{2+} spiking in vascular smooth muscle cells involves phospholipase D. Am. J. Physiol. **280**, H2658–H2664
51 Illingworth, D. R. and Portman, O. W. (1972) The uptake and metabolism of plasma lysophosphatidylcholine *in vivo* by the brain of squirrel monkeys. Biochem. J. **130**, 557–567
52 Portman, O. W. and Illingworth, D. R. (1972) Metabolism of lysolecithin *in vivo* and *in vitro* with particular emphasis on the arterial wall. Biochim. Biophys. Acta **348**, 136–144
53 Savard, J. D. and Choy, P. C. (1982) Phosphatidylcholine formation from exogenous lysophosphatidylcholine in isolated hamster heart. Biochim. Biophys. Acta **711**, 40–48
54 Thies, F., Delachambre, M. C., Bentejac, M., Lagarde, M. and Lecerf, J. (1992) Unsaturated fatty acids esterified in 2-acyl-1-lysophosphatidylcholine bound to albumin are more efficiently taken up by the young rat brain than the unesterified form. J. Neurochem. **59**, 1110–1116
55 Rizza, C., Leitinger, N., Yue, J., Fischer, D. J., Wang, D., Shih, P. T., Lee, H., Tigyi, G. and Berliner, J. A. (1999) Lysophosphatidic acid as a regulator of endothelial/leukocyte interaction. Lab. Invest. **79**, 1227–1235
56 Schultze, C., Smales, C., Rubin, L. L. and Staddon, J. M. (1997) Lysophosphatidic acid increases tight junction permeability in cultured brain endothelial cells. J. Neurochem. **68**, 991–1000
57 Witztum, J. L. and Steinberg, D. (1991) Role of oxidized low density lipoprotein in atherosclerosis. J. Clin. Invest. **88**, 1785–1792
58 Siess, W., Zangl, K. J., Essler, M., Bauer, M., Brandl, R., Corrinth, C., Bittman, R., Tigyi, G. and Aepfelbacher, M. (1999) Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. Proc. Natl. Acad. Sci. U.S.A. **96**, 6931–6936

Received 1 March 2002; accepted 1 May 2002
Published as BJ Immediate Publication 1 May 2002, DOI 10.1042/BJ20020348