methods

Simultaneous separation of lysophospholipids from the total lipid fraction of crude biological samples using two-dimensional thin-layer chromatography

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Abstract **A novel solvent system for two-dimensional thin-layer chromatography was shown to simultaneously separate lysophospholipid standards, including lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidylinositol, lysophosphatidylglycerol, lysophosphatidic acid, lysosphingomyelin (sphingosylphosphorylcholine), and sphingosine-1-phosphate from diradylphospholipids, glycosphingolipids, and neutral lipids. Lysophospholipids contained in the total lipid fraction of activated platelets were also well separated by the same system. The present system is a useful tool for the metabolic and structural analysis of lysophospholipids in biological samples.**—Yokoyama, K., F. Shimizu, and M. Setaka. **Simultaneous separation of lysophospholipids from the total lipid fraction of crude biological samples using two-dimensional thin-layer chromatography.** ***J. Lipid Res.*** **2000.** 41: **142–147.**

Supplementary key words lysophospholipids • two-dimensional thin-layer chromatography • lysophosphatidylcholine • lysophosphatidylethanolamine • lysophosphatidylserine • lysophosphatidylinositol • lysophosphatidylglycerol • lysophosphatidic acid • sphingosylphosphorylcholine • sphingosine-1-phosphate

Lysophospholipids (lysoPLs) have been accepted as intermediate products of the phospholipid deacylation–reacylation pathway (1, 2). Lysophosphatidic acid (lysoPA) is also synthesized de novo by esterification of glycerol-3-phosphate in the glycerolipid biosynthetic pathway (1, 3). Recently, numerous reports have revealed that lysoPLs show various biological activities themselves. For instance, lysophosphatidylcholine (lysoPC) induces intracellular calcium mobilization (4), gene expression of various proteins (5–12), and is a chemotaxin for monocytes (13). LysoPA and sphingosine-1-phosphate (Sp1p) bind to very similar but specific receptors coupled to G-proteins (14–18) and induce various biological responses, such as cell proliferation, platelet aggregation, and intracellular stress fiber formation (14–16). In addition, Sp1P protects cells from apoptosis (16). Sphingosylphosphorylcholine (lysoSM) shares its receptor with Sp1P (19–21) and also acts as a mitogen (22), as a stimulator of smooth muscle contraction (23), as an activator of various protein kinases and an ion exchanger (20, 24, 25), presumably via intracellular calcium mobilization (19). Lysophosphatidylglycerol (lysoPG) inhibits lysoPA (26). Lysophosphatidylinositol (lysoPI) shows mitogenic activity (27). Lysophosphatidylserine (lysoPS) acts as a potent coactivator for mast cells (28). Lysophosphatidylethanolamine (lysoPE) affects the regulated exocytosis (29).

These findings emphasize the need for analysis of the production and localization of lysoPLs at their sites of function, which means that the extraction and identification of lysoPLs from crude preparations of cells and tissues is required. Only a few attempts have been made to detect the lysoPLs present in biological samples, in contrast to the many functional studies. Furthermore, the sites at which lysoPLs were detected in previous studies were not always coincident with the expected target tissues and cells. For example, lysoPCs accumulate in oxidized low density lipoprotein and atherosclerotic aorta (30, 31) and in lesions of ischemia (32). LysoPA and Sp1P are produced and released from activated platelets (33, 34). LysoPI accumulates in stimulated macrophages and oncogene-transformed fibroblasts (27, 35). LysoPS is produced in leukocytes and activated platelets (36, 37).

Abbreviations: Cer, ceramide; Gb_3Cer, Gal(α1-4)Gal(β1-4)Glc(β1-1′)Cer; Gb_4Cer, GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc(β1-1′)Cer; GD_{1a}, NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4) Glc(β1-1′)Cer; GD_{1b}, Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)] Gal(β1-4)Glc(β1-1′)Cer; GlcCer, Glc(β1-1′)Cer; GM_1, Gal(β1-3) GalNAc(β1-4)[NeuAc(α2-3)]Gal(β1-4)Glc(β1-1′)Cer; GM_3, NeuAc(α2-3)Gal(β1-4) Glc(β1-1′)Cer; LacCer, Gal(β1-4)Glc(β1-1′)Cer; GT_{1b}, NeuAc(α2-3)Gal(β1-3)GalNAc(β1-4)[NeuAc(α2-8)NeuAc(α2-3)]Gal (β1-4)Glc(β1-1′)Cer; lysoPA, lysophosphatidic acid; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; lysoPG, lysophosphatidylglycerol; lysoPI, lysophosphatidylinositol; lysoPL, lysophospholipid; lysoPS, lysophosphatidylserine; lysoSM, sphingosylphosphorylcholine; PA, phosphatidic acid; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; Sp1p, sphingosine-1-phosphate; TLC, thin-layer chromatography.

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LysoPE accumulates in serum (38) and ischemic heart (39) and is released from cultured hepatocytes (40). LysoSM is present in meningioma cells (41). LysoPG accumulates in crystalline lens (42). Moreover, various methods were used in these studies such as column chromatography (38, 39), nuclear magnetic resonance spectroscopy (41, 42), and thin-layer chromatography (TLC) with silica gel plates (27, 29–31, 34–37, 40). Each of these methods was designed to separate a specific lysoPL class, which means that a standard method for separating all lysoPLs from other lipids in biological samples has not been available.

We decided to establish a simple and reproducible method for the simultaneous separation of lysoPLs from other lipids using the most widely used system, two-dimensional TLC with silica gel plates. We previously developed a method for the simultaneous separation of glycosphingolipids from other lipids in the total lipid fraction from crude biological samples using two-dimensional TLC (43, 44). Therefore, we began by modifying this system for the separation of lysoPLs. Here, we report a system that simultaneously separates lysoPLs in crude biological samples from diradylphospholipids and sphingolipids on a TLC plate.

MATERIALS AND METHODS

Lipid standards

LysoPC (1-oleoyl-), lysoPE (1-oleoyl-), lysoPS (sodium salt, 1-oleoyl-), lysoPG (sodium salt, 1-oleoyl-), lysoPA (sodium salt, 1-oleoyl-), phosphatidylcholine (PC) (chicken egg), phosphatidylethanolamine (PE) (chicken egg), phosphatidylserine (PS) (sodium salt, 1-palmitoyl-2-oleoyl-), phosphatidic acid (PA) (sodium salt, derived from chicken egg PC), phosphatidylglycerol (PG) (sodium salt, derived from chicken egg PC), and sphingomyelin (SM) (bovine brain) were purchased from Avanti Polar Lipids, Alabaster, AL. Phosphatidylinositol (PI) (ammonium salt, soybeans), lysoPI (ammonium salt, derived from porcine pancreas PI), cholesteryl oleate, oleic acid, monoolein, and triolein were purchased from Doosan Serdary Res. Lab, Englewood Cliffs, NJ. LysoPC (1-caproyl-), lysoPC (1-decanoyl-), cardiolipin (sodium salt, bovine heart), diolein, sphingosine, and ceramide (Cer) (derived from chicken egg SM) were purchased from Sigma, St. Louis, MO. Platelet-activating factor (PAF) and Sp1P were obtained from Cayman Chemical, Ann Arbor, MI. A mixture of neutral glycosphingolipids (GlcCer, LacCer, Gb_3Cer, and Gb_4Cer) and a mixture of bovine brain gangliosides (GM_1, GD_{1a}, GD_{1b}, and GT_{1b}) were products of Iatron, Tokyo, Japan. LysoSM, lysoPAF, GM_3, and free cholesterol were purchased from Biomol Research Lab, Plymouth Meeting, PA, Cascade Biochem, Berkshire, UK, Snow Brand Milk, Tokyo, Japan, and Wako, Osaka, Japan, respectively. CyclicPA (1-oleoyl-) was a gift from Dr. Tetsuyuki Kobayashi (Ochanomizu University). LysoPC (1-melissoyl-) was produced by the action of porcine pancreas phospholipase A_2 from 1-melissoyl-2-oleolyl-PC derived from a mutant strain of *Schizosaccharomyces pombe*. Its structure was confirmed by gas chromatography and mass spectrometry (K. Yokoyama, S. Saitoh, F. Shimizu, K. Nakamura, A. Tokumura, Y. Yamakawa, M. Nishijima, M. Yanagida, and M. Setaka, unpublished results).

Two-dimensional TLC

Authentic standards were spotted in the lower left-hand corner of a TLC plate (Merck, Darmstadt, Germany, #5641, 10 cm × 10 cm). The first chromatographic run was performed with chloroform–methanol–formic acid–water 60:30:7:3 (by vol). The second run was performed with chloroform–methanol–28% ammonia in water–water 50:40:8:2 (by vol) at 90° to the original direction. When needed to separate the lysoPLs from the neutral lipids on the same plate, a third run was performed with diethylether in the opposite direction to the second run. The plate was sprayed with 20% (by vol) H_2SO_4 and the spots were visualized by heating.

Preparation of a biological sample containing lysoPLs

Washed rat platelets were prepared as described as previously (37) with modifications. Blood was drawn from a male Wistar rat by cardiac puncture, anticoagulated with acid citrate dextrose, and platelet-rich plasma was obtained by centrifuging at 300 *g* for 10 min. Platelets were washed with 137 mm NaCl, 12 mm $NaHCO_3$, 0.4 mm Na_2HPO_4, 2 mm citric acid, 2.7 mm KCl, and 0.1% glucose (w/v) (pH 6.5) by centrifuging at 1,500 *g* for 10 min and resuspended in 10 mm Tris-HCl, 137 mm NaCl, 2.7 mm KCl, and 0.1% glucose (w/v) (pH 7.4). The washed platelets were activated with 2 μm A23187 (Calbiochem, La Jolla, CA) in the presence of 2 mm $CaCl_2$ for 1 h and the total lipid fraction was extracted according to Bligh and Dyer (45) three times, adding citric acid to the aqueous phase at the second extraction at a final concentration of 200 μm. The chromatographic run was performed as described above.

RESULTS

Separation of lysoPLs standards from other lipid standards

Each lysoPL (lysoPC, lysoPE, lysoPS, lysoPI, lysoPA, lysoPG, lysoSM, Sp1P) was clearly separated from the phospholipids (PC, PE, PS, PI, PA, PG, SM, and cardiolipin), neutral glycosphingolipids (GlcCer, LacCer, Gb_3Cer, and Gb_4Cer) and gangliosides (GM_3 and bovine brain ganglioside mixture) and were seen as distinct spots on the TLC plate (**Fig. 1A**). Neutral lipids, which were seen in the upper right-hand corner of the plate after the second run, could be roughly separated as larger spots in the third run. When it is not necessary to separate the neutral lipids, the third run can be omitted, because the polar lipids (including the lysoPLs) were developed after the second run and did not migrate during the third run (Fig. 1B). **Table 1** shows the R_f values of the lysophospholipids.

Differences in R_f values among molecular species

The effect of fatty chain length on R_f value was examined. LysoPC with shorter fatty chains (C6:0 and C10:0) migrated to a lesser degree than lysoPC (C18:1). Conversely, a longer chain length (C30:0) resulted in greater migration (Table 1). Then, the different salt forms of acidic lysophospholipids were examined. Sodium salts of lysoPA and lysoPS were converted to the free form or calcium salts by solvent partition (46) (Table 1). Their mobilities in both directions were not affected by the salt forms, although calcium salts showed tailing to some extent (data not shown). Alkyl- and alkenyl-type lysoPC co-migrated with acyl-type lysoPC (Table 1), indicating that the linkage between glycerol and the fatty chain did not affect the R_f value, and thus these lysoPCs including lysoPAF could not be separated in this system. PAF and cyclicPA co-migrated with the lower spot of SM and PE, respectively.

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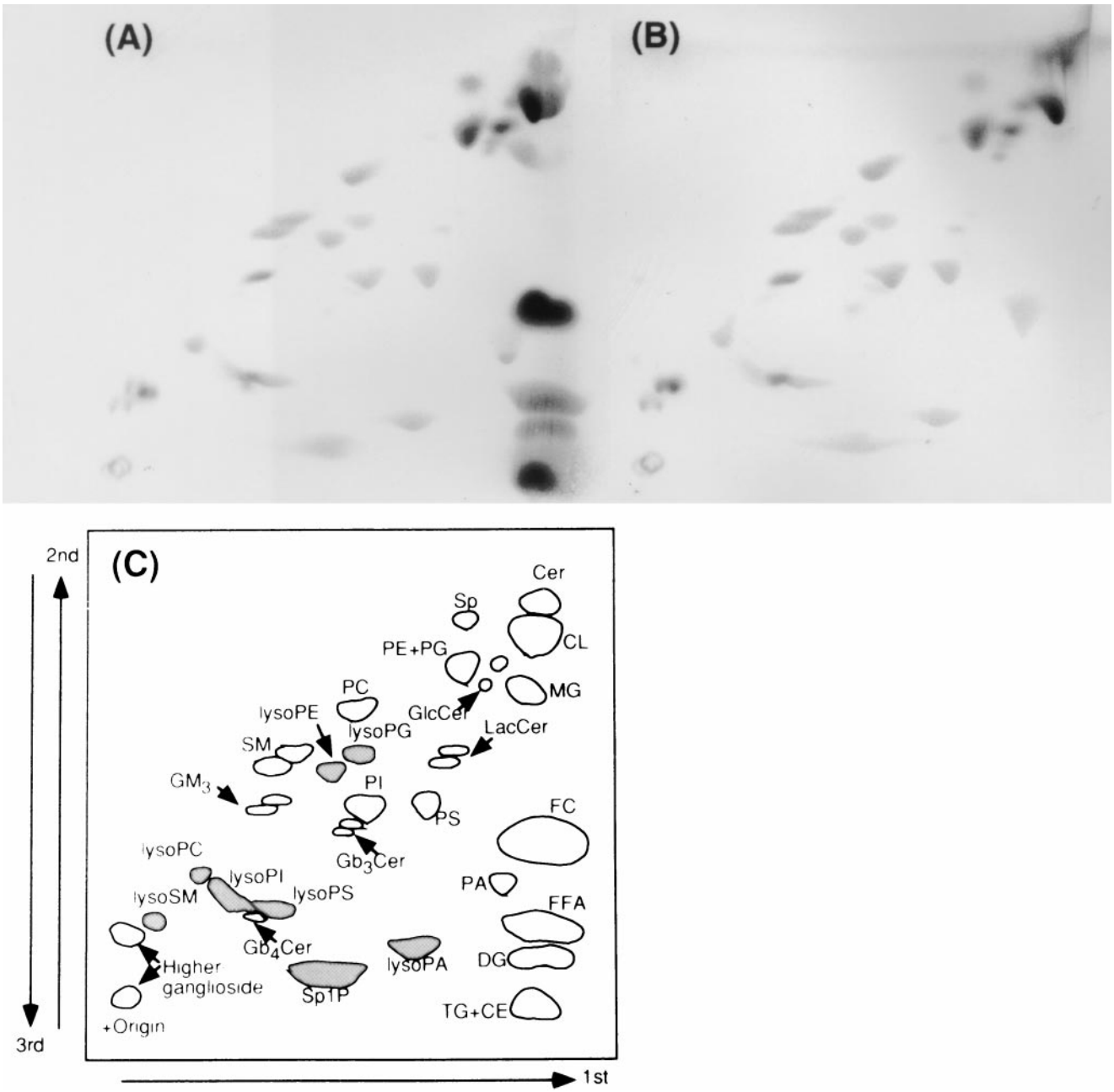

Fig. 1. Separation of lysophospholipids from diradylphospholipids and sphingolipids on a two-dimensional thin-layer chromatography (TLC) plate. (A) Authentic standards were spotted on the lower left-hand corner of a TLC plate. The first chromatographic run was performed with chloroform–methanol–formic acid–water 60:30:8:2 (by vol). Then the plate was developed with chloroform–methanol–28% ammonia in water–water 50:40:7:3 (by vol) for the second dimension. The third run was performed with diethylether in the opposite direction to the second run. The plate was sprayed with 20% (by vol) H_2SO_4 and the spots were visualized by heating. (B) The lipids were spotted and the plate was developed as for (A) except that the third run was omitted. (C) Schematic diagram of the separation pattern. Lysophospholipids are shaded.

Separation of lysoPLs contained in activated platelets

Figure 2B shows the TLC pattern of the total lipids of activated rat platelets. A decrease in PC, PE, and PS and a concomitant increase in lysoPC, lysoPE, lysoPS, and free fatty acid were observed compared with the total lipids of the resting platelets (Fig. 2A), suggesting that platelet phospholipids were degraded by extracellularly released platelet secretory phospholipase As upon platelet activation (37). The lysoPLs even in the biological sample were clearly separated from diradylphospholipids and sphingolipids on a single plate.

DISCUSSION

Here, we report a simple method for the simultaneous separation of lysoPLs in biological samples from diradylphospholipids, glycosphingolipids, and neutral lipids. The

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TABLE 1. R_f values of lysoPLs

LysoPL	First Direction[a]	Second Direction[b]	Fatty Chain
LysoPC[c]	0.19 (0.16–0.22)	0.32 (0.30–0.35)	18:1
LysoPE[c]	0.47 (0.43–0.51)	0.55 (0.52–0.57)	18:1
LysoPS, Na^{+}[c]	0.35 (0.30–0.43)	0.23 (0.22–0.25)	18:1
LysoPI/NH_4^{+}[c]	0.25 (0.20–0.32)	0.26 (0.22–0.30)	18:0, 18:1
LysoPA, Na^{+}[c]	0.65 (0.56–0.70)	0.16 (0.13–0.18)	18:1
LysoPG, Na^{+}[c]	0.53 (0.48–0.56)	0.58 (0.55–0.60)	18:1
LysoSM[c]	0.08 (0.03–0.11)	0.23 (0.20–0.26)	d18:1
Sp1P[c]	0.47 (0.24–0.67)	0.10 (0.08–0.13)	d18:1
Fatty chain length			
LysoPC	0.14 (0.11–0.19)	0.26 (0.22–0.31)	10:0
LysoPC	0.13 (0.11–0.18)	0.23 (0.20–0.29)	6:0
LysoPC	0.34 (0.32–0.38)	0.35 (0.34–0.36)	30:0
Salt forms			
LysoPS, free	0.35 (0.31–0.40)	0.26 (0.23–0.29)	18:1
LysoPS, $Ca2^{+}$[d]	0.34 (0.31–0.38)	0.27 (0.24–0.30)	18:1
LysoPA, free	0.65 (0.58–0.73)	0.16 (0.12–0.19)	18:1
LysoPA, $Ca2^{+}$[d]	0.65 (0.57–0.71)	0.17 (0.14–0.19)	18:1
Linkage between glycerol and fatty chain			
LysoPC, alkyl[e]	0.17 (0.13–0.21)	0.31 (0.27–0.34)	C16
LysoPC, alkenyl	0.18 (0.13–0.24)	0.32 (0.27–0.36)	C16, C18
LysoPL-related molecules			
PAF	0.36 (0.28–0.40)	0.51 (0.47–0.54)	C16
CyclicPA	0.63 (0.61–0.65)	0.76 (0.72–0.81)	18:1

Several nmol of lysophospholipid standards were developed as described in the legend of Fig. 1. The R_f value represented the center of the spot. Parentheses indicate the range of the spot, which depends on the lipid content.

[a] Chloroform–methanol–formic acid–water 60:30:7:3 (by vol).

[b] Chloroform–methanol–28% ammonia in water–water 50:40:8:2 (by vol).

[c] Values deduced from Fig. 1.

[d] Tailing to the origin.

[e] LysoPAF.

lysoPLs isolated on the TLC plate can be recovered by scraping them from the plate with the silica, followed by extraction, and are available for structural analysis such as molecular mass analysis by mass spectrometry, fatty acid analysis, and the quantification of phosphorus. Alternatively, this system could be used to analyze lysoPL metabolism. A metabolic labeling study using various radiolabeled precursors of the phospholipids should be more

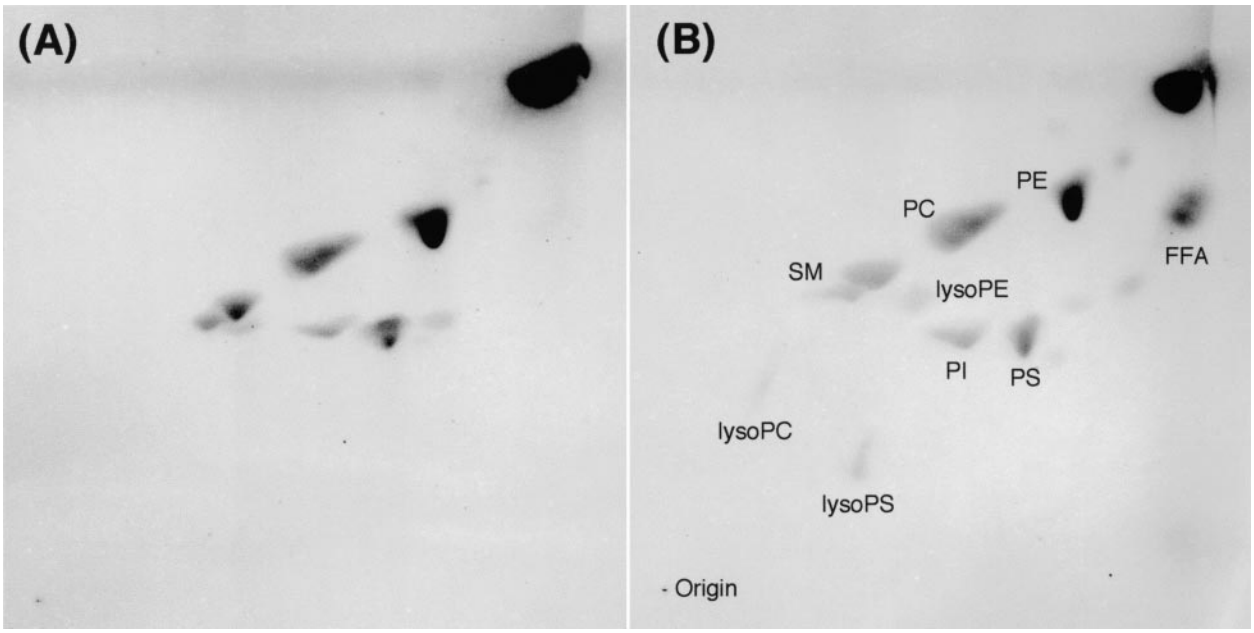

Fig. 2. Separation pattern of lysophospholipids contained in a biological sample. (A) Total lipid fraction of rat washed platelets. (B) The platelets were activated with 2 μm A23187 in the presence of 2 mm $CaCl_2$ for 1 h and the total lipid fraction was extracted. The chromatographic run was performed as described in the legend to Fig. 1.

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informative. As the polar lipids do not migrate during the third run, appropriate neutral lipids, which do not incorporate radioactivity, can be used as internal standards (43). In some cases, the molecular species of the lysoPLs should be considered upon application of this system, as fatty chains of unusual length, such as 6:0 and 30:0, brought about differences in mobility. On the other hand, the linkage between glycerol and the fatty chain and the different salt forms of acidic lysoPLs did not significantly affect the R_f values. Thus, the recently identified biologically functional molecule, alkenyl-type lysoPA (46), might be developed with acyl-type lysoPA. On the other hand, calcium salts of acidic lysoPLs should be avoided by removing calcium ions by extraction under acidic and/or chelating conditions (47, 48), because they show low extraction efficiency (48) and tailing in TLC. The lysoPLs analyzed in this study were only those of the 1-acyl-2-lyso form. Application of this system to 1-lyso-2-acyl form lysoPLs will be complicated by the difference in R_f values and their conversion to the 1-acyl-2-lyso form by acyl residue migration during the treatment. Our preliminary trial to separate PAF and lysoPAF from SM and lysoPC by changing the solvent composition did not succeed, therefore sphingomyelinase-treatment is required for PAF analysis using this system.

LysoPLs with glycerol backbones are degradation products of phospholipase A_2 and these lysoPLs can be metabolized by lysophospholipase and acyltransferase; therefore, their accumulation in cells and tissues indicates phospholipase A_2 activation. Recently, a diverse family of phospholipase A_2 isoforms has been cloned and found to tightly regulate cell signaling via production of the precursors of lipid mediators, that is, lysoPLs and free fatty acids including arachidonic acid (49). Most of them show distinct substrate specificities in vitro (50). Therefore, to better understand phospholipase A_2 isoform activation, we can study lysoPL accumulation in cells and tissues. On the other hand, to determine the in vivo substrate specificity of a phospholipase A_2 isoform, the class of lysoPL that accumulates in recombinant phospholipase A_2-transfected cells can be examined. The present system is a useful tool for analyzing these problems.

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