A Method for Distinguishing 1-Acyl from 2-Acyl Lysophosphatidylcholines Generated in Biological Systems

J. Florin-Christensen,*† J. Narvaez-Vasquez,‡ M. Florin-Christensen,*† and C. A. Ryan‡

*Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, Washington 99164-7040; †Institute of Neuroscience (INEUCI-CONICET), Ciudad Universitaria, Pab. II, 4° Piso, 1428 Buenos Aires, Argentina; and ‡Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340

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Phospholipases A1 and A2 frequently coexist in biological systems. Generation of lysophosphatidylcholine (LPC) in such systems cannot be assigned to any of these types of enzymes unless the position of the fatty acid in the lysocompound can be unambiguously determined. We here present a simple method to achieve this purpose. It is based on the initial chemical acylation of the isolated LPC with a labeled fatty acid, followed by the enzymatic analysis of the resulting phosphatidylcholine (PC), using snake or bee venom phospholipase A2. Thus, if treatment of the PC with this enzyme releases a labeled free fatty acid, it is demonstrated that the initial LPC was acylated at position sn-1, whereas if the product of hydrolysis yields labeled LPC, then the initial LPC was acylated at position sn-2. This is the first method devised to determine the source of LPC in the presence of mixtures of phospholipases A1 and A2 in complex biological systems. © 1999 Academic Press

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ated fatty acids, and selectivity of phospholipases toward particular phospholipid molecular species is a distinct possibility that may not be dismissed. For instance, recent studies on HL-60 cells disclosed a phospholipase A activity that releases predominantly oleic acid, which could come from either position sn-1 or sn-2 in phospholipids and, indeed, this enzyme appears to have both PLA₁ and PLA₂ activities (14). Thus, only the determination of the type of lysocompound formed can ascertain which of these activities is physiologically predominant in these cells.

We here present a simple method for the discrimination between 1-acyl and 2-acyl lysoPC, thus allowing the definition of the type of phospholipase A involved in their generation in complex biological systems.

MATERIALS AND METHODS

Chemicals. Thin-layer silica gel plates, dicyclohexylcarbodiimide, dimethylaminopyridine, amylene-stabilized chloroform, stearic anhydride, bee venom phospholipase A₂, Rhizopus arhizus lipase (suspension in 3.2 M ammonium sulfate), crude soy bean PC (type II-S), and oleic acid were from Sigma Chemical Co. (St. Louis, MO). T. thermophila phospholipase A₁ was purified by the method of Guberman et al. (11). Dioleoyl-PC, egg yolk PC, and egg yolk lysoPC were from Avanti Polar lipids (Alabaster, AL). Analytical grade solvents were purchased from Fisher Scientific Co. (Springfield, NJ). [1-¹⁴C]Stearic acid (58 μCi/μmol) was from New England Nuclear (Boston, MA).

Cells and cultures. T. thermophila, strain CU 399, was cultured in 1% proteose peptone/0.1% yeast extract/0.5% glucose, at 30°C, with continuous shaking for 24 h, at which time culture densities reached 6 × 10⁸ cells/ml. A 50-ml culture was labeled for 12 h with 1 μCi/ml of [¹⁴C]stearic acid, added in ethanol solution (0.1% final concentration). After this labeling period, the cells were exposed to 42°C for 30 min, a lethal condition in which lysocompounds and free fatty acids accumulate. Trypanosoma cruzi epimastigotes, strain RA, were cultured at 30°C for 48 h, in a biphasic medium, as previously described (16). Homogenates obtained by freezing and thawing a 25-ml suspension of parasites washed thrice in PBS (10⁶ cells/ml) were allowed to autolyze for 24 h at 37°C. Under these conditions, lysoPC accumulates in these cells. Bovine erythrocyte ghosts were obtained as described by Dodge et al. (17).

Isolation of lysoPC. The cellular lipids from both organisms were extracted according to Bligh and Dyer (18), dried under nitrogen, and subjected to thin-layer chromatography on silica gel G plates using chloroform/methanol/water/acetic acid (65/35/4/2, v/v). LysoPC was visualized by brief exposure to iodine vapors in authentic standards run on the same plates in parallel lanes. LPC was eluted from the plates with three successive portions of 2 ml of methanol. The solvent was first evaporated under nitrogen, and then under high vacuum. To eliminate any traces of methanol, a few drops of benzene were added to the samples, which were then dried again under high vacuum. LysoPC was then dissolved in amylene-stabilized chloroform. The phosphate concentration was measured in an aliquot of this solution as described by Ames (19).

Preparation of 1-acyl and 2-acyl-lysoPC. The two types of lysoPC were prepared by enzymatic hydrolysis. The incubations were carried out in 5-ml glass vials with Teflon-lined screw caps. For 1-acyl-lysoPC, dioleoyl-PC was hydrolyzed by bee venom phospholipase A₂. The reaction mixture (0.45 ml) contained 1 μmol dioleoyl-PC, 50 μg bee venom phospholipase A₂ (600–1800 U/mg), 50 mM Tris-HCl/100 mM sodium borate, pH 8.1, and 1.5 ml of water-saturated diethylether. After incubation at 37°C for 2 h, diethylether was evaporated under nitrogen and the lipids were extracted according to Bligh and Dyer. For 2-lysoPC, R. arrhizus lipase (100,000 U) was used as a source of phospholipase A₃, acting on dioleoyl-PC. The same conditions as above were used, except that 0.1% Triton X-100 (final concentration) was added to the assay mixtures and the reactions were extended for 2 h. In both cases, lysoPC was purified by thin-layer chromatography as described before.

Acylation of lysoPC. This procedure was based on the method described by Gupta et al. (20). Briefly, stearic anhydride was used to acylate lysoPC in the presence of dimethylaminopyridine. In those cases where radiolabeled stearic anhydride was used to acylate lysoPC, this compound was prepared from [¹⁴C]stearic acid, by reaction with dicyclohexylcarbodiimide, according to Lapidot (21), and diluted with unlabeled stearic anhydride to a specific activity of 5 μCi/μmol. The acylating reactions were carried out in volumes of about 30 μl and the molar proportions used of lysoPC, dimethylaminopyridine, and stearic anhydride were 1:0.55:5, and all reagents were dissolved in amylene-stabilized chloroform, eliminating the need for redistilling chloroform over phosphorus pentoxide. Routinely, 0.1 to 0.2 μmol of lysoPC was employed in the reactions which was carried out at 37°C in the dark, under N₂. The acylation was allowed to proceed for 72 h with constant stirring. This procedure yielded complete conversion of both types of lysoPC to PC, which was purified by TLC using the same method as described above for lysoPC, dissolved in chloroform, and stored at −20°C.

Enzymatic analysis of labeled PC. The isolated PC₅, containing the labeled free fatty acid in either position sn-1 or sn-2, were analyzed with bee venom phospholipase A₂ to release the fatty acid in position
sn-2 and with T. thermophila phospholipase A₂ for position sn-1. The incubation conditions for phospholipase A₁ were as described above. In the case of T. thermophila phospholipase A₁, the labeled PC was diluted with partially purified soy bean PC (0.3 mg per sample), which strongly stimulates the activity of this enzyme (10). The labeled PC and the soy bean PC were mixed in chloroform, the solvent evaporated under N₂, and the lipids were resuspended in 0.1 M sodium acetate/0.1 M sodium borate, pH 4.75, by vigorous vortexing in the presence of glass beads, and finally sonicated for 5 min in a bath sonicator. The assay mixture consisted of 300 μl of substrate and 100 μl of purified enzyme containing 10 μg (specific activity, 300 U/mg). For both enzymes, diethylether in saturating amounts was present during the incubations, which were extended for 2 h at 37°C. At the end of this period, the lipids were extracted according to Bligh and Dyer. Thin-layer chromatographic analysis was performed with the same system as described for the isolation of lysoPC. Autoradiography of the chromatograms was used to locate labeled compounds, identified by comigration with authentic lipid standards of lysoPC, PC, and oleic acid.

RESULTS AND DISCUSSION

The analysis of lysoPC positional isomers is presented here. The lysocompounds are purified and then acylated with a labeled fatty acid (FA). Enzymatic analysis of the resulting PC reveals which position was occupied by the entering FA and, thus, which position was free in the original lysoPC.

The method was applied to the two different lysoPC obtained from synthetic dioleoyl-PC by PLA₂ and by R. arrhizus lipase, which displays PLA₁ activity. Each lysocompound was separately acylated using [1-14C]stearic anhydride, generating 1-oleoyl-2-[1-14C]stearoyl-sn-PC and 1-[1-14C]stearoyl-2-oleoyl-sn-PC. Treatment of these two different PC molecules with PLA₂ was expected to generate only labeled FA in the first case and only labeled lysoPC in the second, and these were indeed the results obtained (Fig. 1). Thus, the procedures used did not result in appreciable acyl migration and appear suitable for the analysis of lysoPCs formed in natural systems.

In the protozoan parasite T. cruzi, large amounts of lysocompounds are formed upon autolysis, including lysoPC (M. Wainszelbaum, E. Isola, S. Wilkowsky, J. Florin-Christensen, and M. Florin-Christensen, unpublished). We have applied the present method to this system to determine the type of phospholipase A responsible for the formation of this lysoPC. The lipids of autolyzing T. cruzi suspensions were separated by TLC and the lysoPC was eluted from the plates and acylated with [1-14C]stearic anhydride as above. As shown in Fig. 1, lane 2, most of the radiolabeled product formed by treatment of the resulting PC with bee venom PLA₂ consisted of lysoPC. Therefore, in the original lysoPC isolated from the trypanosomes, position sn-1 was the one available for esterification. This unambiguously defines a role for a PLA₁ in generating lysoPC in T. cruzi-autolyzing suspensions. Minor amounts (9%) of labeled FA were also observed and may have resulted from the activity of a trypanosomal PLA₂ or by acyl migration during the autolytic process. These results were confirmed using PLA₁ purified from T. thermophila extracellular medium. Figure 2a, lane 3, shows that with this enzyme, the predominant final product is radiolabeled FA. R. arrhizus lipase, as a source of PLA₁, yielded similar results. However, this enzyme has appreciable lysophospholipase activity and incubation must be limited in time and enzyme concentration, to prevent complete decylation of PC. This is not the case for T. thermophila PLA₁, which in the presence of diethylether in the assay mixture displays no detectable lysophospholipase activity (10).

Bovine erythrocyte ghosts contain about 2% of lysoPC. When this natural compound was purified and acylated as described above, digestion of the resulting PC with bee venom PLA₂ gave a pattern opposite to
that observed in the case of trypanosomes, demonstrating a role for a PLA2 in this case (Fig. 2b). Such activity has indeed been previously found in bovine erythrocytes (22).

In the cases analyzed above, the lysoPC used came from unlabeled biological materials. It is possible, however, to metabolically label cells or tissues with radioactive fatty acids and then acylate the purified labeled lysoPC with unlabeled stearic anhydride. The rest of the procedure and the interpretation of results would be similar, except that now release of labeled fatty acid by phospholipase A2 implicates that position sn-2 was vacant in the original lysoPC. We have used this approach to investigate the lysoPC formed in heat-killed T. thermophila cells. These were labeled as described under Materials and Methods. The isolation of the lysoPC and its acylation were carried out as above, except that now the lysoPC is the radioactive component. In this experiment, we found that, after phospholipase A2 digestion, 86% of the radioactivity was recovered as fatty acid and 14% as lysoPC. Thus, 2-acyl-lysoPC is the predominant lysocompound formed upon heat-killing of T. thermophila, pointing out a role for phospholipase A2 activity in this process.

Anhydrides of other fatty acids different from stearate may be employed. Moreover, anhydrides of fluorescent fatty acid analogs could be used, eliminating the need for the handling of radioactivity.

In this work, we show the usefulness of the method in the case of lysoPC. The choline group in these compounds is unaffected by the acylating reagents. In principle, it is possible to apply the method to other phospholipids, such as lysophosphatidylethanolamine or lysophosphatidylinositol. However, acylation of the head group would in these cases take place and, therefore, protection of the head group prior to acylation would be required.

In conclusion, this study provides a simple methodology for determining the type of phospholipase A activated in living tissues or complex biological systems. This problem has hitherto remained unsolved. Our method affords a new tool for analyzing diverse signal transduction and other metabolic events where phospholipases A are involved.

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