The substrate specificities of four different lysophospholipases as determined by a novel fluorescence assay

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A novel fluorescence assay for quantifying lysophospholipase activity is described which utilizes a commercially available acrylodated intestinal fatty-acid-binding protein (ADIFAB) and non-radiolabelled substrate. Quantification of enzyme activity is based on the decrease in ADIFAB fluorescence at 432 nm in the presence of nanomolar concentrations of non-esterified ('free') fatty acids. Lysophospholipase activity measured by the ADIFAB assay and a conventional radiometric assay yield comparable results and have comparable levels of sensitivity (~ 10 pmol/min per ml). The ADIFAB assay has the advantageous features of continuous monitoring of enzyme activity and the availability of a broad range of potential substrates, because non-radiolabelled lysophospholipids can be employed in the assay. The hydrolytic activities of four lysophospholipases were determined, including a bacterial secreted phospholipase A_2/lysophospholipase, the human-eosinophil-secreted lysophospholipase, a human intracellular lysophospholipase (peak 3) isolated from HL-60 cells and a high-molecular-mass cytosolic phospholipase A_2/lysophospholipase from a mouse mammary carcinoma. Each of these enzymes was found to have a distinctive hydrolytic profile as determined by an array of lysophospholipids differing in their polar headgroups and *sn*-1 fatty-acyl substituents.

INTRODUCTION

Lysophospholipids play an important role in the pathogenesis of a broad spectrum of diseases and have been associated with myocardial infarctions, infections, neoplasia and asthma [1–6]. Specific lysophospholipids can participate in the regulation of signal-transduction processes and biological events, including modulation of protein kinase C activity, ion fluxes, G-protein-dependent signalling, mitogenic stimulation of cells and degranulation of mast cells [7–15]. Given the varied functions of lysophospholipids, the concentrations of these lipids must be regulated. One mechanism of regulation is hydrolysis by a lysophospholipase. Increased lysophospholipase activity has been implicated in such functions as protection of cells from the cytotoxic effects of lysophospholipids, attenuation of signal-transduction processes and host-mediated destruction of parasites [4,16,17]. In parallel with the diversity of processes in which lysophospholipases are implicated, they are diverse in their biochemical and physical properties [18–20]. Lysophospholipases have been isolated from a number of different tissues and cell types, including brain, liver, pancreas, neutrophils, eosinophils, macrophages and platelets [21–27]. Some proteins have both lysophospholipase and phospholipase A_2 activities (phospholipase Bs), and these enzymes have been isolated from such varied sources as *Vibrio parahaemolyticus*, and mammalian intestine and macrophages [28–32].

The determination of the substrates hydrolysed by each lysophospholipase is important in elucidating the functions of the individual enzymes. Substrate-specificity studies are limited by the availability of radiolabelled substrates for radiometric analyses and by the availability of sufficient enzyme for pH-stat assays. In the present paper a new lysophospholipase-activity assay is reported which utilizes non-radiolabelled substrates and yet has the sensitivity of a radiometric assay. This assay employs a commercially available and extremely sensitive fluorescent acrylodated derivative of an intestinal fatty-acid-binding protein (ADIFAB), which undergoes a decrease in fluorescence at 432 nm upon binding of non-esterified fatty acid [29]. This assay has been used to define the distinctive lysophospholipid specificities of a *Vibrio* phospholipase A_2/lysophospholipase and three mammalian lysophospholipases. The three mammalian enzymes differ with regard to cells of origin and cellular localization and are likely to have distinct functional roles. These enzymes are: (1) the human eosinophil lysophospholipase, which is released by activated cells; (2) a murine cytosolic phospholipase A_2/lysophospholipase which is observed in many cell types and becomes membrane-associated in response to increased cytosolic Ca^{2+} concentrations [32–35]; and (3) a cytosolic lysophospholipase from the HL-60 human promyelocytic cell line [36]. In the present study the relative hydrolysis of substrates differing in their fatty-acyl substituents and polar headgroups is found to be unique for each enzyme.

MATERIALS AND METHODS

Materials

ADIFAB was obtained from Molecular Probes (Eugene, OR, U.S.A.). The radioactive substrate 1-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (58 mCi/mmol) was obtained from New England Nuclear (Boston, MA, U.S.A.). All other lipids were obtained from Avanti Lipids (Birmingham, AL, U.S.A.), including synthetic lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidic acid and lysophosphatidylserine with various *sn*-1 fatty-acyl substituents (1-palmitoyl, 1-myristoyl, 1-stearoyl, and 1-oleoyl), plus 1-oleoyl-2-acetyl-glycero-3-phosphocholine and lysophosphatidylinositol from brain. Silica-gel G t.l.c. plates were obtained from Analtech (Newark, DE, U.S.A.). The bacterial (*Vibrio*) lysophospholipase (phospholipase

Abbreviations used: ADIFAB, acrylodated intestinal fatty-acid-binding protein; c.m.c., critical micelle concentration.

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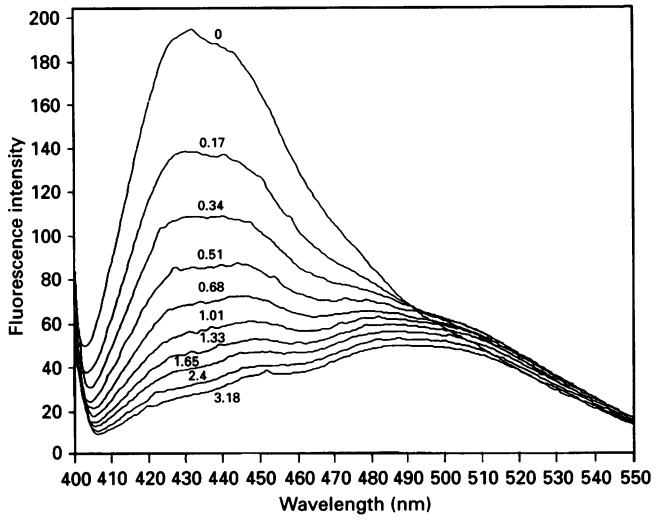

Figure 1 Changes in the fluorescence spectra of ADIFAB at various concentrations of palmitic acid

Fluorescence spectra of ADIFAB with increasing concentrations of palmitic acid are shown. The concentration of fatty acid varied from 0 to 3.18 μM in conjunction with 0.2 mg/ml of ADIFAB in 50 mM Tris, pH 7.5. The fluorescence spectrum was scanned from 400 to 550 nm. The fatty acid concentrations (μM) were as indicated. The highest fluorescence at 432 nm is observed with the sample with no fatty acid; the lowest fluorescence at this wavelength is exhibited by the sample with the highest concentration of fatty acid.

Table 1 The K_d values of ADIFAB for various fatty acids

The K_d value for each fatty acid was determined by performing the analysis described in the Materials and methods section.

	K_d (μM)	
Fatty acid	pH 7.5	pH 8.0
Myristic	1.19	2.81
Palmitic	0.32	0.61
Stearic	0.19	0.33
Oleic	0.28	0.43

B) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents and solvents were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Lysophospholipases

The 22 kDa lysophospholipase observed in HL-60 cells and butyric acid-induced differentiated HL-60 cells, 'peak 3', was purified as previously described [36]. The high-molecular-mass phospholipase A_2/lysophospholipase was partially purified approx. 200-fold from mouse mammary carcinomas by $(NH_4)_2SO_4$ precipitation, centrifugation and hydrophobic and anion-exchange h.p.l.c. [37]. The eosinophil lysophospholipase was purified by affinity chromatography from human eosinophils [25] and was homogeneous as determined by SDS/PAGE. Protein concentrations of enzyme samples were determined by the method of Bradford [38], using the dye reagent obtained from Bio-Rad (Richmond, CA, U.S.A.); BSA (Sigma) was used as a standard.

Lysophospholipase assay using ADIFAB

The standard reaction mixture for the ADIFAB lysophospholipase assay contained ADIFAB (4 mg/ml), buffer [either 50 mM Tris, pH 7.5, for assays of the *Vibrio* enzyme, or pH 8.0 for the assays of the mammalian lysophospholipases], lysophospholipid (as indicated for each experiment) and enzyme, in a final volume of 2.5 ml. Fluorescence was measured at 432 nm with a Perkin–Elmer LS50B fluorimeter with excitation at 390 ± 5 nm. Reactions were initiated by the addition of enzyme and mixtures were incubated at room temperature. The concentration of non-esterified fatty acid, and thereby enzyme activity, was calculated from the following equation:

$$[\text{Non-esterified fatty acid}] = K_d (F_{342} - B)/(F_{max.} - F_{432})$$

where F_{432} is the fluorescence observed at 432 ± 2.5 nm, B is the fluorescent blank observed at 432 nm with ADIFAB without any fatty acid (at zero time), and $F_{max.}$ is the fluorescence at 432 nm where there is maximal binding of non-esterified fatty acid by ADIFAB, i.e. when assayed in the presence of 15 μM fatty acid. The K_d for each fatty acid under each reaction condition was determined experimentally by varying the fatty acid concentration, as illustrated for palmitic acid in Figure 1 and tabulated for myristic, palmitic, stearic and oleic acids in Table 1.

Radiometric lysophospholipase assay

Radiometric assays were performed largely as previously described [19]. Briefly, the assay mixture contained 10 μM 1-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (190 μCi/mmol), buffers as indicated for the ADIFAB assay, and enzyme, in a reaction volume of 0.5 ml. Reactions were initiated by the addition of substrate; mixtures were incubated at room temperature and the reaction terminated by the addition of 1.25 ml of Dole reagent (propan-2-ol/heptane/0.5 M H_2SO_4, 39:10:1, by vol.), 1 ml of water and 1.5 ml of heptane. Radioactivity, in the upper organic phase, was quantified by liquid-scintillation spectroscopy.

RESULTS

Fluorescence assay for the measurement of lysophospholipase activity

The ADIFAB lysophospholipase assay was developed and optimized using a *Vibrio* lysophospholipase, since large amounts of this commercially purified enzyme were available. When this enzyme was incubated with 1-palmitoyl-*sn*-glycero-3-phosphocholine and ADIFAB, there was a decrease in fluorescence, indicative of the release of non-esterified fatty acid (Figure 2a). The magnitude of the decrease in fluorescence increased as a function both of time and of protein concentration. There was also a slight change in fluorescence in the absence of enzyme which increased as a function of time (Figure 2a, curve B). Thus the enzyme activity (release of fatty acid/unit of time per unit of enzyme) was calculated from the non-esterified-fatty-acid value determined with the enzyme minus the 'free fatty acid' value observed without the enzyme. The enzyme activity is indicated in Figure 2(b) as a function of time and protein concentration. Activity was linear over a 30 min incubation period for 3.8–15 ng/ml *Vibrio* lysophospholipase.

Lysophospholipase activities measured by the ADIFAB fluorescence assay and a radiometric assay were compared in order to assess the sensitivity of the fluorescence assay and its suitability relative to an established technique. Both assays were linear over the same range of enzyme concentrations (1.9–20 ng/ml) (Figure

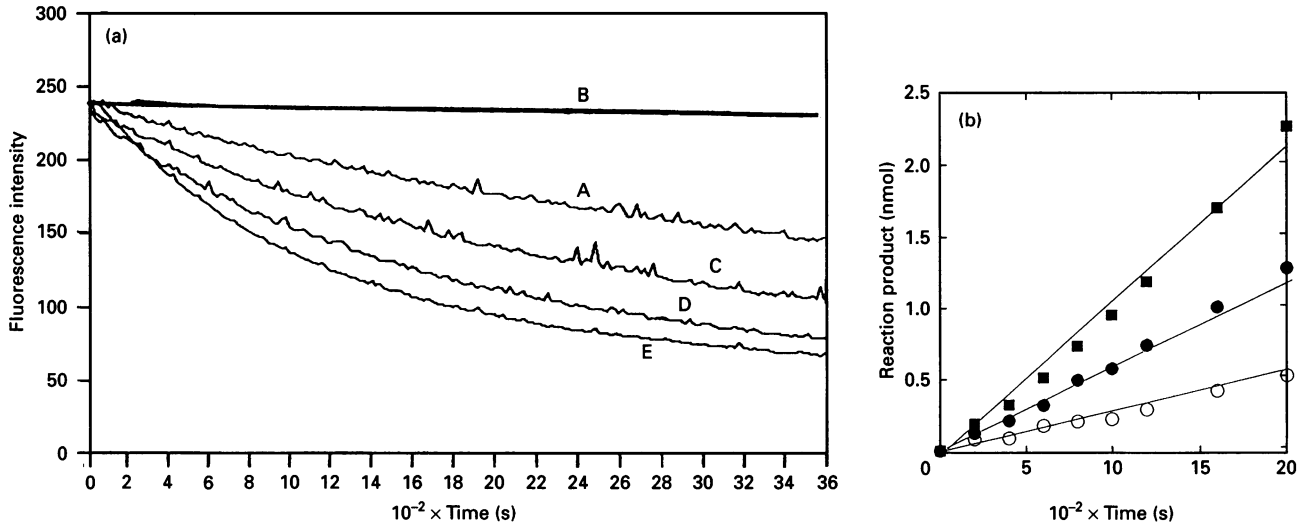

Figure 2 Measurement of fluorescence and determination of activity of *Vibrio* lysophospholipase in the ADIFAB assay as a function of time and enzyme concentration

(**a**) Fluorescence measurement at 432 nm with increasing concentrations of the *Vibrio* enzyme monitored over time. Spectrum A is the blank for the assay, which contained ADIFAB and substrate (10 μM 1-palmitoyl-*sn*-glycerophosphocholine) without enzyme. Spectrum B is the incubation of ADIFAB with buffer. Spectra C, D and E are the result of ADIFAB assays with 3.75, 7.5, and 15 ng/ml of *Vibrio* enzyme respectively. (**b**) The results of the ADIFAB lysophospholipase assay, expressed as pmol of fatty acid per assay. The reaction mixtures contained 3.75 (○), 7.5 (●) or 15 (■) ng/ml *Vibrio* lysophospholipase.

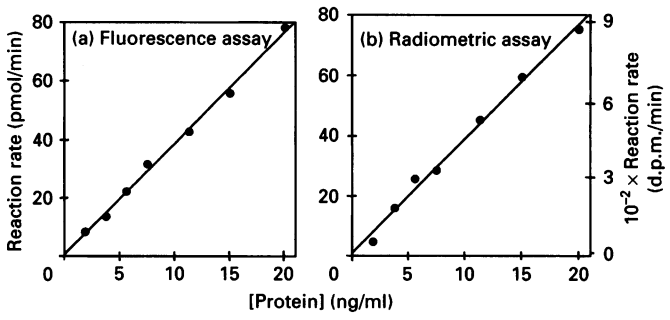

Figure 3 ADIFAB and radiometric analyses of *Vibrio* lysophospholipase activity as a function of enzyme level

The assays were performed with 10 μM 1-palmitoyl-*sn*-glycero-3-phosphocholine, 50 mM Tris, pH 7.5, and 7.5 ng/ml *Vibrio* enzyme, as indicated, plus ADIFAB added to fluorescence-assay reaction mixture. Samples were incubated for 20 min at room temperature and the activity then determined as described in the Materials and methods section. (**a**) ADIFAB assay; (**b**) radiometric assay.

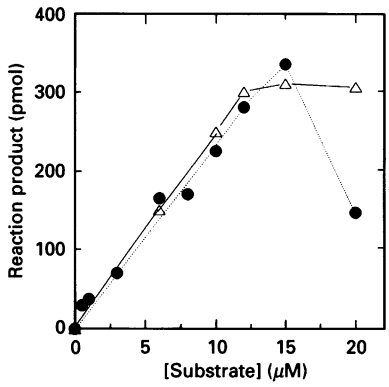

Figure 4 ADIFAB and radiometric analyses of *Vibrio* lysophospholipase activity as a function of substrate concentration

A comparison of lysophospholipase activity as measured using the ADIFAB (●) and radiometric (△) assay methods. The procedures used here were as described in Figure 3, with various substrate concentrations as indicated.

3). Furthermore, virtually identical levels of activity were obtained by the two methods, i.e., 1.55 μmol/min per mg for the fluorescence method and 1.56 μmol/min per mg for the radiometric method. The two assay methods also gave similar activity levels as a function of varying substrate concentrations up to 15 μM (Figure 4). When the substrate concentration was greater than 15 μM, there was a decrease in the detectable activity with the ADIFAB assay, in contrast with the findings with the radiometric assay, even when the radiometric and fluorescence assays are performed in the same reaction vessel. It is likely that the decreased level of activity observed with the ADIFAB assay is due to the fact that ADIFAB can bind only non-esterified fatty acid, i.e. vesicle-associated fatty acid does not bind to ADIFAB [42]. The critical micelle concentration (c.m.c.) of 1-palmitoyl-*sn*-glycero-3-phosphocholine has been estimated to be between 4.3 and 8.3 μM [39–41], and it is likely that non-esterified fatty acid will partition preferentially into lipid vesicles, e.g. 2.4% of 10 μM oleic acid is non-esterified when it is incubated with 100 μM phosphatidylcholine [42]. This suggests that the decrease in the non-esterified fatty acid observed in ADIFAB assays containing more than 15 μM lysophosphatidylcholine relates to association of the non-esterified fatty acid with the lysophospholipid micelles. Thus measurement of lysophospholipase activity with the ADIFAB assay should be performed below the c.m.c. of a potential substrate and/or at several substrate concentrations to ensure that a suitable substrate concentration

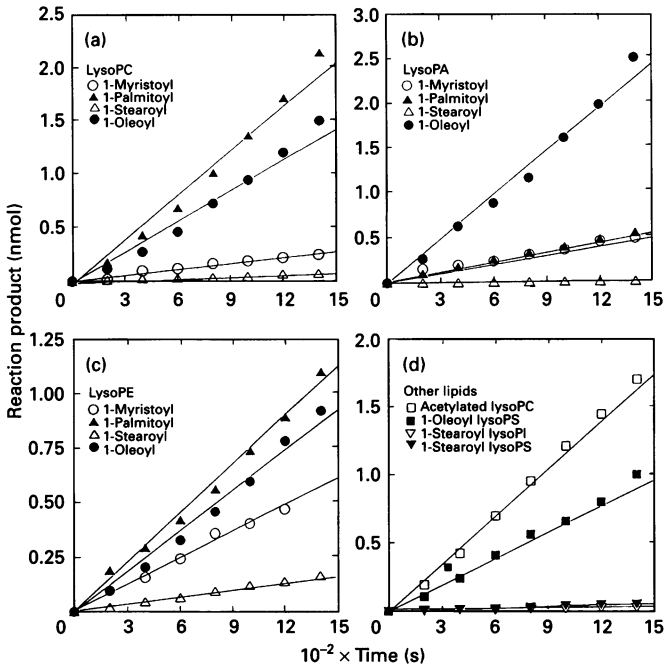

Figure 5 Substrate specificity of *Vibrio* lysophospholipase using the ADIFAB assay

The *Vibrio* enzyme (10 ng/ml) was assayed using ADIFAB (0.5 mg/ml) in 0.1 M Tris, pH 7.5. (**a**) Depicts lysophosphatidylcholine (LysoPC) substrates; (**b**) lysophosphatidic acid (LysoPA) substrates; (**c**) lysophosphatidylethanolamine (LysoPE) substrates. (**d**) Depicts the hydrolysis of acetylated 1-palmitoyl lysophosphatidylcholine, 1-oleoyl and 1-stearoyl lysophosphatidylserine (LysoPS) and 1-stearoyl lysophosphatidylinositol (LysoPI).

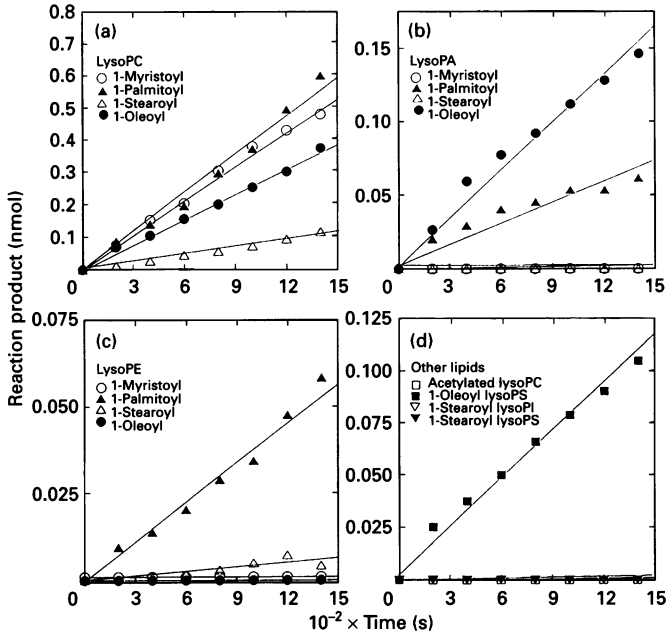

Figure 6 Substrate specificity of the murine phospholipase A_2/lysophospholipase

The murine phospholipase A_2/lysophospholipase (8 ng/ml) lysophospholipase activity was assayed using ADIFAB (0.5 mg/ml) and 0.1 M Tris, pH 8.0. (**a**) Depicts lysophosphatidylcholine substrates; (**b**) lysophosphatidic acid substrates; (**c**) lysophosphatidylethanolamine substrates. (**d**) Depicts the hydrolysis of acetylated 1-palmitoyl lysophosphatidylcholine, 1-oleoyl and 1-stearoyl lysophosphatidylserine and 1-stearoyl lysophosphatidylinositol. For abbreviations, see Figure 5.

is being employed. The c.m.c. of the various substrates is often difficult to determine, and may also be affected by various salt concentrations and pH. Therefore we have found that either performing the assays with several different concentrations of substrate or diluting the assay mixture severalfold prior to quantifying the reaction in the fluorimeter is useful for circumventing this problem. Both types of controls were done for all of the substrates used in these experiments.

Hydrolysis of lysophospholipids by a *Vibrio* lysophospholipase

The ability of the *Vibrio* lysophospholipase to hydrolyse lysophospholipids with differing polar headgroups and differing fatty-acyl substituents was evaluated (Figure 5). The fatty-acyl preference varied as a function of the polar headgroup of the lysophospholipid, although all lysophospholipids assayed with a stearoyl group were poor substrates. Hydrolysis of lysophospholipids with different polar headgroups was observed, and the palmitoyl and oleoyl lysophosphatidylcholines were the preferred substrates (Figure 5).

Comparative substrate specificities of three mammalian lysophespholipases

Lysophospholipases isolated from mouse mammary carcinomas (Figure 6), human eosinophils (Figure 7) and the human promyelocytic HL-60 cells (Figure 8) were compared with respect to

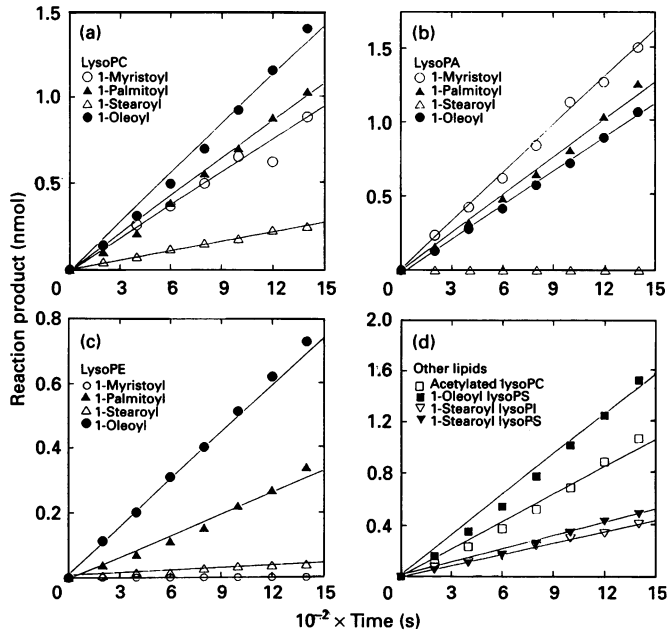

Figure 7 Substrate specificity of human eosinophil lysophospholipase

The purified human eosinophil lysophospholipase (12 ng/ml) was assayed by the ADIFAB method using ADIFAB (0.5 mg/ml), and 0.1 M Tris, pH 8.0. (**a**) Depicts lysophosphatidylcholine substrates; (**b**) lysophosphatidic acid substrates; (**c**) lysophosphatidylethanolamine substrates. (**d**) Depicts the hydrolysis of acetylated 1-palmitoyl lysophosphatidylcholine, 1-oleoyl and 1-stearoyl lysophosphatidylserine and 1-stearoyl lysophosphatidylinositol. For abbreviations, see Figure 5.

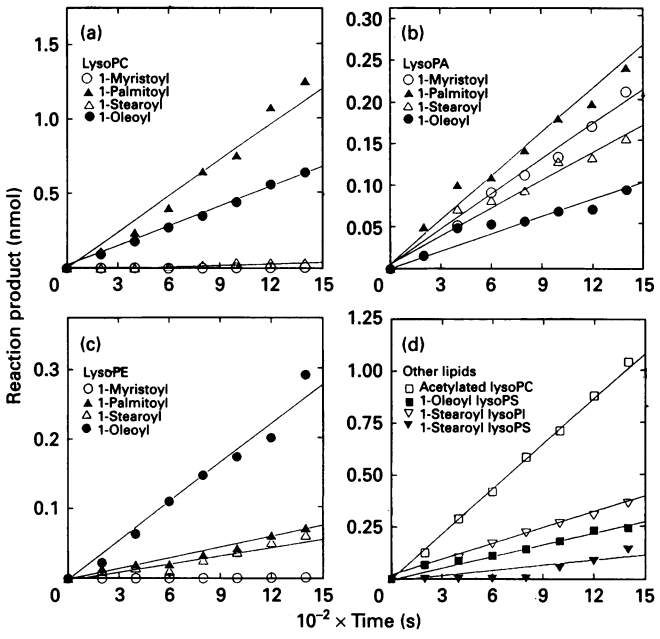

Figure 8 Substrate specificity of human HL-60 'peak 3' lysophospholipase

The intracellular lysophospholipase (0.45 μg/ml) was assayed by the ADIFAB method using ADIFAB (0.5 mg/ml), and 0.1 M Tris, pH 8.0. (**a**) Depicts lysophosphatidylcholine substrates; (**b**) lysophosphatidic acid substrates; (**c**) lysophosphatidylethanolamine substrates. (**d**) Describes the hydrolysis of acetylated 1-palmitoyl lysophosphatidylcholine, 1-oleoyl and 1-stearoyl lysophosphatidylserine and 1-stearoyl lysophosphatidylinositol. For abbreviations, see Figure 5.

their relative hydrolysis of substrates differing in their fatty-acyl substituents and polar headgroups. The data are summarized in Table 2. All three enzymes catalysed significant hydrolysis of lysophosphatidylcholines; however, each enzyme had a distinctive hydrolytic profile with respect to specific lysophosphatidylcholines. All the lysophospholipases effectively hydrolysed lysophosphatidylcholine with either an oleoyl or a palmitoyl group in the *sn*-1 position (Figures 6–8; Table 2). The murine phospholipase A_2/lysophospholipase and the eosinophil enzyme were also highly active with 1-myristoyl-*sn*-glycero-3-phosphocholine and hydrolysed 1-stearoyl-*sn*-glycero-3-phosphocholine, while the HL-60 enzyme did not. Only the eosinophil and HL-60 derived enzymes exhibited significant hydrolysis of 1-oleoyl-2-acetyl-*sn*-glycero-3-phosphocholine (Figures 6d, 7d and 8d; Table 2), while the murine phospholipase A_2/lysophospholipase failed to hydrolyse an *sn*-1 oleoyl group when the *sn*-2 position was acetylated.

In addition to the specificity with respect to lysophosphatidylcholines with various *sn*-1 and *sn*-2 substituents, the three enzymes also exhibited distinctive specificities of hydrolysis with respect to variations in the polar headgroups of lysophospholipids. This was most dramatic with respect to the mitogenic lysophospholipid lysophosphatidic acid (Figures 6b, 7b and 8b). Lysophosphatidic acid (palmitoyl, myristoyl and oleoyl) was the preferred substrate of the eosinophil enzyme, whereas the HL-60 enzyme hydrolysed the lysophosphatidic acid substrates at less than 9 % of the rate of hydrolysis of 1-palmitoyl-*sn*-glycero-3-phosphocholine. The murine phospholipase A_2/lysophospholipase hydrolysed the lysophosphatidic acid substrates very poorly, and only the 1-oleoyl- and 1-palmitoyl-*sn*-glycero-3-phosphates were hydrolysed, at rates that were 28 and 9 % respectively of the rate of hydrolysis of 1-palmitoyl-*sn*-glycero-3-phosphocholine (Figures 6–8; Table 2). Hydrolysis of 1-stearoyl-*sn*-glycero-3-phosphate was observed only with the HL-60 enzyme. Thus, while not exhibiting the highest activity with lysophosphatidic acid substrates, the HL-60 enzyme appeared to be the least selective with respect to the fatty-acyl substituent in the *sn*-1 position.

A similar pattern of a lower level of hydrolysis, but broader range, of substrates for the HL-60 cell enzyme as compared with the other mammalian enzymes was also observed with respect to

Table 2 Comparison of the activities of *Vibrio* and mammalian lysophospholipases with various lysolipid substrates

All substrates were used at 5 μM. Enzyme concentrations and other details of the assays are described in the legends to Figures 5–8. Abbreviations used: PLA_2, phospholipase A_2; LPL, lysophospholipase; lysoPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphoserine; PA, phosphatidic acid; PI, phosphatidylinositol.

	Activity (pmol/min)			
Substrate	*Vibrio* enzyme	Murine PLA_2/LPL	Eosinophil enzyme	HL-60 Pk3 enzyme
Palmitoyl lysoPC	79.0 (100)*	22.25 (100)	40.75 (100)	67.3 (100)
Myristoyl lysoPC	14.0 (17.7)	22.0 (98.8)	35.25 (86.6)	0 (0)
Stearoyl lysoPC	4.75 (6.0)	4.50 (20.2)	10.74 (26)	1.0 (1.5)
Oleoyl lysoPC	59.2 (75)	14.5 (65.2)	58.0 (142.3)	48.0 (71.3)
Palmitoyl lysoPE	44.0 (56)	2.25 (10.1)	13.5 (33)	4.5 (6.7)
Myristoyl lysoPE	31.6 (40)	0 (0)	0 (0)	0 (0)
Stearoyl lysoPE	7.30 (9.2)	0.25 (1.1)	2.25 (5.5)	4.0 (5.9)
Oleoyl lysoPE	38.8 (49)	0 (0)	30.5 (74.8)	10.0 (14.9)
Palmitoyl lysoPA	16.8 (21)	2.0 (9)	50.25 (123)	6.0 (8.9)
Myristoyl lysoPA	15.5 (19.6)	0 (0)	64 (167)	5.39 (8.0)
Stearoyl lysoPA	0 (0)	0 (0)	0 (0)	5.25 (7.8)
Oleoyl lysoPA	103.8 (131)	6.25 (28)	44.0 (108)	3.21 (4.8)
Oleoyl lysoPS	37.0 (46.8)	4.5 (20.2)	62.5 (153.4)	10.75 (16.3)
Stearoyl lysoPS	6.75 (8.5)	0 (0)	21.3 (52.1)	6.5 (9.6)
1-Oleoyl-2-acetyl lysoPC	68.8 (87)	0 (0)	43.5 (106.7)	42.0 (62)
LysoPI	3.75 (4.74)	0 (0)	18.75 (46)	10.79 (16)

* The values in parentheses represent the ratio of the lysophospholipase activity for the respective substrate divided by the activity observed with palmitoyl lysophosphatidylcholine × 100.

lysophosphatidylethanolamine substrates (Figures 6c, 7c and 8c). The HL-60 enzyme hydrolysed the 1-oleoyl-*sn*-glycerophosphoethanolamine substrate at about 15% of the rate of hydrolysis of 1-palmitoyl lysophosphatdiylcholine substrate, and the 1-palmitoyl and 1-stearoyl lysophosphatidylethanolamine substrates were both hydrolysed at about half that rate. The human eosinophil enzyme hydrolysed the 1-oleoyl lysophosphatidylethanolamine at a rate 2.3 times greater than 1-palmitoyl, and more than 10 times greater than the rate with 1-stearoyl-*sn*-glycerophosphoethanolamine substrate. This indicates that the eosinophil enzyme displays a marked selectivity with respect to fatty-acyl substituents of lysophosphatidylethanolamine as compared with the HL-60 enzyme. In contrast, the phospholipase A_2/lysophospholipase hydrolysed only the 1-palmitoyl phosphatidylethanolamine derivative to any appreciable extent.

Similarly, the HL-60 enzyme demonstrated a broader specificity for different fatty-acid substitutions in the *sn*-1 position of the lysophosphatidylserine substrates (Figures 6d, 7d, and 8d). The HL-60 enzyme hydrolysed 1-oleoyl-*sn*-glycero-3-phosphoserine at about 1.7 times the rate of the 1-stearoyl substrate. In comparison, the eosinophil enzyme demonstrated a preference for the 1-oleoyl derivative, which was hydrolysed at almost three times the rate of the 1-stearoyl substrate. The murine phospholipase A_2/lysophospholipase exhibited a preference for the 1-oleoyl-*sn*-glycero-3-phosphoserine and failed to hydrolyse the stearoyl derivative. In general, the phospholipase A_2/lysophospholipase exhibited the most restricted pattern of hydrolysis. That is, only 1-oleoyl phosphatidylserine, 1-oleoyl lysophosphatidic acid and the lysophosphatidylcholines were hydrolysed to any appreciable extent.

DISCUSSION

The newly described ADIFAB lysophospholipase assay provides a sensitive method for determining lysophospholipase activity. This assay has the sensitivity of a radiometric assay ($\sim$ 10 pmol/min per ml), yet has a broader applicability than a radiometric assay, owing to the availability of many more non-radiolabelled lysophospholipids (as compared with radiolabelled lysophospholipids). The ADIFAB assay allows for the continuous monitoring of enzyme activity, and linearity as a function of time can be readily determined. The inclusion of ADIFAB in the assay system may, in fact, assist in the stabilization of the activity and thereby extend the linearity, because exogenous protein has been observed to stabilize the activity of some lysophospholipases [43]. The major limitation of the ADIFAB lysophospholipase assay relates to the requirement of monomolecular fatty acid (and not micelle-associated fatty acid) for the binding by ADIFAB. This is the likely basis for the limitation in the maximal substrate concentration that can be used in the lysophospholipase assay, unless the assay were modified by diluting the terminated enzyme reaction until the fatty acid level is below the c.m.c.

Utilizing the broad range of potential substrates illustrated in Table 2, it was found that the *Vibrio* enzyme effectively hydrolyses a number of different lysophospholipids, including lysophosphatidylcholines (palmitoyl and oleoyl). Choline-containing phospholipids do not comprise a significant portion of the lipids found in *Vibrio* species [44]; however, lysophosphatidylcholine does stimulate protective host-cell functions such as monocyte chemotaxis, generation of superoxide and antibody production [45–47]. Because the *Vibrio* enzyme is a secreted protein, which would have access to host-cell lysophospholipids, the *Vibrio* enzyme may function to reduce protective host defence mechanisms. In a similar manner, *Vibrio*-enzyme-mediated hydrolysis of lysophosphatidylserine may affect mast-cell function, since this lysophospholipid may participate in mast-cell degranulation [15].

The mammalian lysophospholipases also exhibit distinctive substrate specificities. The hydrolysis of 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine by the eosinophil and HL-60 lysophospholipases may play a role in the regulation of inflammation, since inflammatory tissues have increased levels of this phospholipid [51]. These enzymes can also hydrolyse lysophosphatidylcholines and, thus, may participate in the regulation of the host defence mechanisms described above. The function of specific lysophospholipids varies with respect to the individual fatty-acyl substituents in the *sn*-1 position, as well as the composition of the polar headgroups [48–50]; hence, the fatty-acyl and polar-headgroup specificities of the lysophospholipases may regulate the levels and, therefore, the actions, of specific lysophospholipids. The high-molecular-mass murine phospholipase A_2/lysophospholipase enzyme has a more restricted substrate-hydrolysis profile than has the *Vibrio* enzyme. In view of the proposed role of this enzyme in the regulation of non-esterified arachidonic acid levels by its phospholipase A_2 activity, the lysophospholipase activity may serve to remove the other reaction product, the lysophospholipid. Given the involvement of lysophosphatidylcholine in a wide range of biological processes, it would be particularly important to control the level of this lysophospholipid.

In these studies a newly developed continuous lysophospholipase assay has been described. Continuous assays for phospholipase A_1 and phospholipase A_2 have been previously described using a modified substrate [52]. However one of the obvious advantages of this assay is the ability to utilize any natural substrate. As this assay quantifies the production of non-esterified fatty acid, it may be possible to modify this assay for the measurement of phospholipase A_1, phospholipase A_2 or any other enzyme that releases fatty acid from lipids or proteins. These studies illustrate the utility of the newly developed ADIFAB lysophospholipase assay in determining the substrate specificities of individual lysophospholipases. Given the rapidly expanding appreciation of the functional potencies of lysophospholipids, this new lysophospholipase assay should be a useful tool in the study of lysophospholipids.

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Received 21 July 1993/2 September 1993; accepted 13 September 1993