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The effect of *sn*-2 fatty acid substitution on phospholipase C enzyme activities

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The human monocyte cell line U937 expresses phospholipase A_2 and phospholipase C activities and produces eicosanoids. The phospholipase C (PLC) activity exhibits substrate preference for phosphatidylcholine (PC), rather than phosphatidylinositol or phosphatidylethanolamine. In order to characterize the PLC activity found in these cells, the effects of substitution of the *sn*-2 fatty acid on this activity were examined. PC substrates with palmitic acid (PC-2P), oleic acid (PC-2O), arachidonic acid (PC-2A) and linoleic acid (PC-2L) at the *sn*-2 position were used. The *sn*-1 fatty acid was palmitic acid. PC-2L and PC-2A with the longer-chain less-saturated fatty acids linoleic acid and arachidonic acid esterified at *sn*-2 were found to be better substrates for PLC activity than PC-2P or PC-2O in these cells. This preference was maintained even when substrate phospholipid was solubilized in non-ionic, anionic, cationic and zwitterionic amphiphiles. Furthermore, when a 500-fold excess of 1,2-diolein or 1,2-dipalmitin was added to the reaction, the specificity of the PLC activity for PC-2A and PC-2L remained unchanged. When similar experiments were performed with phosphatidylinositol as a substrate, we did not observe any effect when the *sn*-2 position was altered. These data show that the fatty acid constituent at the *sn*-2 position affects the observed PLC activity when phosphatidylcholine, but not phosphatidylinositol, is used as a substrate by these cells.

INTRODUCTION

Eicosanoids are produced by a number of cell types, including monocytes and macrophages, in response to inflammatory stimuli (Bonney *et al.*, 1979; Scott *et al.*, 1980, 1982*a*,*b*; Pawlowski *et al.*, 1983). Arachidonic acid and dietary linoleic acid are the precursors of these compounds. The intracellular concentration of these non-esterified fatty acids is low, and most of eicosanoid precursor fatty acids are esterified to the *sn*-2 position of phospholipids in eukaryotic cells (Flower, 1978; Irvine, 1982). The major phospholipids that contain arachidonic acid and linoleic acid are phosphatidylcholine (PC), phosphatidylethanolamine and phosphatidylinositol (PI).

Previous investigators have shown that eicosanoids are not stored and that the rate-limiting step in the biosynthesis of these compounds is the liberation of the unsaturated fatty acid by phospholipase enzymes (Flower, 1974, 1978). Two different phospholipase enzymes appear to be primarily responsible for fatty acid release (Dennis, 1983; Waite, 1985). The first is phospholipase A_2 (PLA_2), which hydrolyses the fatty acid from the *sn*-2 position of the phospholipid. The second is phospholipase C (PLC), which removes the polar head group from the phospholipid, producing the diacylglycerol, which is subsequently acted on by diacylglycerol lipase or diacylglycerol kinase, followed by a phosphatidic acid-specific PLA_2, thus liberating the fatty acid from the *sn*-2 position. The relative importance of these pathways is at present not well understood. However, if PLC is to be an efficient enzyme in releasing fatty acids that are used in eicosanoid production, it would be expected that the fatty acid at the *sn*-2 position may be important in modulating the observed enzyme activity.

Structure–activity relationships found for bacterial PLC (*Bacillus cereus*) have shown that symmetric modification of both acyl groups affects PLC activities (Little, 1977; Otnaess *et al.*, 1977; El-Sayed & Roberts, 1985; El-Sayed *et al.*, 1985), demonstrating that both the polar head group and the fatty acyl interfacial regions are essential for substrate recognition. PLC (*B. cereus*) also poorly hydrolyses lyso-PC (Otnaess *et al.*, 1977; El-Sayed & Roberts, 1985; El-Sayed *et al.*, 1985). These data suggest further that the *sn*-2 fatty acyl group is critical for bacterial enzyme binding and subsequent hydrolysis.

To date, however, the effects of various fatty acids in the *sn*-2 position on mammalian PLC activity have not been reported. Furthermore, investigations of the effect of various fatty acids in the *sn*-2 position on PLC activity, while keeping the *sn*-1 fatty acid constant, have not been reported for bacterial or mammalian cells. The purpose of the present study was to determine if the substitution of various saturated and unsaturated fatty acids at the *sn*-2 position of PI and PC affects PLC activity. The system chosen for studying this problem is the human monocytic cell line U-937. Because these cells produce a variety of eicosanoids (Cobb *et al.*, 1983; Myers & Siegal, 1984), we hypothesize that, if PLC were responsible for providing unsaturated fatty acids for eicosanoid synthesis, the fatty acid constituents at the *sn*-2 position should affect PLC activity.

Abbreviations used: PC, phosphatidylcholine; PI, phosphatidylinositol; PLA_2, phospholipase A_2; PLC, phospholipase C.

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MATERIALS AND METHODS

Materials

PC and PI with [^{14}C]arachidonic acid in the *sn*-2 position (PC-2A and PI-2A), PC with [^{14}C]linoleic acid in the *sn*-2 position (PC-2L), PC and PI with [^{14}C]oleic acid in the *sn*-2 position (PC-2O and PI-2O) and PC with [^{14}C]palmitic acid in the *sn*-2 position (PC-2P) were purchased from New England Nuclear (Boston, MA, U.S.A.). PI with [^{14}C]linoleic acid in the *sn*-2 position (PI-2L) was purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). The specific radioactivities of these substrates were 48–65 mCi/mmol. Phosphatidylcholine with [^{3}H]choline radiolabel (L-α-dipalmitoyl phosphatidyl[*Me*-^{3}H]choline, specific radioactivity 497 Ci/mmol) was also purchased from New England Nuclear. The solvents used were h.p.l.c. grade and obtained from Fisher Scientific (Philadelphia, PA, U.S.A.). Benzamidine, soya-bean trypsin inhibitor, bacitracin, phenylmethanesulphonyl fluoride, $CaCl_2$, sodium deoxycholate, Triton X-100, cetyltrimethylammonium bromide, NaCl, sodium acetate, Hepes, Tris and diacylglycerols were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Unlabelled phospholipids were obtained from Avanti Lipids (Birmingham, AL, U.S.A.). Silica-gel t.l.c. plates with pre-adsorbent zone were from Analtech Corp. (Newark, DE, U.S.A.). Cell-culture media were obtained from GIBCO (Grand Island, NY, U.S.A.). HP/b scintillation fluid was obtained from Beckman Diagnostics (Fullerton, CA, U.S.A.).

U937 cells

The cells were generously given by Dr. Georgio Trinchieri and Dr. Bruce Freundlich, Wistar Institute and the University of Pennsylvania. They were maintained in RPMI 1640 with 10% (v/v) heat-inactivated fetal-calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml), and were used while in the exponential phase of growth.

PLC assays and stoichiometry of the PLC hydrolysis products

The assay was performed in two different ways, as we have described previously (Bomalaski *et al.*, 1985, 1986*b*,*c*). First, PLC activity was quantified by the formation of diacylglycerol. This method was used to assess the effect of *sn*-2 fatty acid substitution on PLC activities. Cells in media were placed in 1.5 ml polypropylene Microfuge tubes and centrifuged for 1 min in a table-top Microfuge. The resultant supernatants were discarded, and cells were resuspended in phosphate-buffered saline containing proteinase inhibitors and quickly sonicated with six on-off bursts in a Branson Sonifer Cell Disrupter 200.

The organic solvent in which the radioactive phospholipids were dissolved was removed by blowing N_2 across the top of the tube. The residue was then dissolved in deoxycholate (5 mg/ml). The final reaction mixture contained 10 μl of substrate, 20 μl of the sonicated cells, 10 μl of buffer, and 10 μl of 50 mM-NaCl/0.4 mM-$CaCl_2$ solution. The reactions were started by the addition of substrate, incubated at 37 °C for 1 h and terminated by adding 50 μl of chloroform/methanol (1:2, v/v), followed by 50 μl of chloroform and then 50 μl of 4 M-KCl. All assays were performed in triplicate.

A 50 μl sample of the organic layer was spotted on silica-gel t.l.c. plates. Unlabelled phospholipid standards were also added to each lane to enhance detection, by I_2 vapour, of the product. To separate the PLC reaction products, a solvent solution of light petroleum (b.p. 38.7–56.5 °C)/diethyl ether/acetic acid (70:30:1, by vol.) was used. After the iodine stain had disappeared, the spots were scraped from the plates into 20 ml scintillation vials, to which 500 μl of methanol and 10 ml of scintillation fluor were sequentially added. Enzyme activity is expressed as pmol of reaction product (diacylglycerols) produced/min per mg of protein. In most experiments approx. 2000 c.p.m. of radioactive reaction product (diacylglycerol) was produced.

To confirm the stoichiometry of PLC activity as measured by diacylglycerol formation, PLC activity was compared both with the determination of phosphatidic acid, derived from *sn*-2-palmitic acid-labelled PC (phospholipase D activity), and with choline and phosphocholine, derived from polar-head-group-labelled dipalmitoyl PC (PLC activity). Thus PLC activity was measured with *sn*-2-labelled PC and compared with PLC activity measured with choline-labelled PC. The phospholipid reaction products were separated by two different procedures. For phospholipase D activity, phosphatidic acid and other phospholipids were separated by one-dimensional silica-gel t.l.c. with a mobile phase of chloroform/ethanol/triethylamine/water (30:34:35:8, by vol.) as we have described previously (Bomalaski *et al.*, 1986*a*) and by a one-dimensional two-solvent-system method. This latter system employed chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) as the first mobile phase (Bomalaski *et al.*, 1985, 1986*b*). After the mobile phase had run halfway up the plate, the plates were air-dried, and placed in light petroleum (b.p. 38.7–56.5 °C)/diethyl ether/acetic acid (70:30:1, by vol.). The plates were allowed to air-dry, developed with I_2 vapour and processed for scintillation counting as described above. Unlabelled phospholipid standards were also added to each lane, to enhance detection, by I_2 vapour, of the product. For PLC activity on choline-labelled PC, choline and phosphocholine in the alcoholic phase were separated by one-dimensional t.l.c. with a solvent system of methanol/0.5% NaCl/acetic acid (20:20:1, by vol.) (Wolf & Gross, 1986) with detection and scintillation counting as above.

Protein determination

Protein content of cell lysates was determined by the method of Bradford (1976), by using a dye reagent concentration obtained from Bio-Rad Laboratories, with bovine serum albumin as standard.

RESULTS

Kinetics, protein dependence, pH optima and Ca^{2+}-dependence of PLC

PLC activities as a function of pH are shown in Fig. 1. Irrespective of the *sn*-2 fatty acid, maximal activity was observed at neutral pH. Furthermore, irrespective of the pH used, the rank order of substrate is PC-2L > PC-2A > PC-2P > PC-2O (Fig. 1). All subsequent experiments were performed at pH 7.0. The rate of product formation was linear with time (Fig. 2) and protein

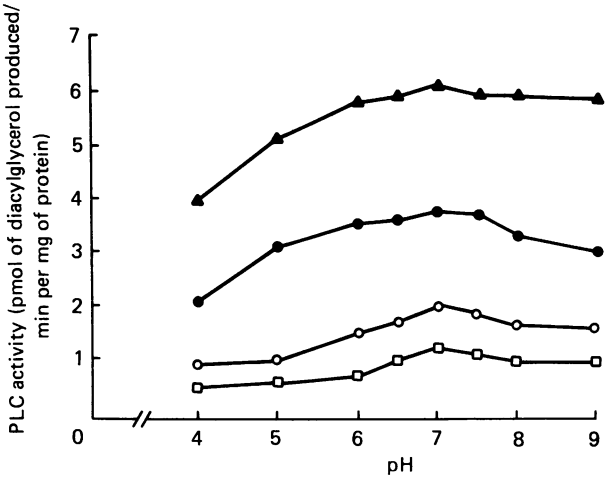

Fig. 1. PLC activities as a function of pH

Substrate phospholipids were prepared as described in the Materials and methods section: ●, PC-2A; ▲, PC-2L; ○, PC-2P; □, PC-2O. The buffers were 100 mM-sodium acetate (pH 4.0–5.5), 100 mM-Hepes/NaOH (pH 6.0–7.0) and 100 mM-Tris/HCl (pH 7.5–9.0). Three experiments were performed, with similar results.

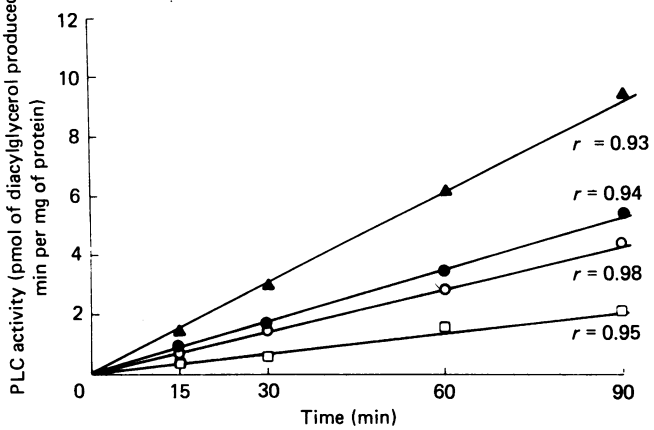

Fig. 2. PLC activities as a function of time

Substrate phospholipids were: ●, PC-2A; ▲, PC-2L; ○, PC-2P; □, PC-2O. Results are shown of a representative experiment, of which three were performed.

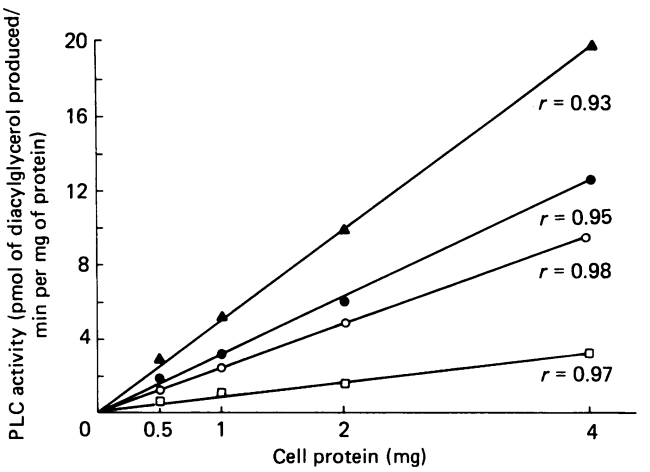

Fig. 3. PLC activities as a function of cell protein

Substrate phospholipids were: ●, PC-2A; ▲, PC-2L; ○, PC-2P; □, PC-2O. Results are shown of a representative experiment, of which three were performed.

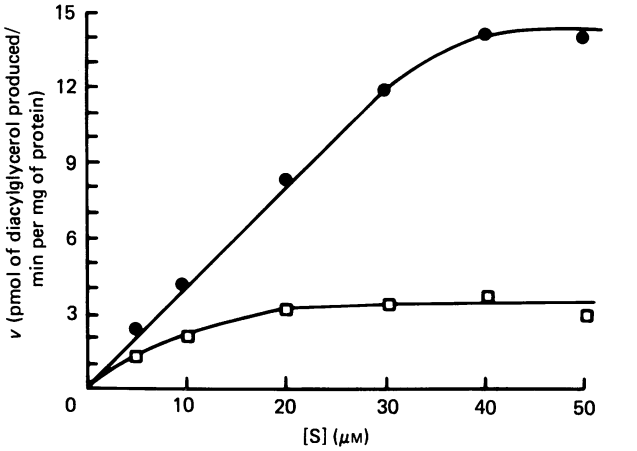

Fig. 4. Effect of substrate concentration on the rate of PLC activity

Substrate phospholipids were: ●, PC-2A; □, PC-2O. Results are shown of a representative experiment, of which three were performed.

(Fig. 3), irrespective of the substrate used under the experimental conditions employed. All experiments were performed in the presence of Ca^{2+} to obtain optimal apparent enzyme activities. Enzyme activity was observed in the absence of added Ca^{2+}, and the rank order of substrate preference was maintained even in the absence of Ca^{2+} (results not shown).

PLC stoichiometry

To confirm that the diacylglycerol produced was not formed by the actions of a phospholipase D and phosphatidate phosphohydrolase, and to confirm the stoichiometry of PLC hydrolysis products, quantification of phosphatidic acid, choline and phosphocholine was performed and compared with diacylglycerol formation. Phospholipase D activity was the same as background control values (results not shown). Incubations containing the polar-head-group-labelled PC were performed in parallel with incubations containing *sn*-2-labelled PC. Only radiolabelled diacylglycerol and phosphocholine were detected, and the molar ratio of phosphocholine to diacylglycerol was 0.80 ± 0.04. These results clearly demonstrate that the enzyme activity quantified by measurement of the diacylglycerol is that of a PLC.

Substrate-concentration–velocity relationship

To characterize PLC activities more fully, PC-2A and PC-2O were used as substrates and substrate phospholipid concentration was varied (Fig. 4). Lineweaver–Burk plots (Fig. 5) were generated. The apparent K_m values of PLC are 69.03 ± 6.30 μM with PC-2A as substrate and 17.62 ± 2.41 μM with PC-2O as substrate. The apparent

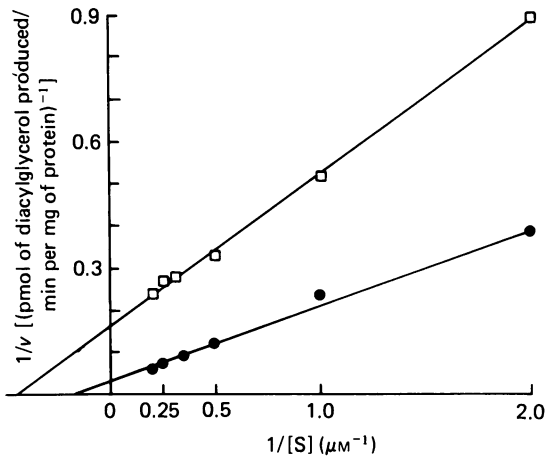

Fig. 5. Lineweaver–Burk plot of the data presented in Fig. 4

Substrate phospholipids were: ●, PC-2A (K_m 69.03 μM; $V_{max.}$ 37.43 pmol/min per mg of protein); □, PC-2O (K_m 17.62 μM; $V_{max.}$ 5.37 pmol/min per mg).

Table 1. Effects of detergents on PLC activities

Data (means ± S.D.) are from a representative experiment, of which three were performed. Sodium deoxycholate, cetyltrimethylammonium bromide (CTMB) and Triton X-100 were all used at their critical micelle concentrations (5 mM, 0.92 mM and 0.24 mM respectively). Lyso-PC was used at 1 mM.

	PLC activity (pmol of reaction product produced/min per mg of protein)			
Amphiphile	PC-2A	PC-2L	PC-2P	PC-2O
Sodium deoxycholate	6.1±2.2	2.3±2.5	0.7±0.3	0.8±0.2
CTMB	12.9±9.0	10.6±1.6	0.6±0.3	0.2±0.1
Triton X-100	1.7±1.0	5.7±0.2	0.7±0.2	0.5±0.1
Lyso-PC	1.1±0.1	1.2±0.4	0.3±0.2	0.3±0.1

$V_{max.}$ values are 37.43±3.29 pmol of substrate hydrolysed/min per mg of protein with PC-2A as substrate and 5.37±0.40 pmol/min per mg of protein with PC-2O as substrate.

Effects of detergents and diacylglycerols

Phospholipase activities may vary, depending on the detergent used to solubilize the substrate (Helenius & Simons, 1975; Lichtenberg *et al.*, 1983). Therefore radiolabelled phospholipids were solubilized in several different amphiphiles. Sodium deoxycholate (anionic), cetyltrimethylammonium bromide (cationic), lyso-PC (zwitterionic) and Triton X-100 (non-ionic) were used at their critical micelle concentrations (Lichtenberg *et al.*, 1983). The results from these experiments are shown in Table 1. Irrespective of the amphiphile, PLC activities with PC-2A or PC-2L as a substrate were appreciably greater than those with PC-2O or PC-2P as a substrate.

In order to determine if the structure of the micelle was important, substrate phospholipid was solubilized in a 100-fold molar excess of 1,2-diolein and 1,2-dipalmitin (Table 2). Regardless of the detergent or diacylglycerol used to suspend the substrate phospholipid, PLC activities with PC-2P or PC-2O as substrate were always appreciably less than those with PC-2A or PC-2L.

Table 2. Effects of micelle composition on PLC activity

Data (means ± S.D.) are from a representative experiment, of which three were performed. Diacylglycerols were used at 1 mM final concentration, and were evaporated under N_2 with the phospholipid substrate before resuspension in sodium deoxycholate, as described in the Materials and methods section.

	PLC activity (pmol of reaction product produced/min per mg of protein)			
Diacylglycerol	PC-2A	PC-2L	PC-2P	PC-2O
1,2-Diolein	1.1±0.4	0.8±0.1	0.5±0.3	0.4±0.2
1,2-Dipalmitin	1.4±0.2	3.5±2.0	0.7±0.2	0.6±0.1

Table 3. Effect of *sn*-2 substitution on PLC activity with PI as substrate

Data (means ± S.D.) are from a representative experiment, of which three were performed. PLC activities determined as described in the Materials and methods section were the same as those employed with PC as substrate. These conditions also produced optimal PLC activities with PI as substrate.

Substrate	PLC activity (pmol of reaction product produced/min per mg of protein)
PI-2A	0.69±0.02
PI-2L	0.69±0.02
PI-2O	0.67±0.08

Effects of *sn*-2 fatty acid substitution on PLC activities with PI as substrate

Although the preferred substrate for the PLC activity in these cells was PC, PLC activity with PI as substrate was also observed. The effect of *sn*-2 fatty acid substitution in PI on apparent PLC activities was also investigated. The substrates used were PI-2A, PI-2L and PI-2O (PI-2P was not available). The results from these experiments are shown in Table 3. Unlike the PLC activity observed with PC as substrate, PLC activity with PI was unaffected by *sn*-2 substitution.

DISCUSSION

The apparent PLC activity of the human monocyte cell line U937 exhibits greater activities against PC when longer-chain less-saturated eicosanoid precursor fatty acids are esterified at the *sn*-2 position. This preference for PC-2L and PC-2A over PC-2P and PC-2O occurs when substrate PC was presented in non-ionic, cationic, anionic and zwitterionic amphiphiles, and in the presence of excess diacylglycerol.

The enhanced PLC activities seen with PC-2A and PC-2L occurred when enzyme activities were examined as a function of pH, time and protein concentrations (Figs. 1–3). The pH–activity curves are flat and express different pH optima from those observed with PLC activities in resident mouse peritoneal macrophages (Wightman *et al.*, 1981) and Bacillus Calmette Guerin-induced alveolar macrophages (Eisen *et al.*, 1984). Also, the mouse macrophage P388D1 cell line does not express appreciable PLC activity (Ross *et al.*, 1985). Species and mononuclear phagocyte lineage differences may account for these variations.

Enhanced PLC activity with PC-2A as substrate, containing an unsaturated eicosanoid precursor fatty acid, compared with PC-2O, containing a less-saturated fatty acid that is not an eicosanoid precursor, is seen when substrate phospholipid concentration is varied, and Lineweaver–Burk plots are generated (Figs. 4 and 5). Similar enhanced PLC activity was observed with PC-2L, containing an unsaturated dietary fatty acid that must be elongated and desaturated to form arachidonic acid. Thus these cells express enhanced apparent PLC enzyme activities when the *sn*-2 fatty acid has a longer chain and is less saturated. These findings are not due to diacylglycerol formation by the combined activity of phospholipase D and phosphatidate phosphohydrolase, as appreciable phospholipase D activity is not detected under the experimental conditions employed, and PLC activity, as measured with both *sn*-2-labelled and choline-labelled substrate, shows excellent correlation.

Little information is available as to how acyl-group substitution influences phospholipase activity. Using *Naja naja* PLA_2, which prefers PC as substrate, Roberts *et al.* (1978) demonstrated increasing activity with decreasing fatty acid chain lengths when the same fatty acid was in both *sn*-1 and *sn*-2 acyl chain groups. Similar results were observed for *Bacillus cereus* PLC activity. Thus, for both *Naja naja* PLA_2 and *B. cereus* PLC, an increase in the rate of hydrolysis ratios was observed on going from dipalmitoyl ($C_{16:0}$) to dilauryl ($C_{12:0}$) PC. More recent studies with *B. cereus* PLC also indicate that enhanced enzyme activity occurs with an increase in the number of methylene groups in phenylalkanoyl phosphatidylcholines (El-Sayed & Roberts, 1985; El-Sayed *et al.*, 1985). These data suggest that the degree of unsaturation in the acyl groups may regulate PLC activity, and correlates with the enhanced activity seen with the long-chain less-saturated PC-2A and PC-2L PLC activity in U937 cells observed under the experimental conditions employed.

These data shown suggest that there may be multiple PLC enzymes with different substrate specificities in U937 cells. There is precedence for this hypothesis, as *B. cereus* contains both a PI-specific PLC enzyme and a PC-preferring PLC enzyme (Little, 1981; Ikezawa & Taguchi, 1981), rat tissues contain heterogeneous PLC (Irvine *et al.*, 1979; Hirawasa *et al.*, 1982*a*,*b*), human platelets contain two PI-specific PLC enzymes (Chau & Tai, 1982; Lenstra *et al.*, 1984), sheep seminal-vesicular glands contain three immunologically distinct PLC enzymes (Hofmann & Majerus, 1982*a*,*b*), and bovine heart contains four distinct PLC forms (Low & Weglicki, 1983; Low *et al.*, 1984).

As phospholipase activities may vary, depending on phospholipid presentation in an amphiphile (Helenius & Simons, 1975; Lichtenberg *et al.*, 1983), the phospholipids were solubilized in detergents with different ionic strengths and PLC activities were examined (Tables 1 and 2). Regardless of the detergents used, PLC activities with PC-2A and PC-2L as substrate were always greater than with PC-2O and PC-2P as substrate.

As a control, we also examined the effect of *sn*-2 fatty acid substitution on apparent PLC activities with PI as substrate (Table 3). PLC activities with PI as substrate were not affected by the *sn*-2 fatty acid, in contrast with PC. PI-specific PLC from pig and human platelets also is not affected by changing the fatty acyl groups (Holub & Celi, 1984). Thus our results could not be explained by specific diacylglycerol lipases or diacylglycerol kinases, as the diacylglycerol substrate for these enzymes is the same once the polar phospho head group (choline or inositol) is cleaved by PLC.

In conclusion, the human monocyte cell line U937 expresses different PLC activities, depending on the *sn*-2 fatty acid of the substrate phospholipid, and specifically expresses greater apparent activities when the longer-chain less-saturated fatty acids arachidonic acid and linoleic acid are esterified at the *sn*-2 position, in contrast with palmitic acid and oleic acid. This preference occurs even when substrate phospholipids are presented in a variety of amphiphiles at different concentrations. These enhanced activities are not seen when the *sn*-2 fatty acids of PI were varied. These data suggest that multiple PLC enzymes may be present and that the *sn*-2 carbonyl groups and resultant adjacent interfacial regions are important in the regulation of PLC activity in these cells.

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