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Mass Spectrometric Analysis of Arachidonyl-containing Phospholipids in Human U937 Cells

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The human histiocytic lymphoma U937 cell line contains a rich source of the 85 kDa cytosolic phospholipase A_2 (cPLA$_2$). DMSO-differentiated U937 cells were used as a model to investigate the free arachidonic acid release, the arachidonate distribution and the phospholipid source of arachidonate upon Ca^{2+} ionophore stimulation. A combination of several chromatographic and mass spectrometric techniques was employed in this study. The amount of free arachidonic acid (AA) released upon stimulation, the arachidonate content in total lipids and in each of the phospholipid classes were determined by gas chromatography/mass spectrometry (GC/MS). Glycerophosphoethanolamine (GPE) was found to be the major pool of arachidonate in differentiated human U937 cells (55%) and glycerophosphocholine (GPC) and glycerophosphoinositol (GPI) contributed 22 and 8%, respectively. Upon Ca^{2+} ionophore stimulation, GPE class lost the largest amount of arachidonate, followed by GPC class. GPI class, however, gained a substantial amount of arachidonate. Most of the arachidonate depleted from GPE and GPC was recovered as free AA, some of which was rapidly esterified into GPI species. GC/MS with electron capture negative chemical ionization provided excellent sensitivity for the measurement of arachidonic acid which was derivatized to its pentafluorobenzyl ester. Intact phospholipid molecular species including the arachidonyl-containing phospholipid species were identified using capillary high-performance liquid chromatography/continuous-flow liquid secondary ion mass spectrometry (CF-LSIMS). No specificity was found for releasing free AA among the arachidonyl-containing GPE and GPC species upon Ca^{2+} ionophore stimulation. CF-LSIMS provided a sensitive and effective means of detecting intact phospholipid species. **Copyright © 1999 John Wiley & Sons, Ltd.**

KEYWORDS: phospholipids; arachidonic acid; gas chromatography/mass spectrometry; capillary high-performance liquid chromatography; continuous-flow liquid secondary ion mass spectrometry

INTRODUCTION

Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is an essential fatty acid and plays a unique role as a precursor molecule for potent biological mediators. Arachidonic acid is metabolized through two major pathways. The cyclooxygenase pathway of arachidonic acid metabolism leads to the formation of prostaglandins, thromboxanes and prostacyclin.[1] The lipoxygenase pathway leads to the production of leukotrienes.[2] Oxygenated arachidonic acid metabolites are mediators of broncho-constriction, vaso-constriction and inflammatory reactions. They are implicated in the pathology of diseases in humans, e.g. asthma, arthritis, psoriasis and inflammatory bowel disease.[3] The rate limiting step in the biosynthesis of these mediators such as prostaglandins and leukotrienes is believed to be initiated by the activation of a specific phospholipase A_2 (PLA$_2$), which releases arachidonic acid from the *sn*-2 position of endogenous membrane phospholipids.[4,5] However, it remains unknown which are the exact phospholipid sources of endogenous arachidonic acid utilized for production of these metabolites during cell activation. Most of the studies[6–9] trying to address this question involved using cells prelabeled with arachidonic acid followed by cell stimulation. Other studies involved measurement of the quantity of arachidonate converted into eicosanoids and the quantity released from phospholipids following cell activation.[8–10] Major discrepancies were found between the two approaches to determine sources of arachidonate for eicosanoid biosynthesis.[9] In the radiolabeled experiments, little was generally known about the size of various arachidonate pools or rates of incorporation of exogenously added arachidonic acid into those pools. The total PLA$_2$

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activity was often underestimated,[10] and it was very difficult to determine the contribution of arachidonate from a particular species during cell activation. The measurements of the quantities of endogenous arachidonate was a more direct approach for determining PLA_2 activities; however, in these early studies the arachidonate mobilization upon stimulation was investigated only among the phospholipid classes, not at the individual phospholipid molecular species level.

In our early efforts searching for PLA_2 inhibitors as potential anti-inflammatory agents, DMSO-differentiated human U937 cells (dU937 cells) were used as the primary cell line for assaying arachidonic acid release. The U937 cell is a human histiocytic lymphoma cell line which is a rich source of the 85 kDa cytosolic phospholipase A_2 ($cPLA_2$). The arachidonic acid distribution in the dU937 cell membrane phospholipids has become of interest to us. A more direct and detailed approach was employed in the present study to address the question of the specific phospholipid sources of arachidonate. The total cellular arachidonate content and free arachidonate released from differentiated human U937 cells upon Ca^{2+} ionophore challenge were measured by gas chromatography mass spectrometry (GC/MS). The arachidonic acid distribution in each of the major phospholipid classes before and after ionophore challenge was also determined. Phospholipid molecular species composition of the human dU937 cells was analyzed by capillary high-performance liquid chromatography (HPLC)/continuous-flow liquid secondary ion mass spectrometry (CF-LSIMS). The analysis of molecular species within a phospholipid class has been a challenging and time-consuming task owing to the diversity and number of phospholipid molecular species. In a previous publication,[11] we demonstrated that CF-LSIMS offered clear advantages over electrospray LC/MS and traditional probe fast atom bombardment (FAB). Using sub-nanogram amounts of phospholipid, information regarding the molecular mass, the polar head group and the fatty substituents can be obtained from the positive and negative ion full-scan LSI mass spectra, allowing the phospholipid species to be readily identified. This paper presents the direct application of the CF-LSIMS technique to the analysis of complex phospholipid species within a biological system. The extremely good sensitivity in detecting intact phospholipids was further demonstrated. This high-sensitivity technique for the analysis of phospholipids is valuable for the lipid research. Analysis of arachidonyl-containing phospholipid molecular species before and after Ca^{2+}-ionophore challenge can provide an insight into whether or not there is a specific phospholipid precursor pool within the cell that preferably releases arachidonic acid.

EXPERIMENTAL

Materials

U937 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). RPMI 1640 medium, fetal bovine serum (FBS), and penicilin/streptomycin were purchased from GIBCO (Ivine, UK). Hank's balanced salt solution (HBSS) and calcium ionophore A23187 were purchased from Sigma (St Louis, MO, USA). Calcium ionophore stock solution and U937 cell differentiation were prepared with dimethyl sulfoxide (DMSO) obtained from J. T. Baker (Philipsburg, NJ, USA). Phospholipid standards were obtained from Avanti Polar Lipids (Alabaster, AL, USA), diisopropylethylamine and pentafluorobenzyl bromide from Pierce (Rockford, IL, USA), potassium hydroxide from American Chemicals (Montreal, Quebec, Canada), ammonium acetate and pure arachidonic acid from Aldrich (Milwaukee, WI, USA) and deuterated arachidonic acid (d_8-arachidonate) from Cayman Chemical (Ann Arbor, MI, USA). All organic solvents were of HPLC grade, from either Accusolv (Toronto, Ontario, Canada) or Omnisolv (Gibbstown, NJ, USA). Water was deionized with a Millipore filtration system.

Cell growth, differentiation and activation

The U937 cell is a human histiocytic lymphoma cell line. The cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicilin/streptomycin. The cells were grown at 37 °C with 6% CO_2 and maintained at a density of (0.15–1.5) $\times$ 10^6 cells ml^{-1}. Cell counting and viability were determined by trypan blue exclusion. The cells were diluted with fresh complete media every 4 days, when the cell density reached the upper limit. The cells were induced to differentiate to a neutrophil-like phenotype by culture in 1.3% DMSO for 4 days. The differentiated U937 cells were grown in suspension in a 500 ml spinner flask (62 rpm) at 37 °C, with 6% CO_2.

Differentiated U937 cells were harvested in polypropylene centrifuge tubes, centrifuged for 5 min at 1200 rpm and resuspended in HBSS and adjusted to 2.0 $\times$ 10^6 cells ml^{-1}. Cells (2 ml each) were challenged with vehicle (DMSO in HBSS) or with A23187 (10 μM in HBSS) for 3 min at 37 °C.

Two sets of experiments were carried out in parallel; one set was used for free arachidonate measurements and the other for phospholipid analysis.

Free arachidonic acid measurement

One set of incubations was terminated by adding 3.3 ml of Dole and Meinertz's solvent[12] (120 ml of propan-2-ol, 30 ml of heptane, 3 ml of 1 M H_2SO_4). A 50 ng amount of d_8-AA was added as an internal standard. After mixing, an additional 2 ml of heptane were added. The top heptane phase containing free fatty acids was taken into a glass vial and dried under vacuum. A set of arachidonic acid standards ranging from 1 ng to 1 μg spiked in HBSS were also prepared the same way.

The free fatty acids were derivatized to their pentafluorobenzyl (PFB) esters by a modification of Blair *et al.*'s method.[13] Briefly, 100 μl of the derivatizing reagent (10% diisopropylethanolamine, 0.1% pentafluorobenzyl bromide in CH_3CN) were added to each sample. The reaction was held at 60 °C for 15 min. The excess

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reagents were removed under vacuum and the derivatized arachidonic acid was resuspended in 1 ml of dodecane for GC/MS analysis.

Isolation of phospholipids from dU937 cells

Simultaneously with the Dole and Meinertz extraction procedure, the other set of incubations was terminated by adding 7.5 ml of $CHCl_3$–CH_3OH (1:2, v/v). Lipids were extracted according to the method of Bligh and Dyer.[14] Briefly, chloroform (2.5 ml) and then water (2.5 ml) were added, each time mixing vigorously. Following a 10 min centrifugation at 3000 rpm, the $CHCl_3$ phase containing lipids was removed and evaporated to dryness under vacuum. The lipids were redissolved in 400 μl of hexane–propan-2-ol–water (6:8:1, v/v/v) before HPLC separation.

Phospholipid classes were isolated by normal-phase HPLC on a LiChrosorb Si-60, 5 μm, 250 × 4.6 mm i.d. silica column (Alltech). A gradient separating at a flow-rate of 1 ml min^{-1} was employed with mobile phases A (hexane–propan-2-ol–25 mM aqueous ammonium acetate, 300:400:30, v/v/v) and B (hexane–propan-2-ol–25 mM aqueous ammonium acetate, 300:400:70, v/v/v). The gradient started with 90% A for 10 min, increased linearly to 100% B over 20 min, and held at 100% B for 15 min. The gradient was then returned to its initial conditions and equilibrated for 5 min. The absorbance was monitored at 206 nm. Major phospholipid classes, PE, PI, PS and PC fractions, were collected manually based on the retention times of commercial phospholipid standards, and were stored at −80 °C before further analysis.

Cellular arachidonate content and arachidonate distribution

In addition to the measurement of free arachidonic acid released from differentiated human U937 cells upon activation, the amounts of arachidonate in the total lipid pool and also in each of the phospholipid classes were determined. An aliquot of the total lipid extract and each collected fraction of phospholipid classes was dried under vacuum. Lipids were hydrolyzed using the standard base hydrolysis procedure.[15] Briefly, d_8-AA (50 ng) was added as an internal standard, 1 ml of 2 M KOH in ethanol–water (3:1, v/v) was added and vortex mixed and the reaction was held at 60 °C for 30 min. After cooling, additional water (1 ml) was added and the pH was adjusted to 3 with 6 M HCl. The free fatty acids were extracted with two volumes of hexane and were converted to their PFB esters as described above for GC/MS analysis. A set of arachidonic acid standards ranging from 1 ng to 1 μg spiked in HBSS were also prepared the same way. These standards were used to construct a calibration curve to quantify the arachidonate contents in lipid fractions.

GC/MS analysis

A Finnigan SSQ7000 mass spectrometer equipped with a Varian gas chromatograph was used for arachidonic acid quantification. A 1 μl volume of the sample solution was injected on-column into a 1 m retention gap attached to a fused-silica capillary column (Rtx-1, 25 m × 0.25 mm i.d., 0.25 μm film thickness, from Restek). The initial column temperature was 210 °C and was increased to 310 °C at a rate of 20 °C min^{-1}. Helium was used as the carrier gas with a head pressure of 10 psig. Electron capture negative chemical ionization was employed with methane as reagent gas. Under such conditions, predominant carboxylate anions at m/z 303.2 (AA) and 311.2 (d_8-AA) were detected for AA-PFB and d_8-AA-PFB, resulting from the loss of a pentafluorobenzyl radical.[13] Selected ion monitoring of the two ions at m/z 303.2 (AA) and 311.2 (d_8-AA) was therefore used. A calibration curve ranging from 1 to 1000 ng AA was constructed, and the peak area ratios were linear over this concentration range. The amount of arachidonic acid was determined using the peak area ratio and regression parameters from the calibration curve.

CF-LSIMS characterization of phospholipid molecular species

Intact phospholipid molecular species within each phospholipid class were analyzed using capillary HPLC/CF-LSIMS on a JEOL HX110A double focusing mass spectrometer.[11] The HPLC-isolated phospholipid classes were dried under vacuum and dissolved in 1 ml of methanol–propan-2-ol (80:20, v/v) containing 1.5% glycerol. The capillary HPLC flow was supplied by splitting the main flow (1.0 ml min^{-1}) from a Waters 600MS HPLC pump to 3 μl min^{-1} using an open split at a Valco tee (Valco Instruments, Houston, TX, USA). The flow was directed through a Valco injector (with a 10 μl loop) and then through a KAPPA Hypersil BDS C_{18} capillary column (100 mm × 0.30 mm i.d.) (Keystone Scientific, Bellefonte, PA, USA) to the frit probe of the JEOL mass spectrometer. The mass spectrometer was operated in the LSIMS mode. Ions were produced by bombardment with a beam of Cs^+ ions (10 keV for the positive ion mode and 15 keV for the negative ion mode), with the ion source accelerating voltage at 10 kV. The resolution was set at 1000. Data acquisition was in either the negative or positive ion mode and the mass spectrometer was scanned at a rate of 4 s from m/z 0 to 1000 Da. The background produced by the glycerol matrix was fairly stable in our system. The background-subtracted mass spectra were therefore obtained by averaging a few scans associated with the maximum of a specific peak in the reconstructed ion chromatogram and subtracting an average matrix ion spectrum from the adjacent chromatographic background.

CF-LSIMS characterization of phospholipid molecular species was described in detail previously.[11] Distinguishing between the 1-*O*-alkyl-2-acyl and 1-*O*-alk-1′-enyl-2-acyl (plasmalogen) phospholipid species was accomplished by acid hydrolysis. Plasmalogen species are acid labile and were converted to lysophospholipids upon acid treatment (with 2 M HCl at room temperature for 30 min), whereas the diacyl and 1-*O*-alkyl-2-acyl species were acid stable.[16]

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Tandem mass spectrometric measurement of phospholipid molecular species

Parent ion scanning and multiple reaction monitoring (MRM) measurements were performed on a JEOL HX110A hybrid mass spectrometer with an EBQQ geometry. The magnetic sector was used as MS-1 and the second quadrupole was used as MS-2. Ions were mass selected by the magnetic sector and deaccelerated before passing through the first quadrupole. The first quadrupole was operated in the r.f.-only mode and was used as the collision cell. Argon was used as the collision gas. In the parent ion scanning experiments, the second quadrupole was fixed to detect the fragment ion at m/z 303, which was diagnostic for arachidonate, and the magnet was scanned to detect any precursor ion which gave rise to the fragment ion at m/z 303 upon collision with argon gas. In the MRM measurements, the parent ions were mass selected by the magnet, and the fragment ion at m/z 303 was monitored by the second quadrupole; the dwell time for each MRM channel was 50 ms.

RESULTS AND DISCUSSION

Free arachidonic acid released upon Ca^{2+} ionophore stimulation in differentiated human U937 cells

The human histiocytic lymphoma U937 cells contain a rich source of the 85 kDa cytosolic phospholipase A_2 ($cPLA_2$), and had high $cPLA_2$ enzymatic activity.[17] This enzyme catalyzes the release of arachidonic acid from membrane phospholipids, thereby initiating the biosynthesis of prostaglandins and leukotrienes. The DMSO-differentiated human U937 cells were used in this study to investigate the mobilization of arachidonic acid within the membrane phospholipids. Although the events taking place during cell differentiation are not well understood, it was known that differentiation of U937 cells with DMSO led to a neutrophil-like cell line and an increase in intracellular calcium.[18] Intracellular calcium helps $cPLA_2$ translocate to the membrane, where it meets with its substrate phospholipids. Furthermore, it was found that differentiation with DMSO led to activation of protein kinase C, which then phosphorylates and activates $cPLA_2$.

The amounts of free arachidonic acid in the dU937 cells before and after Ca^{2+} ionophore stimulation were determined. The free fatty acids were extracted by the Dole and Meinertz's procedure, converted to their PFB esters and detected by GC/MS with electron capture negative chemical ionization. GC/MS selected ion chromatograms of PFB esters of arachidonic acid and d_8-arachidonic acid (internal standard) in the dU937 cells before and after Ca^{2+} ionophore stimulation are shown in Fig. 1. Very little free arachidonate was detected in the dU937 cells before Ca^{2+} ionophore stimulation (resting cells). Upon stimulation with Ca^{2+} ionophore, free arachinonic acid was rapidly released from membrane phospholipids, leading to an increase in the cellular levels of free AA (typically a 50–200-fold increase, depending on the cell viability), as summarized in Table 1. The time course of free arachidonic acid release in dU937 cells upon Ca^{2+} ionophore stimulation is shown in Fig. 2. The free arachidonic acid level increased rapidly during the first few minutes of stimulation, and decreased thereafter, presumably owing to rapid reincorporation into phospholipids. Thereafter, a 3 min Ca^{2+} ionophore cell stimulation was used for further studies of arachidonic acid mobilization.

Table 1. Free arachidonic acid levels in human dU937 cells before and after Ca^{2+} ionophore stimulation for 3 min, as measured by GC/MS[a]

	Free AA (ng per 10^6 cells)
Before Ca^{2+} ionophore stimulation	1.4 ± 0.1
After Ca^{2+} ionophore stimulation for 3 min	242.4 ± 8.1

[a] Data are normalized to ng per 10^6 cells, and are the means ± SEM of five replicate experiments.

Similar studies with undifferentiated U937 cells showed a very low level of free AA release upon Ca^{2+} ionophore stimulation at all time points (data not shown). This is also consistent with the results obtained by Rzigalinski *et al.*[18] It was suggested that the $cPLA_2$ regulatory elements, such as calcium and phosphorylation, may be lacking or non-functional in undifferentiated U937 cells. DMSO differentiation of U937 cells may induce these regulatory elements, thereby resulting in a dramatic increase in the free AA released upon Ca^{2+} ionophore stimulation.

Normal-phase HPLC separation of phospholipid classes

In order to determine the arachidonate and phospholipid molecular species distribution, the total lipid extract was separated into phospholipid classes. A number of chromatographic methods for the separation of phospholipid classes have been reported.[19–21] Patton *et al.*'s method[19] gave a good separation between the lipid classes, but we found that the method was not reproducible and often resulted in column clogging due to precipitation of the phosphate buffer. We modified Rivnay's method[20] by replacing water with 25 mM ammonium acetate. The chromatographic separation was improved and phospholipid classes were consistently resolved in a relatively short time. Figure 3 shows the HPLC traces of the phospholipid classes from commercial standards and from the lipid extract of differentiated human U937 cells. Each of the phospholipid classes was collected based on the retention times of the commercial standards.

Total cellular arachidonate content and arachidonate distribution

In order to determine the total cellular arachidonate content and arachidonate distribution in each of the

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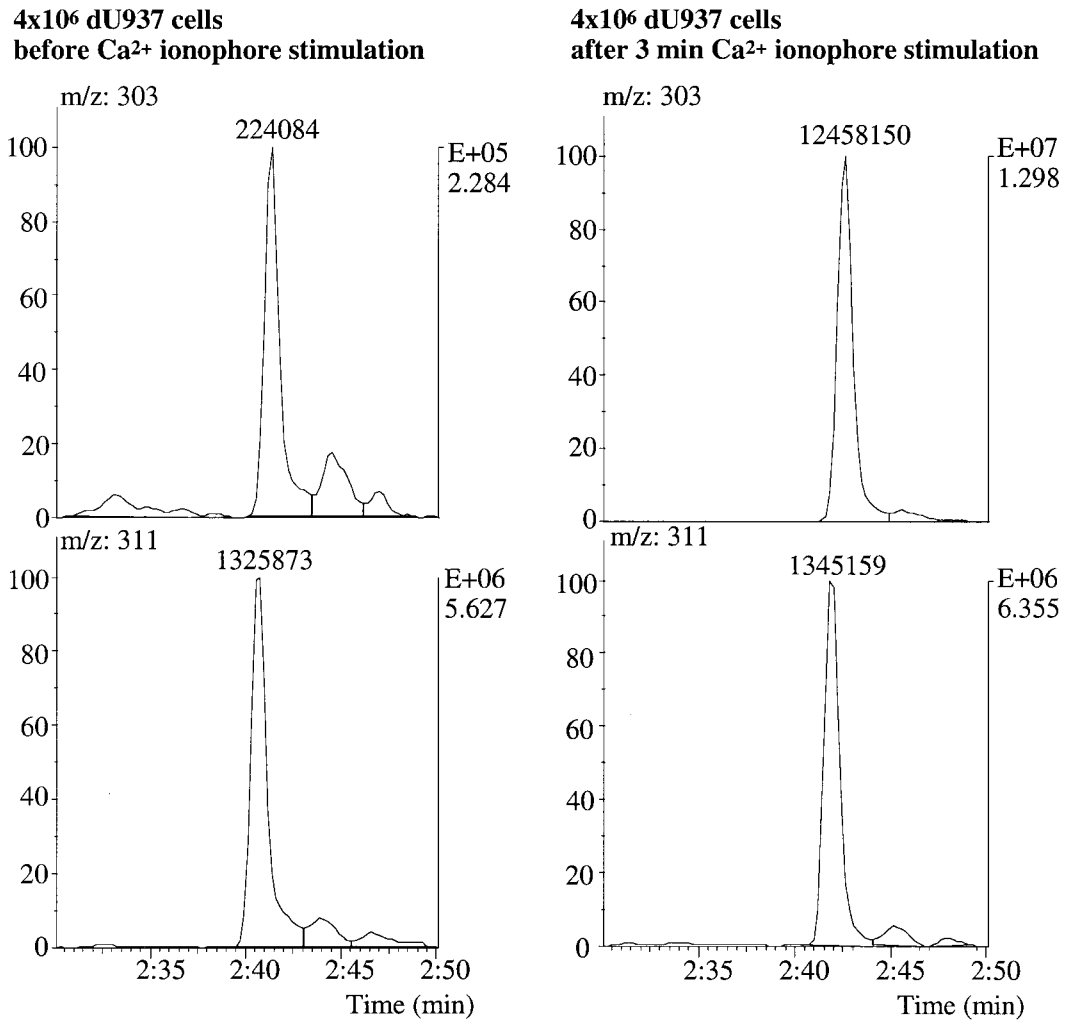

Figure 1. Free arachidonic acid level in dU937 cells before and after and Ca^{2+} ionophore stimulation. Free AA was derivatized to PFB ester and quantified by GC/MS. SIM chromatograms of AA-PFB and d_8-AA-PFB in dU937 cells (a) before Ca^{2+} ionophore stimulation and (b) after Ca^{2+} ionophore stimulation for 3 min.

phospholipid classes, an aliquot of the total lipid extract and each isolated phospholipid class was subjected to base hydrolysis to liberate the free fatty acids. The free fatty acids liberated were then extracted and converted to their PFB esters, and the amounts of arachidonate were quantified by GC/MS. The total cellular arachidonate content and the arachidonate distribution in each of the phospholipid classes in dU937 cells before and after Ca^{2+} ionophore stimulation are shown in Table 2. In differentiated human U937 cells, glycerophosphoethanolamine (GPE) was found to be the major pool of arachidonate; it contained ~55% of total cellular arachidonate. Glycerophosphocholine (GPC) and glycerophosphoinositol (GPI) consisted of 22% and 8% of the total cellular AA, respectively. Glycerophosphoserine (GPS) contained very little arachidonte. Upon Ca^{2+} ionophore stimulation, there is a clear class specificity for releasing arachidonate. Arachidonate was lost from both GPE and GPC, and GPE being the major source of liberated arachidonate. Interestingly, arachidonate was not lost from GPI; instead, a substantial gain of arachidonate content was found in GPI. This suggested that arachidonate was rapidly released from and shuttled between specific phospholipid pools during cell activation. A portion of AA released from GPE or GPC may be rapidly esterified into GPI, consistent with the finding that the rate of AA incorporation follows the order: GPI > GPC > GPE in human neutrophil.[15]

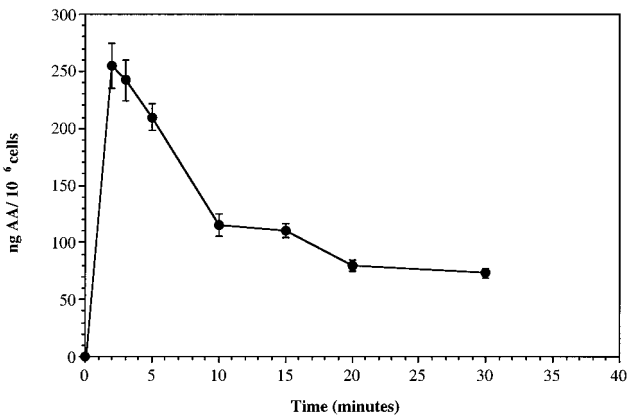

Figure 2. Time course of free arachidonate release in dU937 cells upon Ca^{2+} ionophore stimulation.

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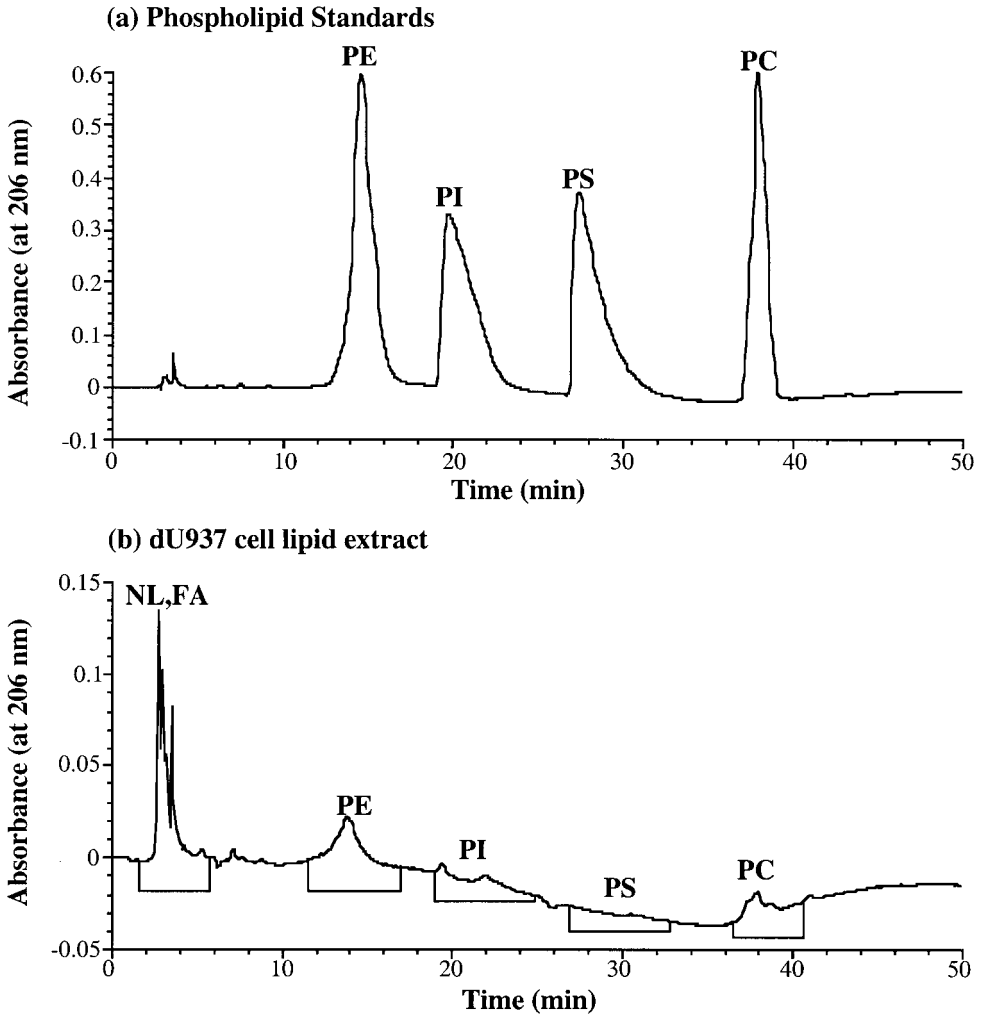

Figure 3. Normal-phase HPLC separation of (a) phospholipid classes for commercial standards and (b) lipid extract from human differentiated U937 cells. The brackets in (b) show the time range during which each phospholipid class was collected.

The arachidonate content in the neutral lipid/fatty acid fractions isolated from dU937 cells was determined both without and with base hydrolysis, followed by derivatization to PFB esters. Without base hydrolysis, only the free fatty acids were derivatized to their PFB esters; the arachidonate esterified to neutral lipids did not react with the derivatization reagent. The amount determined, therefore, represented only the free arachidonic acid. The amounts of free AA in dU937 cells before and after Ca^{2+} ionophore stimulation were determined, and are shown in column 2 of Table 2. Free arachidonic acid was found to be very minor in the dU937 cells before Ca^{2+} ionophore stimulation (3.2 ± 0.4 ng per 10^6 cells), and increased dramatically (226.8 ± 10.5 ng per 10^6 cells) upon Ca^{2+} ionophore stimulation. This result also agrees well with the separate free AA measurements as shown in Table 1. The arachidonate contents in the neutral lipid/free fatty

Table 2. Total cellular arachidonate content and arachidonate distribution in human dU937 cells, before and after Ca^{2+} ionophore stimulation for 3 min[a]

	Free AA[b]	NL + free FA[c]	PE[c]	PI[c]	PS[c]	PC[c]	Total
Resting	3.2 ± 0.9	89.2 ± 4.8	653.0 ± 58.5	94.8 ± 6.8	18.8 ± 2.8	254.8 ± 9.5	1179.2 ± 83.5
Stimulated	226.8 ± 10.5	322.0 ± 37.0	402.5 ± 21.5	134.8 ± 6.5	22.8 ± 2.0	232.8 ± 6.0	1197.2 ± 125.8
Change	223.6 ± 10.5	232.8 ± 37.3	−250.5 ± 62.3	40.0 ± 9.4	4.0 ± 3.4	−22.0 ± 11.2	

[a] ~10% of lipid extract of 4×10^6 cells was used for measurements. Data are normalized to ng per 10^6 cells, and are the means ± SEM of four separate experiments. Neutral lipids (NL) includes free fatty acids, triglycerides, diglycerides and cholesterol esters.

[b] Amount of free arachidonate was determined by direct derivatization of the neutral lipid and free FA fraction without prior base hydrolysis.

[c] Arachidonate in these fractions was base hydrolyzed to liberate free AA, and quantified after derivatization to PFB ester.

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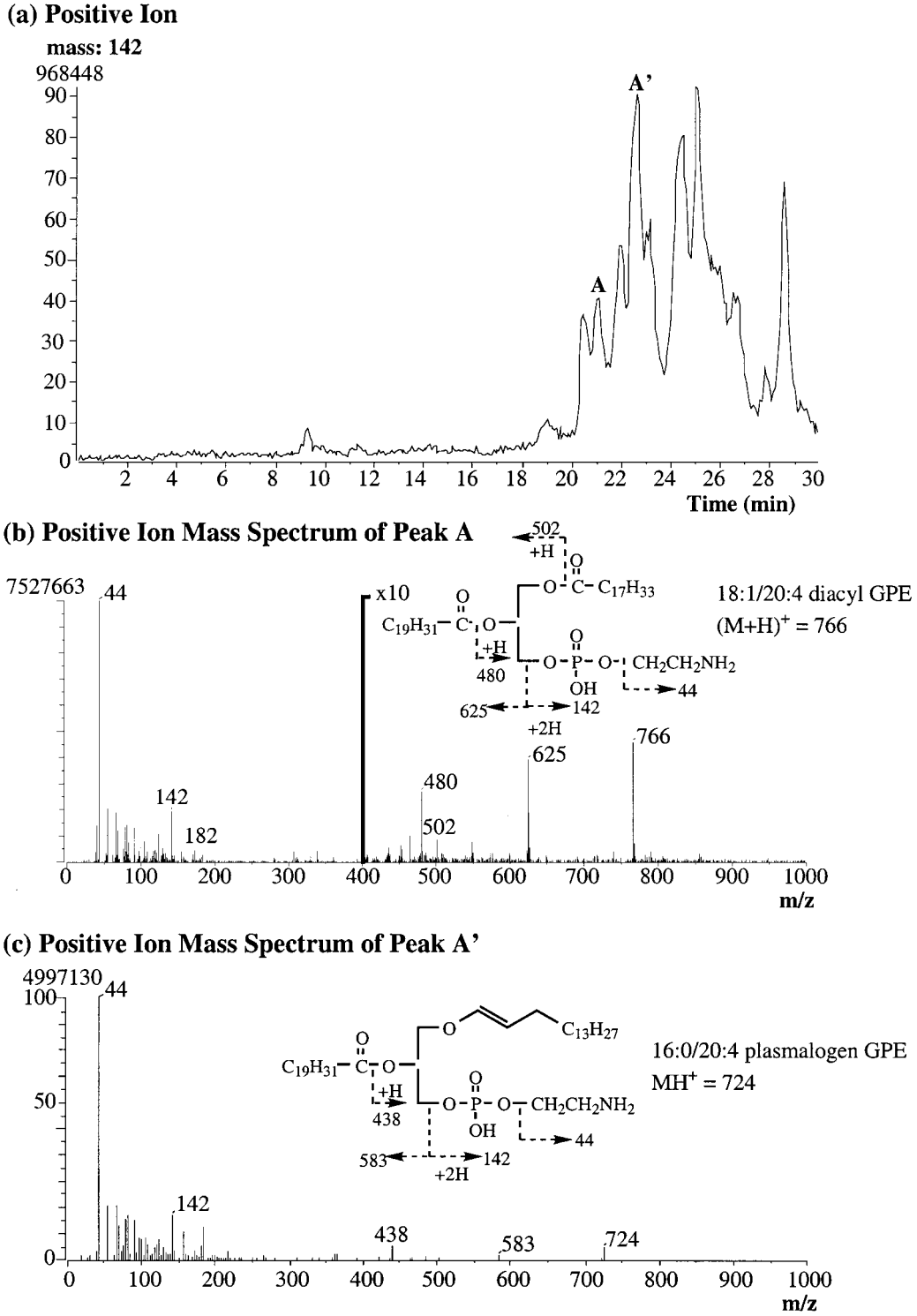

Figure 4. Positive ion CF-LSIMS of glycerophosphoethanolamine (GPE) isolated from human dU937 cells. (a) Reconstructed ion chromatogram of protonated phosphoethanolamine ion (*m/z* 142); (b) positive ion mass spectrum of 18:1/20:4 diacyl GPE; (c) positive ion mass spectrum of 16:0/20:4 plasmalogen GPE.

acid fractions as determined after base hydrolysis are shown in column 3 of Table 2. These amounts represented the sum of free arachidonic acid and arachidonate content in neutral lipids. A significant amount (89.2 ± 1.9 ng per 10^6 cells) of arachidonate was found in this fraction of the dU937 cells before Ca^{2+} ionophore stimulation. Because only minor free AA (3.2 ± 0.4 ng per 10^6 cells, column 2 of Table 2) was detected, most of the arachidonate (~86 ng per 10^6 cells) was in the neutral lipids, such as diglycerides, triglycerides and cholesterol esters. Upon Ca^{2+} ionophore stimulation, the arachidonate level in this fraction increased 232.8 ± 37.2 ng per 10^6 cells. This increase in AA level again represented the sum of free arachidonic acid liberated from other phospholipid classes by $cPLA_2$ and arachidonate content in any new neutral lipid products

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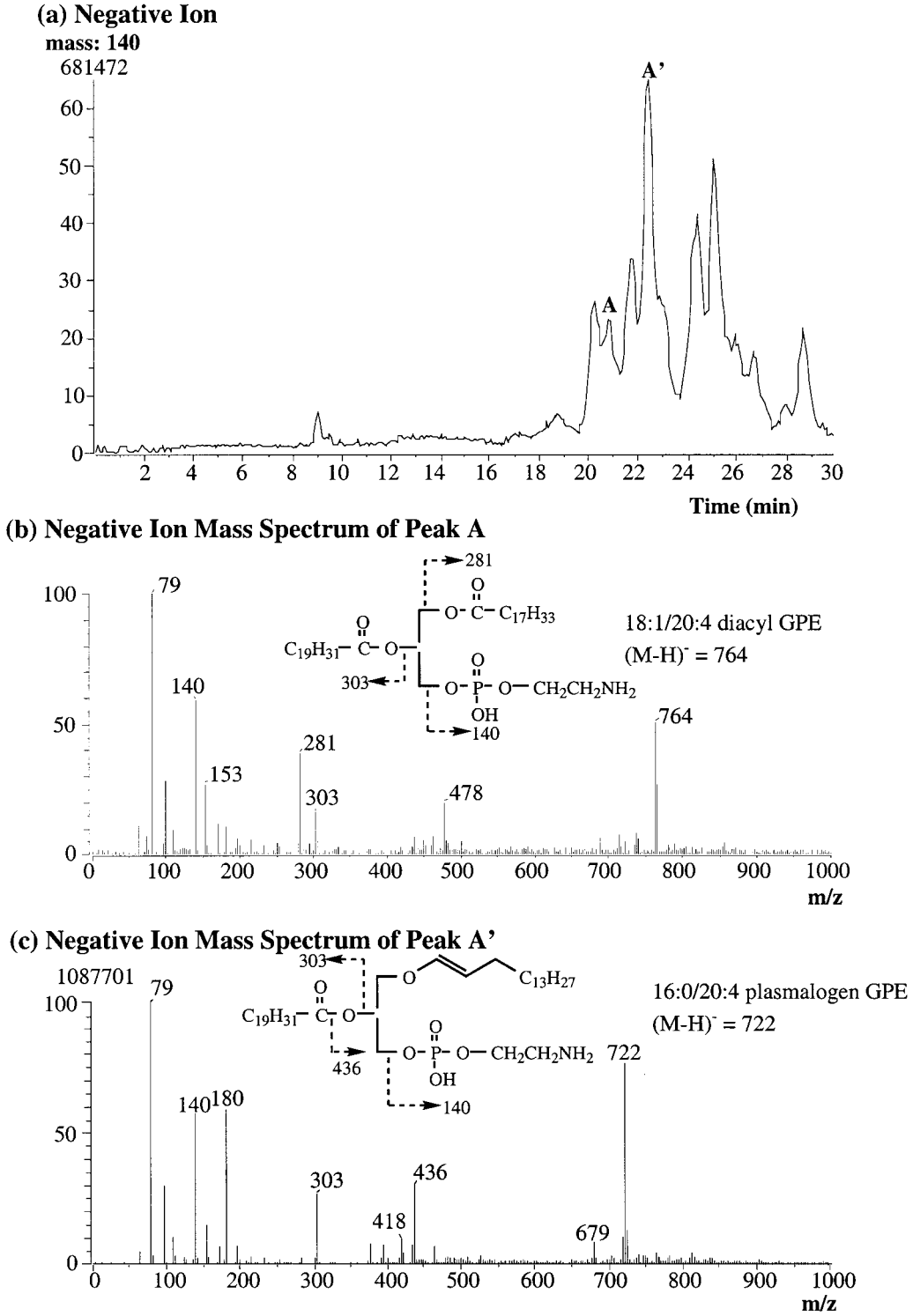

Figure 5. Negative ion CF-LSIMS of glycerophosphoethanolamine (GPE) isolated from human dU937 cells. (a) Reconstructed ion chromatogram of phosphoethanolamine anion (*m/z* 140); (b) negative ion mass spectrum of 18:1/20:4 diacyl GPE; (c) negative ion mass spectrum of 16:0/20:4 plasmalogen GPE.

as a result of phospholipase C. The increase in the free arachidonic acid (223.6 $\pm$ 10.5 ng per 10^6 cells, column 2 of Table 2) accounted for essentially all of the arachidonate increase in the neutral lipid/free AA fraction (column 3 of Table 2) upon Ca^{2+} ionophore stimulation. Therefore, the contribution from the phospholipase C mediated conversion of phospholipid classes such as GPE to diglycerides was minimal.

Phospholipid molecular species analysis using CF-LSIMS

The phospholipid molecular species composition in each of the phospholipid classes was investigated using capillary HPLC/CF-LSIMS. CF-LSIMS provided a useful and effective way of detecting intact phospholipid

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Table 3. Identification of phospholipid molecular species in human dU937 cells by CF-LSIMS[a]

GPE species			GPI species	GPS species	GPC species		
Diacyl	Alkylacyl	Plasmalogen	Diacyl	Diacyl	Diacyl	Alkylacyl	Plasmalogen
14:0/16:1			14:1/18:1	16:1/18:1	16:1/16:1		
16:1/16:1			16:1/18:2	32:1	16:1/18:2		
16:1/22:6			18:1/20:5	40:5	16:0/16:1		
36:5			18:1/16:1	18:0/16:1	16:1/18:1		
16:1/18:2			32:1	18:1/18:1	**16:0/20:4**		
18:1/20:5		34:4	**18:1/20:4**	18:0/22:6		30:0	
16:0/20:5		36:5	18:1/18:2	18:0/22:5		32:1	32:0
16:1/18:1		16:0/20:5	**18:0/20:4**	18:0/20:3	18:1/22:6		
14:0/18:1			16:0/18:1	18:0/18:1	**18:1/20:4**		
16:0/22:6			18:1/18:1	38:1	18:1/22:5		
18:1/22:6			18:1/20:3	40:1			**16:0/20:4?**
16:0/20:4			18:0/20:3		36:3		
18:1/20:4			18:0/18:1			36:4	
18:1/18:2			18:0/20:2		16:0/8:1		
18:1/22:5		16:0/22:6	18:0/20:1		18:1/18:1		
		16:0/20:4			16:0/16:0		
		16:0/22:5			18:1/20:2		
18:1/18:1		**18:1/20:4**				34:2	34:1
16:0/18:1						34:1	34:0
18:0/22:6						32:0	
18:0/20:4		16:0/18:1			18:1/20:1		
		18:0/20:4			18:0/18:1		
18:0/22:5						36:2	36:1
18:0/20:3						36:1	36:0
	36:3	36:2			18:1/20:0		
	38:4	38:3					
38:2							
18:0/18:1							

[a] *n*1 :*d*1/*n*2 :*d*2 represents the assignment of the *sn*-1 and *sn*-2 carbon chain (*n*1, *n*2) with the total number of unsaturations in the two radyl groups (*d*1, *d*2); *n* :*d* represents the total number of carbon atoms in both radyl groups (*n*) and total number of unsaturations in both groups (*d*). The vinyl ether degree of unsaturation in plasmalogens is not added to the value of *d*. Phospholipid molecular species identified by CF-LSIMS are listed in the order of their RP-HPLC retention times. Arachidonate-containing phospholipid species are highlighted in bold.

molecular species.[11] It offers significant advantages over the conventional FAB measurements.[16,22] The background noise associated with matrix adduct ions is significantly reduced and the ion suppression effect is diminished. The sensitivity of this technique is enhanced by several orders of magnitude. Coupling capillary HPLC with CF-LSIMS allowed the separation of many molecular species within each phospholipid class, and the identification of minor species in the complex mixture became more feasible. CF-LSIMS also offers clear advantages over LC/electrospray ionization (ESI) MS.[23–27] With LC/ESI-MS, the full-scan ESI mass spectra of phospholipids were not very informative, giving exclusively abundant molecular ions. The identification of phospholipid species often requires extensive MS/MS experiments on each molecular species. However, with CF-LSIMS, the positive and negative ion full-scan mass spectra were much more informative. The molecular mass, polar headgroup and *sn*-1, *sn*-2 substituents of phospholipid species can be readily obtained from the full-scan LSI mass spectra.

LSIMS and FAB characterization of phospholipid molecular species were described in detail previously.[11,28] In brief summary, positive ion full-scan LSI mass spectra of phospholipids generally showed diagnostic ions of polar head groups and abundant protonated molecules. Negative ion full-scan LSI mass spectra of phospholipids usually exhibited abundant carboxylate anions corresponding to *sn*-1 and *sn*-2 fatty acyl groups, and also abundant $[M - H]^-$ except for GPC species. The following results and discussion will further illustrate the use of these diagnostic ions for the identification of phospholipid molecular species, and specifically detail the characterization of AA-containing phospholipids in dU937 cells.

GPE species in dU937 cells. Positive and negative ion CF-LSIMS analysis of the isolated GPE class from dU937 cells revealed many phosphoethanolamine molecular species. Figure 4(a) shows the reconstructed positive ion chromatogram of protonated phosphoethanolamine ion (*m*/*z* 142) for the GPE species in dU937 cells. Figure 5(a) shows the corresponding reconstructed negative ion chromatogram of phosphoethanolamine anion (*m*/*z* 140). Figures 4(b) and 5(b) show representative positive ion and negative ion full-scan mass spectra of a GPE molecular species (labeled peak A). Both positive and negative ion LSI mass spectra could be divided into three regions, the molecular ion region (typically >600 amu), the fatty acid region (200–500 amu) and the polar head group region (<300 amu). For component A, the molecular mass could be easily obtained from both $[M + H]^+$ at *m*/*z* 766 and $[M - H]^-$ at *m*/*z* 764. The *sn*-1 and *sn*-2 fatty groups were identified as acyl-16 :0

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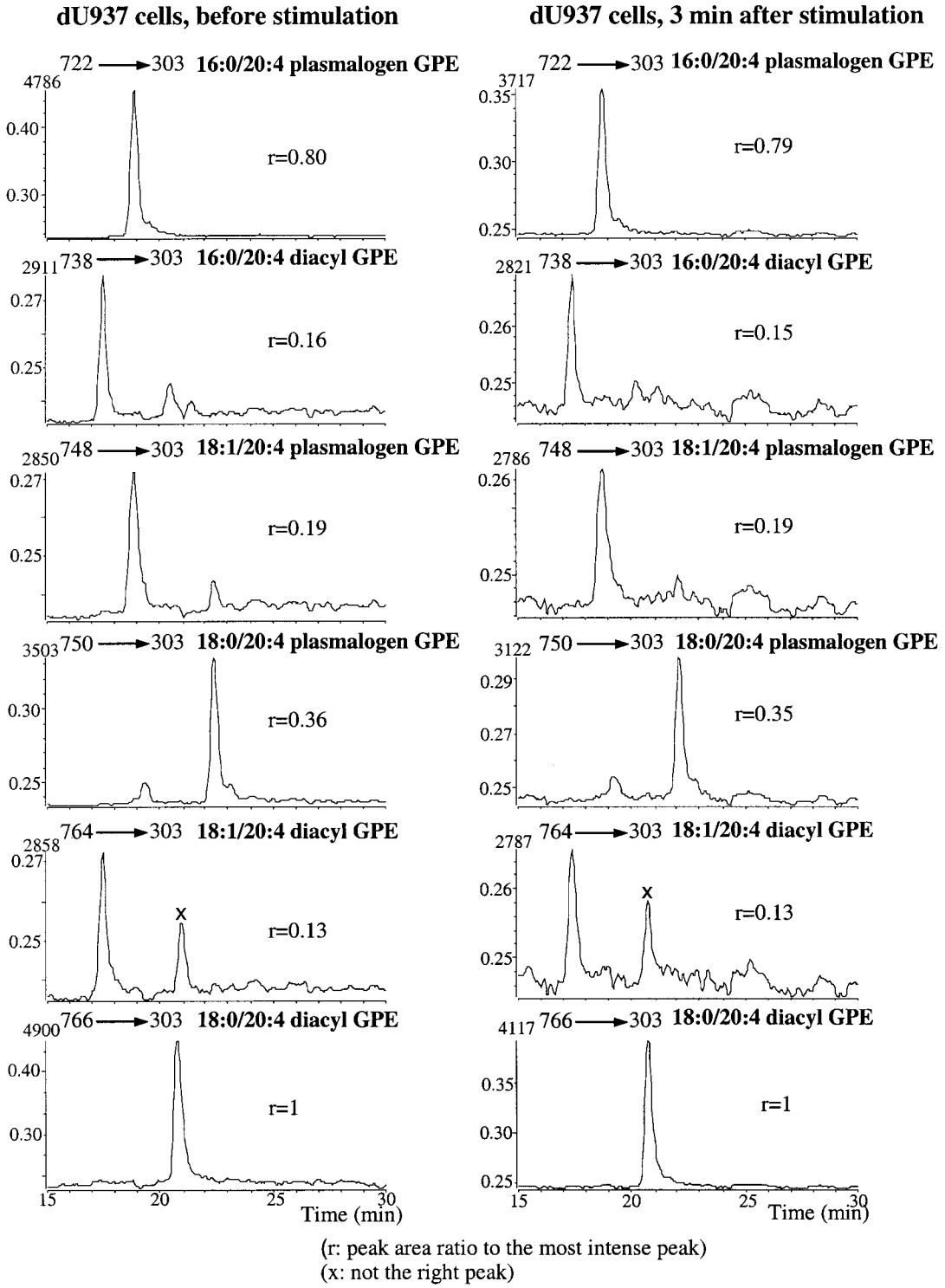

Figure 6. MRM measurements of six arachidonyl-containing GPE molecular species (a) before Ca^{2+} ionophore stimulation and (b) after Ca^{2+} ionophore stimulation for 3 min.

and acyl-20:4 because of the two abundant anions observed at *m/z* 281 and 303 in the fatty acid region [Fig. 4(b)]. The polar head group phosphoethanolamine was confirmed by the diagnostic ions in the polar head group regions, i.e. *m/z* 142 and 44 in Fig. 4(b) and *m/z* 140 and 180 in Fig. 5(b). This component A was therefore identified as diacyl-16:0/20:4 GPE. The positive and negative ion full-scan mass spectra of another GPE molecular species (labeled peak A′) are shown in Figs 4(c) and 5(c). For component A′, only one carboxylate anion at *m/z* 303 was observed in the fatty acid region of Fig. 5(c), suggesting an acyl group at *sn*-2 and an alkyl group at *sn*-1. This could be either a 1-*O*-alkyl-2-acyl GPE or 1-*O*-alk-1′-enyl-2-acyl (plasmalogen) GPE species. Upon acid treatment, this component A′ was converted to a lyso-PE species, suggesting it was the plasmalogen species.[16] The acyl substituent was identified as arachidonate, and the *sn*-1 substituent was then

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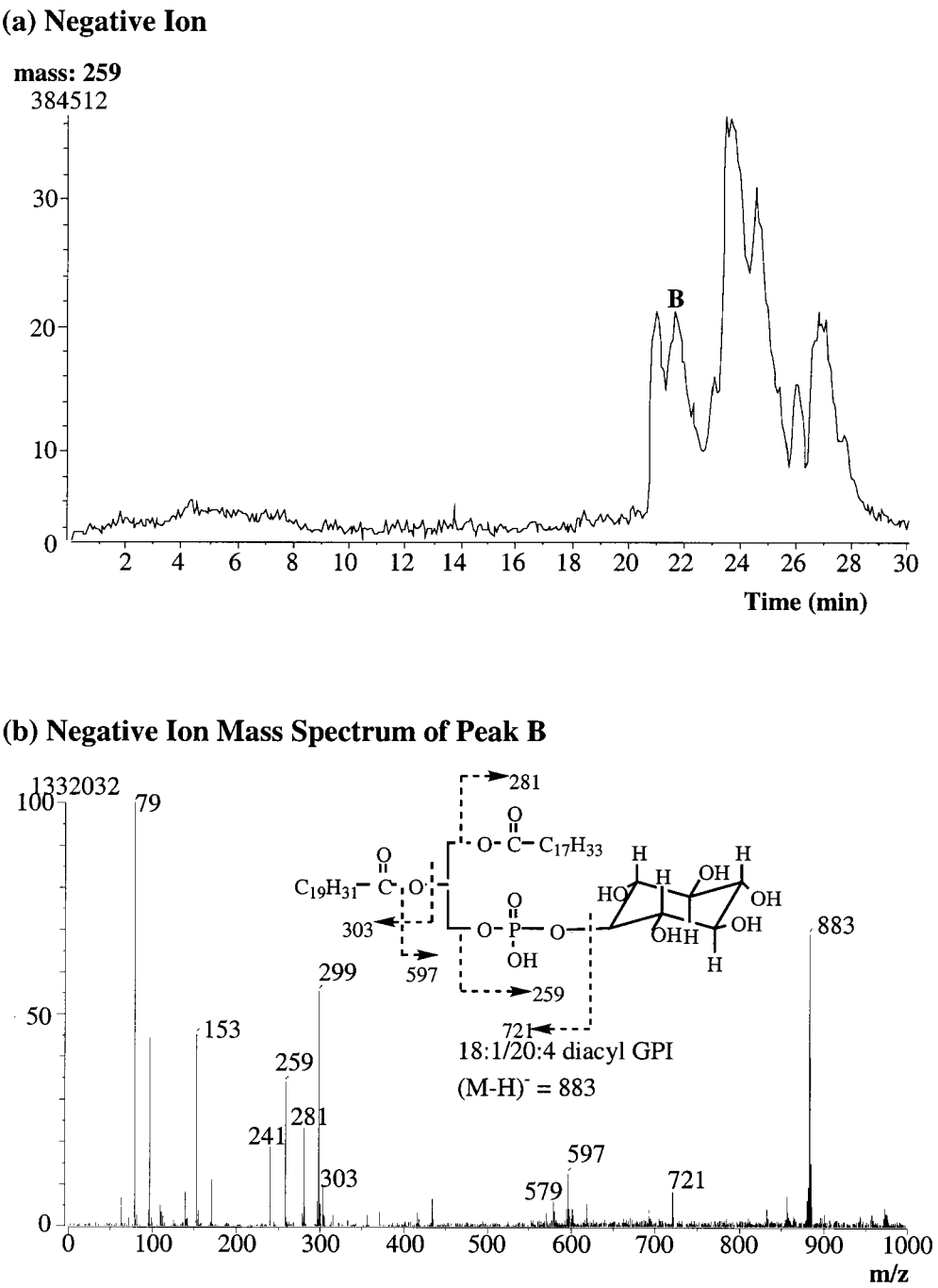

Figure 7. Negative ion CF-LSIMS of glycerophosphoinositol (GPI) isolated from human dU937 cells. (a) Reconstructed ion chromatogram of phosphoinositol anion (*m/z* 259); (b) negative ion mass spectrum of 18:1/20:4 diacyl GPI.

identified by the mass difference between $[M - H]^-$ (722) and PE backbone (180) plus *sn*-2 acyl carboxylate anion (303). It was therefore identified as 16:0/20:4 plasmalogen GPE. More than 30 different PE species were identified in this manner, and are listed in Table 3. These species are listed in the order of their retention times on reversed-phase HPLC.

In the negative ion mode, collision-induced dissociation (CID) of $[M - H]^-$ for GPE species produced abundant carboxylate anions corresponding to the fatty acyl substituents. Parent ion scanning of arachidonyl carboxylate anion (*m/z* 303) would therefore reveal the AA-containing species. Six arachidonyl-containing GPE species were confirmed by the parent ion scanning of *m/z* 303 on the JEOL HX110 hybrid mass spectrometer. These AA-containing GPE species are highlighted in bold in Table 3; three were diacyl GPE species and other three were plasmalogen GPE species. In order to identify whether there is a selectivity of releasing free AA upon CA^{2+} ionophore stimulation among the six GPE species, MRM measurements were carried out. Six CID transitions of $[M - H]^-$ (722, 748 and 750 for 16:0/20:4, 18:1/20:4 and 18:0/20:4 plasmalogen GPE; 738, 764 and 766 for 16:0/20:4, 18:1/20:4 and 18:0/20:4 diacyl GPE) to fragment ion at *m/z* 303 were chosen for MRM of the six AA-containing GPE species.

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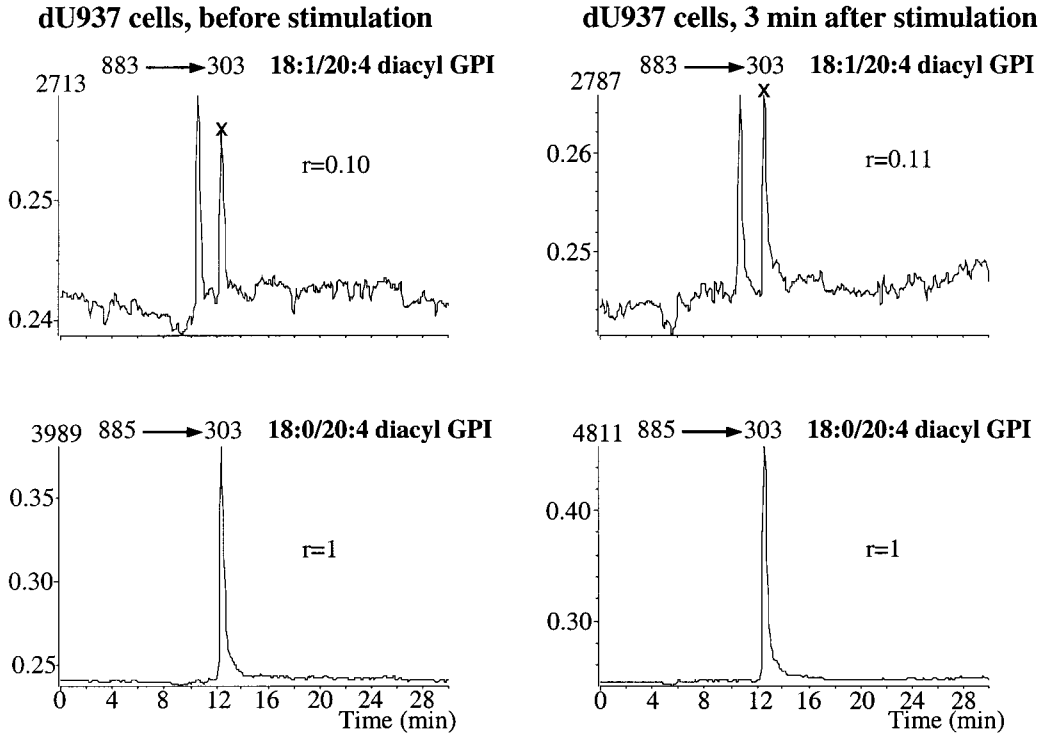
The MRM measurements were carried out for dU937 cells before and after Ca^{2+} ionophore stimulation. Absolute quantification of these arachidonyl-containing phospholipids was impossible owing to the lack of synthetic standards. The MRM measurements only represent the relative distribution of the six AA-containing GPE species, and the relative change in distribution upon Ca^{2+} ionophore stimulation. As shown in Fig. 6, the peaks corresponding to each MRM transition were integrated and were normalized to the most intense peak. The normalized peak area ratios remained the same before and after Ca^{2+} ionophore stimulation. If there were AA-containing GPE species selectively releasing free AA upon Ca^{2+} ionophore stimulation, one would expect that the normalized peak area ratios for those species would change significantly after stimulation. Our results suggested that there was no selectivity releasing free AA upon stimulation among the AA-containing GPE species. This probably reflects the non-specific nature by which Ca^{2+} ionophore stimulated the dU937 cells.

GPI species in dU937 cells. CF-LSIMS analysis of the GPI class isolated from human dU937 cells also showed complex mixtures of GPI molecular species, as shown in Fig. 7(a). Figure 7(b) shows the negative ion mass spectrum of an arachidonate-containing species diacyl 18:1/20:4 GPI. IN the molecular ion region, abundant deprotonated molecule $[M - H]^-$ at *m/z* 883 was observed. In the fatty acid region, two carboxylate anions (*m/z* 281 and 303) were detected, allowing the assignment of the two acyl groups at *sn*-1 and *sn*-2 as 18:1 and 20:4, respectively. In the polar head group region, several GPI-related negative ions at *m/z* 259 (inositol phosphate), 241 (inositolphosphate $- H_2O$) and 299 $[M - H - R^1COO - R^2COO]$ were produced. The GPI molecular species in the differentiated human U937 cells identified by CF-LSIMS are listed in Table 3. Only two arachidonyl-containing GPI species were identified, i.e. diacyl-18:0/20:4 GPI.

Similar MRM measurements of the AA-containing GPI species were carried out before and after Ca^{2+} ionophore stimulation. Two CID transitions of $[M - H]^-$ (i.e. 883 and 885 for diacyl 18:1/20:4 and 18:0/20:4 GPI) to fragment ion at *m/z* 303 were chosen for MRM. Again, the peaks corresponding to each MRM transition were integrated and were normalized to the most intense peak. As shown in Fig. 8, the normalized ratios did not change after Ca^{2+} ionophore stimulation. From the measurements of arachidonate content in GPI after base hydrolysis, arachidonate was enriched substantially after Ca^{2+} ionophore stimulation (see Table 2). Free arachidonic acid released may be rapidly esterified into these two diacyl GPI species (18:1/20:4 and 18:0/20:4 GPI). The constant peak area ratio observed in MRM measurements implied that there was no preference in the esterification of free AA into the two AA-containing GPI species.

GPS species in dU937 cells. In the isolated GPS fraction, the molecular species were also identified readily from their positive and negative ion CF-LSI mass spectra. Figure 9(a) shows the reconstructed negative ion chromatogram of the class characteristic phosphoserine ion



(r: peak area ratio to the most intense peak)
(x: not the right peak)

Figure 8. MRM measurements of two arachidonyl-containing GPI molecular species (a) before Ca^{2+} ionophore stimulation and (b) after Ca^{2+} ionophore stimulation for 3 min.

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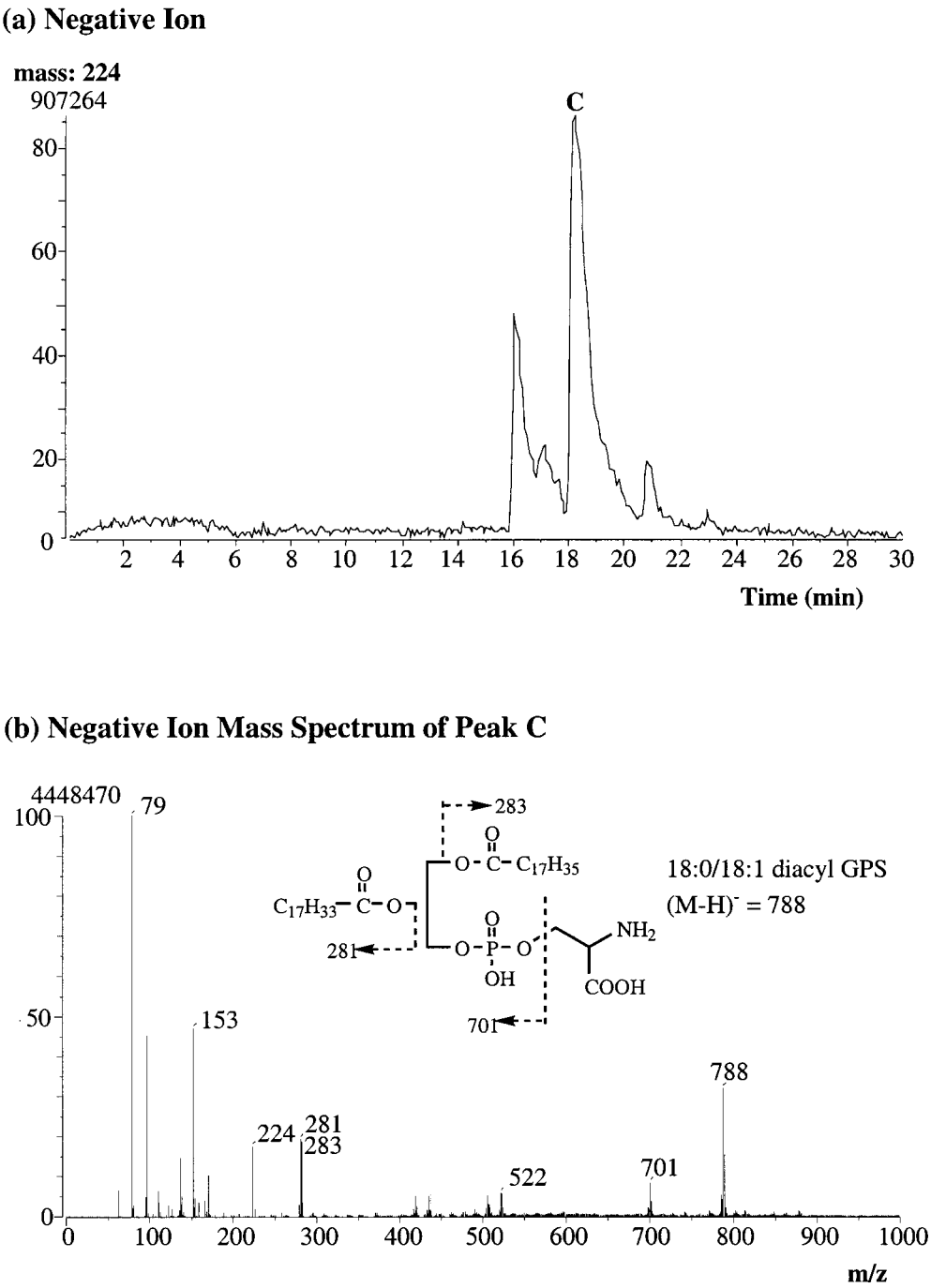

Figure 9. Negative ion CF-LSIMS of glycerophosphoserine (GPS) isolated from human dU937 cells. (a) Reconstructed ion chromatogram of phosphoserine anion (*m/z* 224); (b) negative ion mass spectrum of 18:1/18:0 diacyl GPS.

(*m/z* 224) for the GPS species isolated from human differentiated U937 cells. The representative negative ion mass spectrum of one major GPS molecular species (labeled C, 18:0/18:1 diacyl GPS) is shown in Fig. 9(b). Abundant $[M - H]^-$ ions at *m/z* 788 and $[M - 88]^-$ at *m/z* 701, corresponding to the loss of serine, were observed. Carboxylate anions at *m/z* 283 and 281 from the two fatty acyl groups were also detected. The GPS molecular species identified by CF-LSIMS are listed in Table 3. A very small amount of arachidonate content was detected from the base hydrolysis of the GPS fraction (see Table 2). However, no arachidonyl-containing GPS species were positively identified by full-scan or parent ion scanning of *m/z* 303. If arachidonyl-containing GPS species were present, they would have been very minor components of the GPS fraction (<1% of total GPS species), based on previously demonstrated CF-LSIMS detection of minor intact phospholipid molecular species within a complex mixture.[11]

GPC species in dU937 cells. Molecular species analysis of the isolated GPC fraction was more complex, as shown in Fig. 10(a). The negative ion CF-LSI mass spectrum of a minor GPC species 18:1/20:4 diacyl GPC [labeled peak D in Fig. 10(a)] is shown in Fig. 10(b). In the

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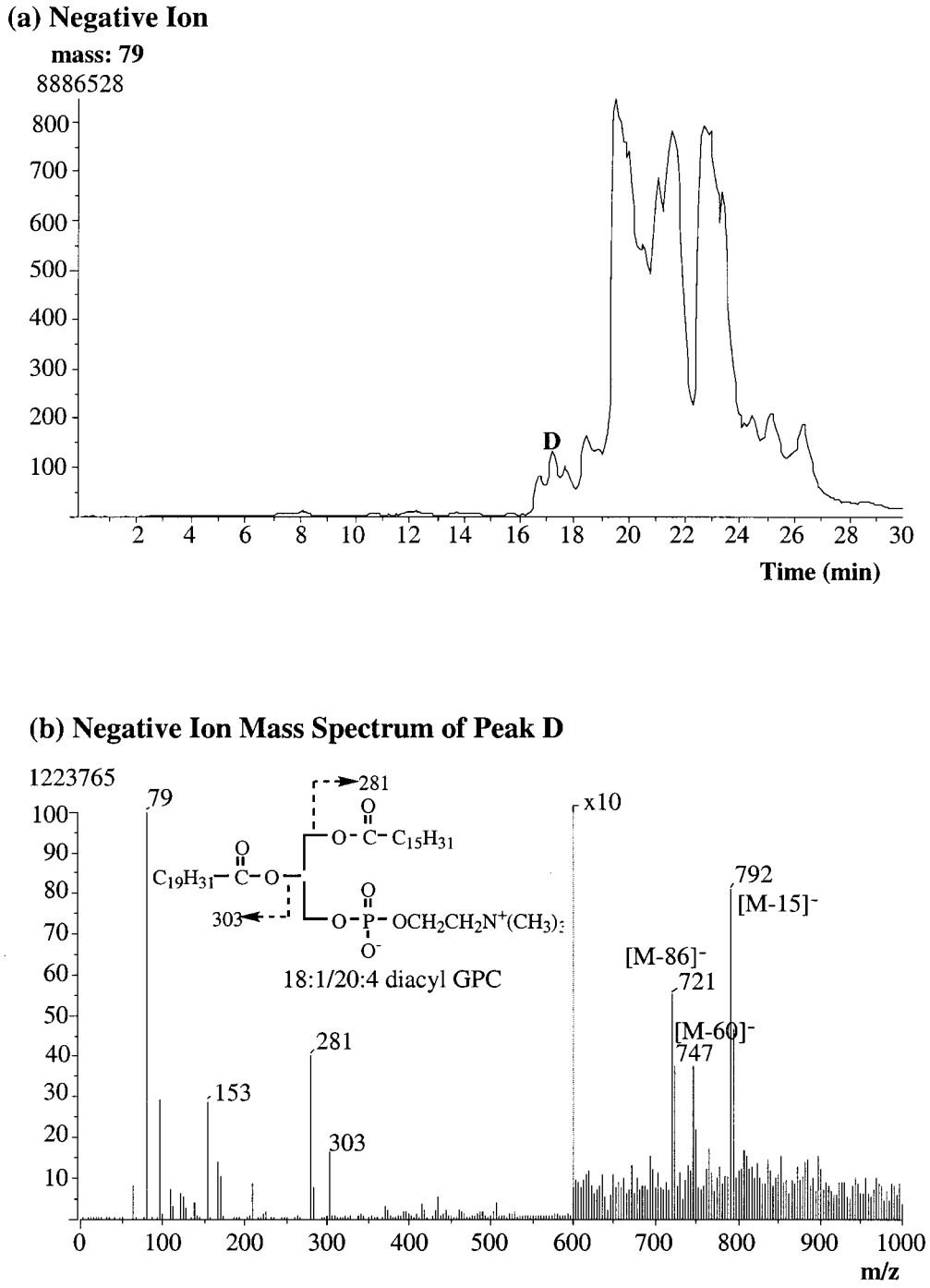

Figure 10. Negative ion CF-LSIMS of glycerophosphocholine (GPC) isolated from human dU937 cells. (a) Reconstructed ion chromatogram of PO_3^- (*m/z* 79); PO_3^- is not characteristic for phosphocholine species, however the specificity comes from the prior HPLC isolation for GPC class; (b) negative ion mass spectrum of 18:1/20:4 diacyl GPC.

molecular ion region, the deprotonated molecule $[M - H]^-$ at *m/z* 806 was absent from the spectrum and, instead, ions at *m/z* 721, 747 and 792 corresponding to $[M - 15]^-$, $[M - 60]^-$ and $[M - 86]^-$, respectively, were observed, consistent with other FAB measurements.[28–30] In the fatty acid region, the two abundant anions at *m/z* 281 and 303 facilitated the identification of the fatty acyl groups 18:1 and 20:4 at *sn*-1 and *sn*-2 positions. In the low-mass region, PO_3^- at *m/z* 79 and the dehydrated glycerophosphate ($C_3H_6PO_5$) ion at *m/z* 153 were present. These two ions were not diagnostic for phosphocholine; the specificity for GPC came from normal phase HPLC isolation. The positive ion CF-LSI mass spectra of the GPC species showed abundant characteristic phosphocholine ion at *m/z* 184, in addition to protonated molecules. The ions at the fatty acid region were usually weak; information regarding the fatty groups at *sn*-1 and *sn*-2 positions can be obtained better from negative ion LSI mass spectra. The GPC molecular species in differentiated human U937 cells identified by CF-LSIMS are listed in Table 3. Owing to the large number and diversity of GPC

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molecular species, the identification of minor arachidonyl-containing GPC species was extremely difficult. There were several arachidonate-containing GPC species indicated from the full scan mass spectra (see Table 3); however, the confirmation of these species was difficult owing to the interference from co-eluting major species and the complex molecular ion clusters.

Two arachidonyl-containing GPC species positively identified by parent ion scanning of m/z 303 were diacyl-18:1/20:4 and 16:0/20:4 GPC. CID of $[M\text{-}15]^-$ of arachidonyl-containing GPC showed an abundant fragment ion at m/z 303. CID transitions of $[M-15]^-$ (792 and 794 for diacyl-18:1/20:4 and 18:0/20:4 GPC) to fragment ion at m/z 303 were chosen for MRM measurements. The normalized peak area ratios of these species before and after stimulation remained unchanged, similarly to the observation with GPE and GPI arachidonyl-containing species. This result again implied that there was no selectivity in releasing free AA upon Ca^{2+} ionophore stimulation among the AA-containing GPC species.

CONCLUSION

A combination of several chromatographic and mass spectrometric techniques was used to investigate the arachidonic acid release and distribution in the membrane phospholipids of differentiated human U937 cells. A direct and detailed approach was demonstrated for the understanding of arachidonate mobilization and distribution upon a stimulus challenge.

Compared with undifferentiated U937 cells, DMSO-differentiated U937 cells showed a dramatic increase in free arachidonic acid release upon Ca^{2+} ionophore stimulation. The release of free AA reached a maximum after 2–3 min stimulation. The total cellular arachidonate content and arachidonate distribution in each of the major phospholipid classes before and after Ca^{2+} ionophore stimulation were also determined. The major phospholipid classes were isolated by normal-phase HPLC. The total lipids and each of the phospholipid classes were base hydrolyzed to liberate free fatty acids, which were derivatized to their PFB esters. The arachidonate content in each class was then quantified by GC/MS with electron capture negative chemical ionization. Glycerophosphoethanolamine (GPE) was found to be the major source of arachidonate, containing ~55% of total cellular arachidonate. Glycerophosphocholine (GPC) and glycerophosphoinositol (GPI) consisted of 22 and 8% of total cellular arachidonate, respectively. Glycerophosphoserine (GPS) contained very little arachidonate. Upon Ca^{2+} ionophore stimulation, GPE class lost the largest amount of arachidonate, followed by GPC class. Most of the arachidonate depleted from GPE and GPC was recovered as free arachidonic acid, as detected in the neutral lipid/free fatty acid fraction. A substantial increase in arachidonate content in the GPI class was also detected; some of the free AA release from GPE and GPC may be rapidly esterified into GPI species.

Phospholipid molecular species in each of the major lipid classes were analyzed by capillary HPLC/CF-LSIMS. Arachidonyl-containing phospholipid species were identified using regular magnetic scanning and parent ion scanning. MRM measurements of arachidonyl-containing phospholipids before and after Ca^{2+} ionophore stimulation revealed that there was no specificity for releasing free AA among the GPE and GPC AA-containing species, or esterification into GPI AA-containing species. The selectivity for free AA release was observed only between the different phospholipid classes, and not between the molecular species within each phospholipid class. Using this technique, it should be possible to investigate further whether a more physiological stimulus releases arachidonate selectivity from a given phospholipid precursor in human dU937 cells or other cell types.

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