papers on methodology

Analysis of glycerophosphocholine molecular species as derivatives of 7-[(chlorocarbonyl)-methoxy]-4-methylcoumarin

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Abstract A method has been developed for the analysis of derivatized diradylglycerols obtained from glycerophosphocholine (GPC) of transformed murine bone marrow-derived mast cells that provided high performance liquid chromatography (HPLC) separation of GPC subclasses and molecular species separation with on-line quantitation using UV detection. In addition, the derivatized diradylglycerol species were unequivocably identified by continuous flow fast-atom bombardment mass spectrometry. GPC was initally isolated by thin-layer chromatography (TLC), the phosphocholine group was hydrolyzed, and the resultant diradylglycerol was derivatized with 7-[(chlorocarbonyl)-methoxy]-4-methylcoumarin (CMMC). After separation of the derivatized subclasses by normal phase HPLC, the individual molecular species of the alkylacyl and diacyl subclasses were quantitated and collected during a subsequent reverse phase HPLC step. With an extinction coefficient of 14,700 l mol^{-1} cm^{-1} at a wavelength detection of 320 nm, the CMMC derivatives afforded sensitive UV detection (100 pmol) and quantitation of the molecular species. Continuous flow fast-atom bombardment mass spectrometry of the alkylacyl CMMC derivatives yielded abundant $[MH]^+$ ions and a single fragment ion formed by loss of alkylketene from the *sn*-2 acyl group, $[MH-(R=C=O)]^+$. No fragmentation of the *sn*-1 alkyl chain was observed. Diacyl derivatives also produced abundant $[MH]^+$ ions plus two fragment ions arising from loss of RCOOH from each of the acyl substituents and two fragment ions from the loss of alkylketene from each acyl group. Individual molecular species substituents were assigned from these ions.—**Wheelan, P., J.A. Zirrolli, and K.L. Clay.** Analysis of glycerophosphocholine molecular species as derivatives of 7-[(chlorocarbonyl)-methoxy]-4-methylcoumarin. *J. Lipid Res.* 1992. **33:** 111–121.

Supplementary key words continuous flow fast-atom bombardment mass spectrometry • HPLC-UV quantitation

The possibility of a link between the production of the lipid mediators, prostaglandins and leukotrienes, and the production of platelet-activating factor, 1-*O*-hexadecyl-2-acetyl glycerophosphocholine (PAF) has received considerable attention in studies with polymorphonuclear leukocytes (1–4). Phospholipase A_2 hydrolysis of a single glycerophosphocholine molecular species, 1-*O*-hexadecyl-2-arachidonoyl-GPC, would yield the immediate precursors, arachidonic acid and lyso-PAF, for both leukotrienes and PAF, respectively. In addition, the findings of *sn*-2 acetylated GPC molecular species that contain *sn*-1 acyl linkages (5–8) and an arachidonic acid-specific PLA_2 (9) that does not discriminate on the basis of the *sn*-1 linkage may suggest a relationship between precursor GPC species that contain AA in the *sn*-2 position and *sn*-2 acetylated GPC. These studies have revealed the complexity of phospholipid metabolism in cells and demonstrated the need for both qualitative and quantitative analysis of all possible precursors of *sn*-2 acetylated GPC and leukotrienes.

Several methods have been developed for HPLC analysis of GPC molecular species (10–16) and these methods generally use similar strategies. Initially, the polar head group is hydrolyzed and the resultant glycerides are derivatized with a chromophore suitable for UV detection and quantitation. After separation into subclasses of alkylacyl-, alkenylacyl-, and diacylglycerides with normal phase chromatography, the individual molecular species are separated and quantitated using reverse phase HPLC. Specific

Abbreviations: GPC, glycerophosphocholine; CMMC, 7-[(chlorocarbonyl)-methoxy]-4-methylcoumarin; CF-FAB/MS, continuous flow fast-atom bombardment mass spectrometry; AA, arachidonic acid; HPLC, high performance liquid chromatography; TLC, thin-layer chromatography; PAF, platelet-activating factor; GC, gas chromatography; DMAP, 4-dimethylaminopyridine. Letters "a" and "e" in numeric nomenclature for molecular species refer to *sn*-1 acyl- and alkyl-linked substituents, respectively.

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identification of the glycerol radyl substituents is based either on HPLC retention time or by hydrolysis of the individually collected diradylglycerol derivatives and subsequent analysis of the derivatized fatty acids and fatty aldehydes by gas chromatography (GC) or by gas chromatography/mass spectrometry (GC/MS). Both procedures suffer from a lack of direct analysis of the intact diradylglycerol which may introduce artifacts and errors.

The performance of mass spectrometry in the analysis of phospholipid molecular species has yielded moderate successes. High performance liquid chromatography coupled with chemical ionization mass spectrometry has been used to identify molecular species of GPC without the necessity of prior derivatization (17). More recently, fast-atom bombardment and tandem mass spectrometry have been used to identify individual molecular species of underivatized GPC mixtures (18–20). While these methods offer the high specificity of mass spectrometric analysis, they do not quantitatively determine the amounts of individual molecular species present.

A combination of the above procedures using dinitrobenzoate derivatives of diradylglycerols, which allowed reverse phase HPLC separation and quantitation of molecular species, followed by direct negative chemical ionization mass spectrometric identification of the derivatized diradylglycerols has been reported by Haroldsen and Murphy (21). However, due to the lack of fragmentation of the molecular anion, only the total carbon number and degree of unsaturation could be determined for each molecular species. Identification of individual substituents at the *sn*-1 and *sn*-2 positions required additional hydrolysis and analysis of the fatty acid methyl esters by GC/MS.

We have developed a method for GPC molecular species analysis that combines the advantage of quantitation by reverse phase HPLC separation of derivatized diradylglycerols with the specific identification of intact derivatives by continuous flow fast-atom bombardment mass spectrometry (CF-FAB/MS). Fragment ions formed during CF-FAB/MS also allowed direct identification of the *sn*-1 and *sn*-2 substituents. We have applied this method to the identification and quantification of molecular species of GPC in the bone marrow-derived murine mast cell.

MATERIALS AND METHODS

All solvents were HPLC grade and were purchased from Fisher Scientific Co. (Denver, CO). Dipalmitoyl [2-palmitoyl-9, 10-^{3}H(N)]-phosphatidylcholine (50.0 Ci/mmol) was purchased from NEN (Dupont Co, Boston, MA). Phospholipase C (*Bacillus cereus* Type XIII), 4-dimethylaminopyridine (DMAP), arachidonic acid (AA), and standard diacyl GPC were purchased from Sigma (St. Louis, MO). 7-[(Chlorocarbonyl)-methoxy]-4-methyl coumarin (CMMC) was purchased from Kodak (Rochester, NY). Silica Gel G thin-layer chromatography (TLC) plates were purchased from Analtech, Inc. (Newark, DE). Methylene chloride was dried just prior to use by passing it through a column of anhydrous sodium sulfate.

Murine bone marrow-derived mast cells were obtained from Dr. E. Razin (Hadassah Medical School, Jerusalem, Israel). These cells had been transformed by Abelson-MuLV virus to a cell that could be carried in culture (22). Cells were grown to a density of approximately 1×10^6 cells/ml with 90–95% viability in RPMI media supplemented with 10% fetal calf serum. Cells were subcultured each Monday by adding 10 ml of cells to 20 ml of fresh media. Fresh media (20 ml) was again added on Wednesday and Friday of each week. For cells supplemented with arachidonic acid, AA in 3 μl ethanol was added with the first addition of media to give an AA concentration of 16.4 μM. Arachidonic acid in 9 μl of ethanol was added at the second addition of media to give a concentration of 29.6 μM AA and molecular species determinations were made on the following day. Control cultures were grown in media in the presence of ethanol but not AA.

Lipids were extracted from pelleted mast cells (50×10^6) by the method of Bligh and Dyer (23). A tracer amount of [^{3}H]dipalmitoyl-GPC was added for recovery analysis and an aliquot was removed for counting by a Packard TRI-CARB 4430 Liquid Scintillation Counter. GPC was isolated by Silica Gel G TLC using the solvent system chloroform–methanol–acetic acid–water 50:25:8:4 and visualized with iodine or by radioactivity measured by a Berthold TLC Linear Analyzer (Berthold Instruments, Nashua, NH). The area corresponding to GPC was extracted by the method of Bligh and Dyer (23) and the lipid extract was dried under nitrogen. Phospholipase C (10 units) in 1 ml 0.1 N phosphate buffer at PH 7.1 with 30 mM boric acid, to prevent acyl migration (11), was added with 1 ml diethyl ether. The sample was mixed at room temperature for 1 h. Hexane (2 ml) was added and the organic layer was removed and evaporated under nitrogen, followed by further drying under vacuum for 15 min. DMAP (10 mg) was vacuum-dried for 30 min and then dissolved in 0.5 ml dry methylene chloride. The DMAP solution was added to vacuum-dried CMMC (20 mg) and immediately added to the dried diradylglycerols. The solution was heated at 60°C for 30 min and then allowed to stand at room temperature overnight. Methylene chloride (3 ml) was added and the solution was washed with 4 ml 0.1 N KH_2PO_4 (pH 4.8). The organic layer was further washed with 4 ml H_2O and then

dried under nitrogen. The CMMC derivatives were purified by TLC using the solvent system chloroform–methanol–acetic acid (98:2:1) and visualized by UV or by radioactivity measurement. Diradylglycerol CMMC derivatives have an R_f value of 0.8 in this solvent system and no separation of subclasses was observed. TLC radioactivity measurements showed radioactivity only in the UV active area at R_f 0.8, indicating 100% derivatization. The CMMC derivatives were extracted by the method of Bligh and Dyer, dried under nitrogen, and redissolved in 1.0 ml of HPLC normal phase eluent.

CMMC derivatives were separated into subclasses using normal phase HPLC. Aliquots of sample (10–100 nmol) were injected onto an Ultremex column (4.6 × 250 mm, 5 μ silica; Phenomenex, Rancho Palos Verdes, CA) and eluted with hexane–cyclohexane–methyl-*t*-butyl ether–tetrahydrofuran 100:100:20:5 at a flow rate of 2 ml/min using a Beckman 110B solvent delivery system. The eluent was monitored at 320 nm using a Shimadzu SPD-2A variable wavelength UV detector. Collected subclasses were evaporated to dryness under nitrogen and redissolved in 1.5 ml acetonitrile–isopropanol–methyl-*t*-butyl ether 90:15:8. Absorbance at 320 nm was measured using a Hewlett-Packard 8450A diode array spectrometer. GPC concentrations (moles/liter), as the CMMC derivatives for each subclass, were calculated from the absorbance values using an extinction coefficient of 14,700 l mol^{-1} cm^{-1} which was determined in the same solvent using standard solutions of coumarin reagent. Overall recovery yields were determined from the amount of radioactivity measured after subclass separation compared to the initial radioactivity added. The amounts of each GPC subclass present, expressed as moles/10^7 cells, were calculated from the recovery data and the UV absorbance values. This corrects for losses during isolation as well as losses during phospholipase C hydrolysis and subsequent derivatization.

The isolated subclasses of CMMC derivatives were separated into molecular species using reverse phase HPLC. Samples (5–20 nmol) were injected onto an Ultremex column (4.6 × 250 mm, 5 μ C-18; Phenomenex, Rancho Palos Verdes, CA) and eluted with acetonitrile–isopropanol–methyl-*t*-butyl ether 90:15:8 at a flow rate of 1 ml/min and monitored at 320 nm. Individual molecular species were measured using peak height times half width and expressed as percent peak area of total subclass. The mole amount of individual molecular species can then be calculated using this percent area times the total subclass amount determined by UV absorbance. The separated molecular species were collected, evaporated to dryness under nitrogen, and stored at -20°C until CF-FAB/MS analysis.

Diacyl GPC standards (0.1–0.2 mg) were derivatized and the CMMC derivatives were purified by TLC as described for mast cell GPC. The standard solutions were used to determine the minimum level of detection by reverse phase HPLC-UV and HPLC retention times.

The collected CMMC derivatives were redissolved in 20–30 μl of the reverse phase solvent system containing glycerol (2%) and analyzed by continuous flow FAB mass spectroscopy using a double focusing VG 7070E (VG Analytical, Altrincham, UK). Samples were introduced into the mass spectrometer through a 1 meter × 50 μm untreated fused silica transfer capillary using an injector equipped with a 1 μl loop. The solvent system was acetonitrile–isopropanol–glycerol 90:10:2 and was maintained at a flow rate of 5 μl/min using a μLC-500 Micro Flow pump (ISCO, Lincoln, NE). Linear scans were obtained for the 200–1000 amu range with a scan rate of 7.5 sec/scan. The FAB gun (FAB11NFF, Ion Tech Ltd., Teddington, England) was operated at 7 kV and xenon was used as the particle source. Data were analyzed with a Vector One (Teknivent, St. Louis, MO) data system.

Tandem FAB/MS/MS experiments were performed with a TSQ-70B triple quadrupole (Finnigan Corporation, San Jose, CA). The FAB gun was operated at 6 kV and xenon was used as the particle source. Product ion spectra of $[MH]^+$ ions were obtained from collisional-induced dissociations with argon as the collision gas in the second quadrupole cell (0.3 Torr) and 5 ev collision cell offset energy.

RESULTS

Derivatization of diradylglycerols

7-[(Chlorocarbonyl)methoxy]-4-methylcoumarin has been used for the derivatization and HPLC fluorescent detection of steroids and prostaglandins (24). However, conditions required for the complete derivatization of diradyl glycerides were found to be different. In the CMMC derivatization of steroids and prostaglandins (24), optimum derivatization yields were obtained when the concentration of the base catalyst, dimethylaminopyridine (DMAP), was approximately equal to the concentration of CMMC. Derivatization was carried out at ambient temperature and no time dependancy was observed. In the present work, the derivatization of diradylglycerols proceeded to 100% completion (as assayed by TLC radioactivity measurements) only when the concentration of DMAP exceeded the concentration of CMMC by a factor of 2. The use of DMAP was critical as substitution of other bases, such as pyridine or triethylamine, resulted in yields of less than 10%, in

agreement with the earlier work involving steroids and prostaglandins. The derivatization of diradylglycerides also required higher reaction temperatures. Complete derivatization occurred only after reaction at 60°C for 0.5 h followed by 3–4 h at room temperature. As continued derivatization at room temperature neither increased nor decreased yields, reactions were routinely continued overnight to allow convenient workup of the derivatives. Yields of only 50–70% were obtained when the derivatives were isloated by TLC immediately after the initial 60°C treatment for 0.5 h.

HPLC separation of subclasses and molecular species

Diradylglycerol-CMMC derivatives of GPC obtained from mast cells were resolved into subclasses by normal phase HPLC as shown in **Fig. 1.** No separate peak corresponding to 1-alkenyl-2-acyl-GPC was detected. To check for possible contamination of the alkylacyl subclass by 1-alkenyl-2-acyl-CMMC, the subclasses were collected and respotted on TLC plates followed by exposure of the plate to HCl fumes for 20 min (25). Subsequent development in chloroform–methanol–acetic acid 98:2:1 resulted only in the detection of the diacyl and alkylacyl derivatives at the expected R_f 0.8. No additional compounds were detected at lower R_f values which would have indicated the acid catalyzed hydrolysis product of the 1-alkenyl-2-acyl-CMMC subclass. This indicates that the cultured murine bone marrow-derived mast cell does not contain 1-alkenyl-2-acyl-GPC in quantities detectable by this method.

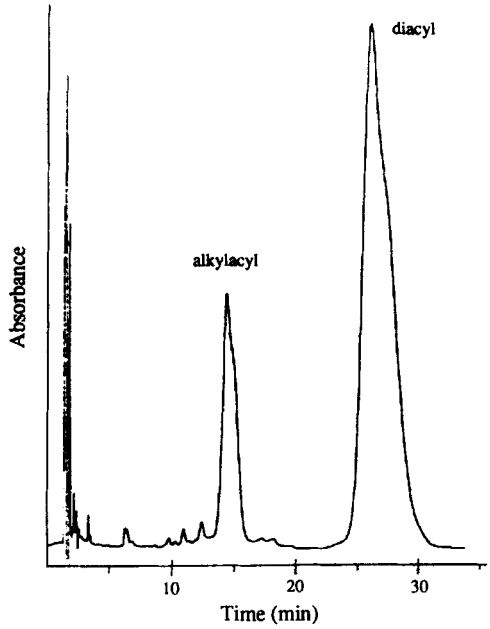

Fig. 1. Normal phase HPLC separation of alkylacyl and diacyl CMMC derivatives prepared from mast cell GPC as described in Materials and Methods and detected by UV absorbance at a wavelength of 320 nm.

Absorbance measurements, corrected for overall recovery, of subclasses from three different cell preparations gave values of $7.9 \pm 0.5 \times 10^{-8}$ mol diacyl-GPC/10^7 cells and $2.7 \pm 0.5 \times 10^{-8}$ mol alkylacyl-GPC/10^7 cells. Overall yield based on radioactivity recovery of [^{3}H]-dipalmitoyl GPC was $40 \pm 6\%$. No significant differences in the absolute amounts of total GPC were detected between cells grown in unsupplemented media and those in media supplemented with AA. Cell cultures supplemented with arachidonic acid showed no change in the ratio of alkylacyl- to diacyl-GPC as compared to unsupplemented cells. Also, control cells grown in media containing ethanol but no AA showed no change in total amounts of subclasses present when compared to normal or AA-supplemented cultures.

Reverse phase separation of standard diacyl CMMC derivatives is shown in **Fig. 2.** At a full scale absorbance 0.005, a signal-to-noise ratio of 10 was obtained with 5–10 pmol of standards. Chromatographic retention of these nonpolar derivatives is a function of both total carbon number and of number of radyl double bonds (11, 12). As expected, the retention times of the disaturated acyl species, ditetra- (peak 1), dipenta- (peak 2), dihexa- (peak 5), dihepta- (peak 7), and dioctadecanoylglycerol-CMMC (peak 8), increased in a nonlinear fashion during isocratic elution. Also, chromatographic retention was greatly decreased by the de-

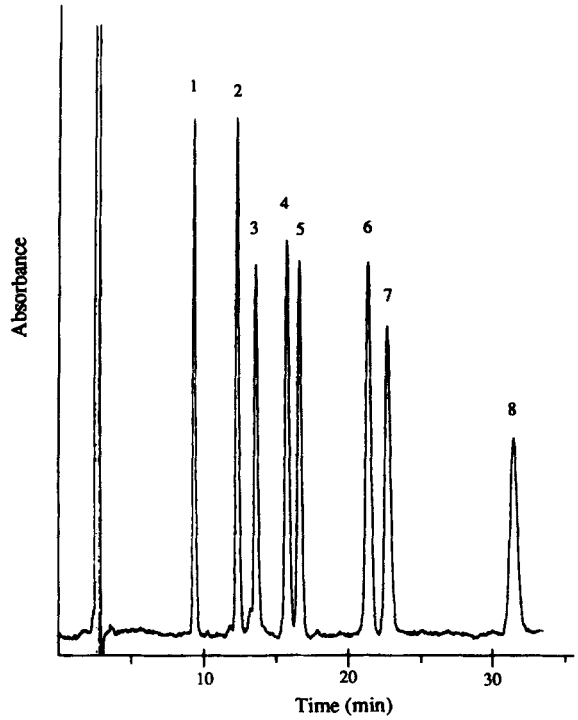

Fig. 2. Reverse phase HPLC separation of 80 to 90 pmol of diacyl CMMC derivatives. Full scale absorbance was 0.005. Peak numbers correspond to (1) 14:0a/14:0, (2) 15:0a/15:0, (3) 18:0a/20:4, (4) 18:0a/18:2, (5) 16:0a/16:0, (6) 18:0a/18:1, (7) 17:0a/17:0, (8) 18:0a/18:0.

gree of unsaturation, as shown in the 18:0a/18:1- (peak 6) and 18:0a/18:0-CMMC (peak 8) series. The decrease in retention time due to an increase of one double bond (18:0a/18:2 compared to 18:0a/18:1) was approximately the same as a decrease of two methylene moieties in the saturated diacyl derivatives (16:0a/16:0 as compared to 17:0a/17:0).

The reverse phase separations of molecular species of diacyl- and alkylacyl-CMMC derivatives obtained from mast cells are shown in **Fig. 3** and **Fig. 4,** respectively. Due to the complex mixture of species present and the opposing influences of total carbon number and degree of unsaturation on retention times, unequivocal identification of individual molecular species was not possible based on specific retention times alone. Therefore, mass spectrometry was used to identify individual molecular species.

FAB/MS identification of CMMC derivatives

Continuous flow fast-atom bombardment mass spectral data was obtained for the mast cell diacyl- and alkylacyl-CMMC derivatives. The diacyl-CMMC derivatives yielded abundant $[MH]^+$ ions as well as four fragment ions corresponding to losses of the free acids. R_1COOH and R_2COOH [(A) and (C) in **Fig. 5a,** respectively] and alkylketene, R_1=C=O and R_2=C=O [(B) and (D) in Fig. 5a, respectively] from the *sn*-1 and *sn*-2 acyl substituents. For the example shown in Fig. 5a (peak 9, Fig. 3), 1-stearoyl-2-arachidonoyl-*sn*-glycerol-CMMC, the molecular weight is indicated by the $[MH]^+$ at *m/z* 861. Neutral loss of eicosatetraenylketene from the *sn*-2 substituent with back transfer of a proton produces the protonated lyso 18:0 CMMC derivative (*m/z* 575), a loss of 286 u. Analogous fragmentation occurred from the *sn*-1 position, loss of octadecylketene, to yield the ion observed at *m/z* 595. Additional fragment ions involving the loss of stearic acid from the *sn*-1 and arachidonic acid from the *sn*-2 position are observed at *m/z* 577 and *m/z* 557, respectively. Preferential losses from either the *sn*-1 versus the *sn*-2 position were not observed.

The alkylacyl derivatives also yielded diagnostic structural ions by FAB/MS. A representative continuous flow FAB mass spectrum obtained after reverse phase separation of the mast cell alkylacyl-CMMC derivatives (peak 6, Fig. 4) is shown in Fig. 5b. The 18:1e/16:1 alkylacyl derivative showed an abundant protonated molecular ion $[MH]^+$, *m/z* 795, and fragmentation of the *sn*-2 acyl chain, producing the protonated lyso species, *m/z* 559, by loss of hexadecenylketene (fragment ion (E) in Fig. 5b). Unlike the diacyl derivatives, loss of neutral acid from the *sn*-2 position was not ob-

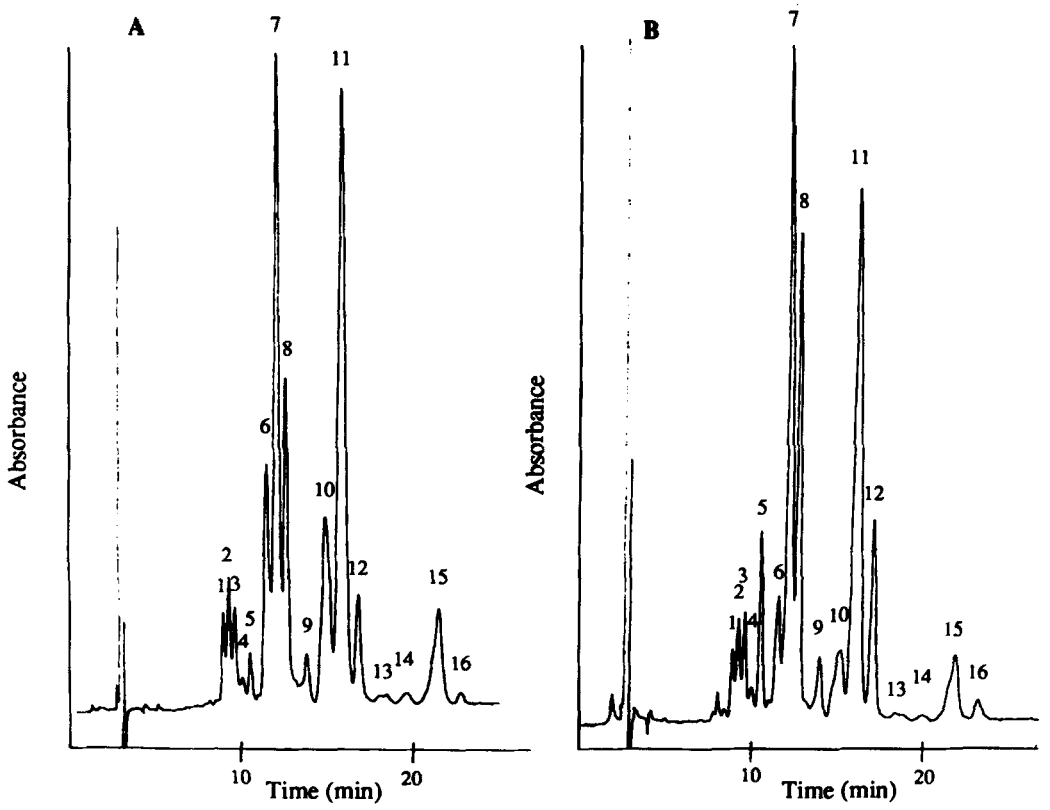

Fig. 3. Reverse phase HPLC separation of diacyl CMMC derivatives prepared from mast cell GPC. Cells were grown in A, normal media, or B, arachidonic acid-supplemented media. Peak numbers refer to molecular species as listed in Table 3.

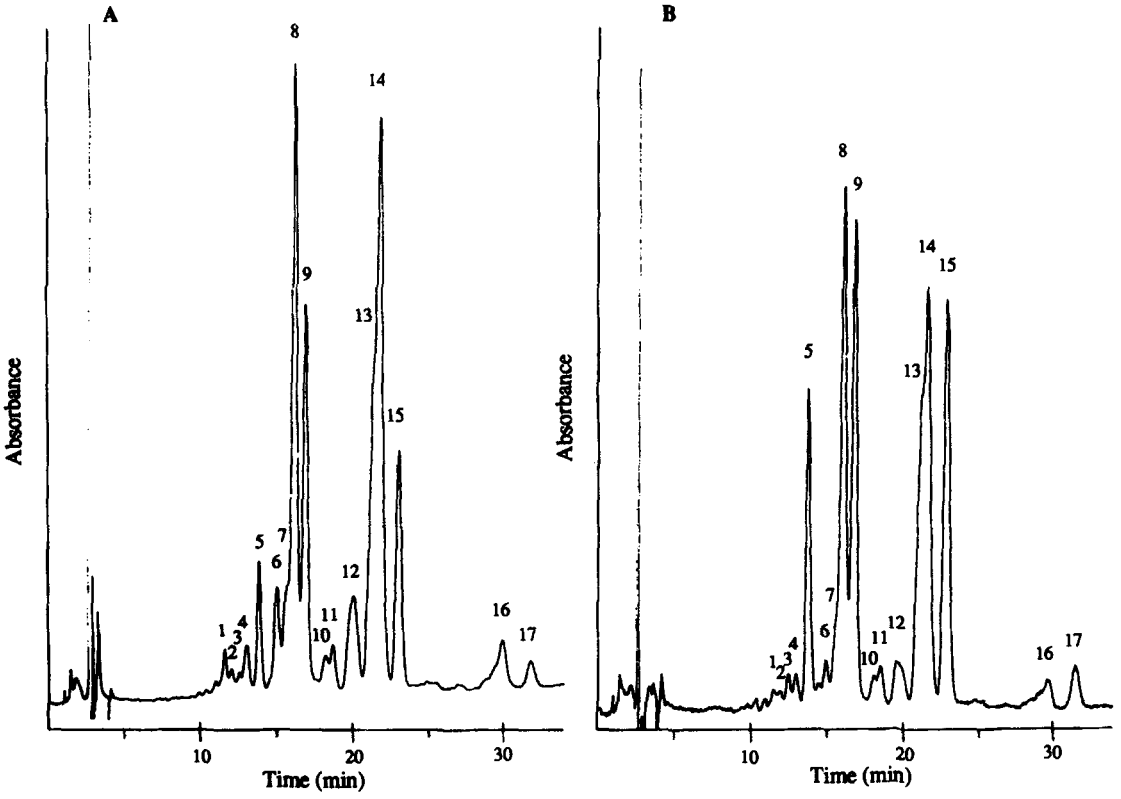

Fig. 4. Reverse phase HPLC separation of alkylacyl CMMC derivatives prepared from mast cell GPC. Cells were grown in A, normal media, or B, arachidonic acid-supplemented media. Peak numbers refer to molecular species as listed in Table 4.

served. No fragmentation of the ether-linked substituent was observed.

To further study the formation of these fragment ions, collisional-activated decompositions (CAD) studies were performed with standard diacyl-CMMC derivatives and several representative alkylacyl derivatives obtained from mast cells. Product ion spectra obtained by tandem FAB/MS/MS analysis of the decomposition of $[MH]^+$ ions produced qualitatively similar spectra as obtained from FAB/MS. Product ion spectra are generated by isolating the protonated molecular ion, $[MH]^+$, of a specific molecular species by the first quadrupole analyzer, decomposing this ion in the second RF only collision quadrupole cell, and then identifying the product ions in the third quadrupole analyzer. These product ion spectra demonstrated that these acyl-specific fragment ions were derived from the intact diradyl derivatives and were not from contaminants in the sample or probe matrix. The MS/MS analysis also reduced the background chemical interference due to FAB matrix ions and product ion spectra had ion currents with significantly higher signal-to-noise ratios.

Based upon these fragment ions, mass spectrometric analysis of the diradylglycerol-CMMC derivatives allowed the structural assignment of the intact individual molecular species, as listed in **Table 1** and **Table 2** for the diacyl- and alkylacyl derivatives, respectively, eliminating the need for a subsequent derivatization and additional analysis of the methyl esters by gas–liquid chromatography.

Analysis of GPC molecular species of murine bone marrow-derived mast cells

Analysis of murine mast cell diacyl-GPC molecular species from cells grown in normal media and from cells grown in AA-supplemented media are shown in Fig. 3. Mole percent of individual species, as measured by UV absorbance (320 nm) and identified by FAB/MS, are listed in **Table 3.** Since the absolute mole amount of diacyl-GPC was unaffected by AA supplementation, these percent changes in individual molecular species are directly related to changes in the absolute amount of individual species due to AA supplementation. After AA supplementation, the diacyl molecular species analysis showed a greater than threefold increase in the 16:0a/20:4 molecular species (peak 5, Fig. 3B, Table 3). This increase in arachidonoyl species was apparently compensated for by 50% decreases in the doubly unsaturated species, 16:1a/18:1 (peak 6, Fig. 3B, Table 3) and 18:1a/18:1 (peak 10, Fig. 3B, Table 3). At the same time, there was an increased in the saturated 16:0a/16:0 species (peak 12, Fig. 3B, Table 3).

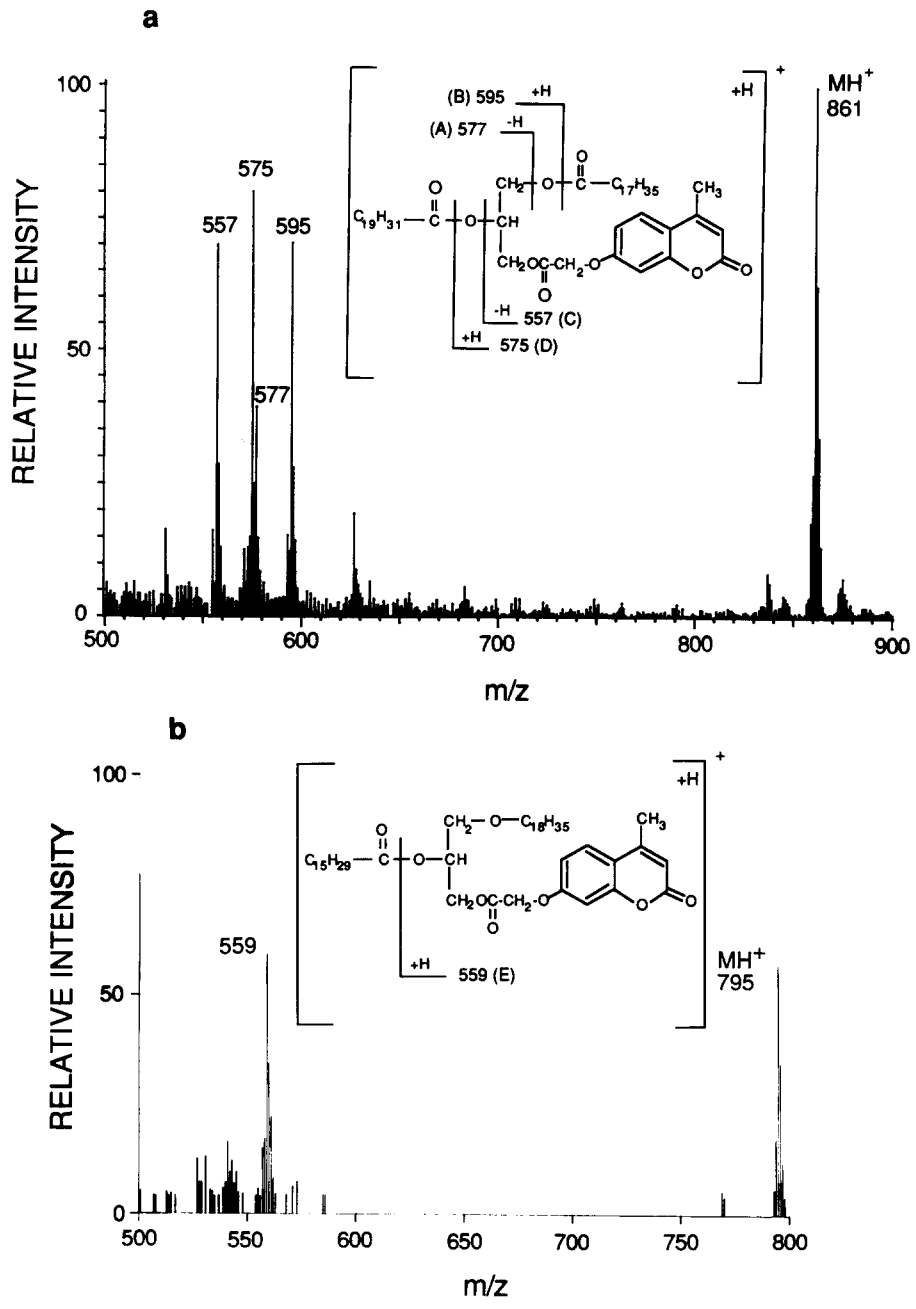

Fig. 5. a) CF-FAB/MS chromatogram of 18:0a/20:4 CMMC derivative prepared from standard GPC. the MH^+ ion has observed at *m/z* 861. Fragment ions were observed corresponding to side chain losses shown in the inset. b) CF-FAB/MS chromatogram of 18:1e/16:1 CMMC obtained from reverse phase HPLC separation of alkylacyl CMMC derivatives prepared from mast cell GPC. This was collected as peak #6 in Fig. 4. The MH^+ ion was observed at *m/z* 795 and the fragmention at *m/z* 559 allowed complete structural identification.

The changes in the alkylacyl GPC molecular species with AA supplementation were similar to the changes observed in diacyl-GPC as illustrated in Fig. 4 and listed in **Table 4.** The 16:0e/20:4 molecular species (peak 5, Fig. 4B, Table 4) showed a 250% increase while the diunsaturated species, 18:1e/16:1 (peak 6, Fig. 4B, Table 4) and 18:1e/18:1 (peak 12, Fig. 4B, Table 4) decreased during AA supplementation as did 16:0e/18:1 (peak 14, Fig. 4B, Table 4). However, the saturated species, 16:0e/16:0 (peak 15, Fig. 4B, Table 4) also showed a significant increase during AA supplementation.

Control cell cultures grown in media containing ethanol but no AA showed no change in molecular species in either diacyl or alkylacyl subclasses as compared to cell cultures grown in normal media.

TABLE 1. FAB/MS fragment ions of diacyl-CMMC derivatives

HPLC peak[a]	Diacyl-CMMC	MH^{+}[b]	A	B	C	D
1	14:0a/18:2	781	553 (36)[c]	571 (20)	501	519
2	14:0a/16:1	755	527	545	501	519
3	14:0a/14:0	729	501 (51)	519 (106)[d]	501	519
4	18:1a/20:4	859	577 (31)	595	555 (27)	573 (48)
5	16:0a/20:4	833	577 (28)	595 (53)	529 (61)	547 (77)
6	16:1a/18:1	809	555 (50)	573 (50)	527 (40)	545 (72)
7	16:0a/16:1	783	527	545	529 (39)	547 (86)
8	14:0a/16:0	757	529	547	501 (22)	519 (36)
9	18:0a/20:4	861	577 (40)	595 (71)	557 (70)	575 (80)
	16:0a/17:1	797	541	559 (63)	529 (48)	547 (75)
10	18:1a/18:1	837	555 (43)	573 (78)[d]	555	573
11	16:0a/18:1	811	555 (25)	573 (94)	529 (35)	547 (35)
12	16:0a/16:0	785	529 (63)	547 (93)[d]	529	547
13	17:0a/18:1	825	555	573	543	561
14	18:1a/20:1	865	583 (32)	601 (45)	555 (52)	573 (56)
15	18:0a/18:1	839	555 (19)	573 (38)	557 (24)	575 (30)
16	16:0a/18:0	813	557	575	529	547

[a]HPLC peak numbers are identified in Fig. 3. The letter "a" refers to acyl linkage in the *sn*-1 position.
[b]Ion *m/z* values. Letters correspond to ion structures discussed in the text and in Fig. 5a.
[c]Numbers in parentheses are ion abundance values relative to MH^{+} = 100. No abundance values are given for overlapping HPLC fractions that contained common ions or for very low abundance ions.
[d]For symmetrical species, identical sets of fragment ions are formed. Abundance values are listed as the sum.

DISCUSSION

Arachidonic acid is in a dynamic flux throughout the phospholipid classes of a cell. When added to culture media, AA is rapidly taken up by mast cells and processed into phospholipids (26). This previous study also showed that in a time- and concentration-dependent process, AA initially appears in glycerophosphocholine (GPC) and subsequently is remodeled to other classes, primarily to glycerophosphoethanolamine (GPE) and to a less extent glycerophosphoinositol (GPI) and glycerophosphoserine (GPS). To assess the role of arachidonoyl-containing GPC molecular species as immediate precursors for both PAF and leukotrienes requires a sensitive, quantitative, and specific determination of GPC molecular species. Our approach used a quantitative spectrophotometric method based on HPLC separation and quantitation of carbonylmethoxymethylcoumarin derivatives of diradylglycerides coupled with specific identification by mass spectral analysis.

When CMMC derivatization combined with fluorescent detection was used in the analysis of steroids and prostaglandins, favorable detection limits in the high picogram range were obtained but were found to vary with solvent composition due to variation in quantum yield, with higher quantum yields obtained in aqueous solvent systems (24). That study and a similar one involving coumarin derivatives of fatty acids (27) also demonstrated a strong UV absorption maxima at 320 nm with an extinction coefficient independent of derivatized species and solvent conditions. In the present application, complete derivatization required stronger reaction conditions due to the nonpolar nature of the glyceride hydroxyl group. Also, the reverse phase solvent system that resulted in the best separation of CMMC-derivatized molecular species was nonaqueous and UV detection was superior to fluorescent detection. Nonetheless, the relatively high extinction coefficient (14,700 l mol^{-1} cm^{-1}) combined with UV detection at 320 nm resulted in high sensitivity (10 pmol) for on-line HPLC quantitation.

TABLE 2. FAB/MS fragment ions of alkylacyl-CMMC derivatives

HPLC Peak[a]	Alkylacyl-CMMC	MH^{+}[b]	E
1	unidentified		
2	14:0e/16:1	741	505
3	14:0e/14:0	715	505
4	18:1e/20:4	845	559 (51)[c]
5	16:0e/20:4	819	533 (96)
6	18:1e/16:1	795	559 (81)
7	18:1e/14:0	769	559 (82)
8	16:0e/16:1	769	533 (136)
9	16:0e/14:0	743	533
10	16:0e/17:1	783	533 (66)
11	16:0e/15:0	757	533
12	18:1e/18:1	823	559 (112)
13	18:1e/16:0	797	559
14	16:0e/18:1	797	533 (103)
15	16:0e/16:0	771	533 (95)
16	18:0e/18:1	825	561 (86)
17	18:0e/16:0	799	561

[a]HPLC peak numbers are identified in Fig. 4. The letter "e" refers to ether linkage in the *sn*-1 position.
[b]Ion *m/z* values. E corresponds to the ion structure discussed in the text and in Fig. 5b.
[c]Numbers in parentheses are ion abundance values relative to MH^{+} = 100. No abundance values are given for overlapping HPLC fractions that contained common ions or for very low abundance ions.

TABLE 3. Diacyl molecular species of glycerophosphocholine in transformed mast cells

Peak#[a]	Molecular Species[b]	Normal Media[c] n = 5[d]	AA-Supplemented[c] n = 2[e]
1	14:0a/18:2	2.2 ± 0.1	1.6 ± 0.1
2	14:0a/16:1	3.2 ± 0.4	2.6 ± 0.5
3	14:0a/14:0	2.2 ± 0.6	2.7 ± 0.5
4	18:1a/20:4	0.8 ± 0.2	1.2 ± 0.3
5	16:0a/20:4	1.3 ± 0.2	4.7 ± 0.6
6	16:1a/18:1	9.8 ± 1.3	4.1 ± 0.4
7	16:0a/16:1	18.7 ± 1.4	19.8 ± 0.9
8	14:0a/16:0	10.3 ± 1.6	14.7 ± 0.1
9	18:0a/20:4	1.9 ± 0.1	1.9 ± 0.1
	16:0a/17:1		
10	18:1a/18:1	9.8 ± 1.9	4.9 ± 0.5
11	16:0a/18:1	28.4 ± 1.6	27.8 ± 0.8
12	16:0a/16:0	4.1 ± 0.6	7.6 ± 0.5
13	17:0a/18:1	0.6 ± 0.1	0.5 ± 0.3
14	18:1a/20:1	0.5 ± 0.1	0.3 ± 0.1
15	18:0a/18:1	5.4 ± 0.7	4.9 ± 0.3
16	16:0a/18:0	0.5 ± 0.1	0.9 ± 0.1

[a]Peak number refers to numbered peaks in Fig. 3A and 3B.
[b]The first number refers to the length of the carbon chain and the second number to the number of double bonds in the chain. The shorter chain is assumed to be the *sn*-1 position. The letter "a" refers to acyl linkage in the *sn-1* position.
[c]% mol composition based on reverse phase HPLC peak area.
[d]% mol values are listed as the mean ± SD.
[e]% mol values are listed as the mean ± range.

Fast-atom bombardment mass spectrometry has become a routine and powerful tool for the analysis of large, nonvolatile biomolecules. Continuous-flow FAB simplifies both the sample introduction procedure and reduces the amount of matrix (hence background ions) required for ionization and desorption. Under continuous-flow FAB conditions, the diradylglyceride CMMC derivatives yielded simple and diagnostic fragment ions from acyl substituents. Combined with good RP-HPLC separation and sensitive UV absorption, these derivatives form the basis of a specific and quantitative analysis of GPC molecular species.

TABLE 4. Alkylacyl molecular species of glycerophosphocholine in transformed mast cells

Peak#[a]	Molecular Species	Normal Media[b] n = 4	AA-Supplemented[b] n = 3
1	unidentified	1.3 ± 0.4	0.5 ± 0.2
2	14:0e/16:1	0.9 ± 0.3	0.6 ± 0.2
3	14:0e/14:0	0.9 ± 0.4	1.1 ± 0.2
4	18:1e/20:4	1.1 ± 0.3	1.0 ± 0.1
5	16:0e/20:4	3.4 ± 0.4	8.6 ± 0.4
6	18:1e/16:1	4.0 ± 0.3	1.9 ± 0.4
7	18:1e/14:0	4.1 ± 0.7	2.3 ± 0.3
8	16:0e/16:1	17.4 ± 0.5	16.6 ± 0.2
9	16:0e/14:0	11.6 ± 0.6	15.1 ± 0.5
10	16:0e/17:1	1.5 ± 0.2	1.0 ± 0.2
11	16:0e/15:0	1.4 ± 0.1	1.4 ± 0.1
12	18:1e/18:1	5.4 ± 0.3	3.1 ± 0.3
13	18:1e/16:0	10.0 ± 2.2	12.4 ± 1.0
14	16:0e/18:1	25.5 ± 1.4	15.4 ± 1.3
15	16:0e/16:0	8.0 ± 0.6	14.8 ± 0.9
16	18:0e/18:1	2.6 ± 0.4	1.9 ± 0.4
17	18:0e/16:0	1.2 ± 0.2	2.1 ± 0.1

[a]Peak number refers to numbered peaks in Fig. 4A and 4B. The letter "e" refers to ether linkage in the *sn*-1 position.
[b]% mol composition based on reverse phase HPLC peak area. Values are listed as the mean ± SD.

This analysis readily distinguishes isomeric diacyl molecular species of the same molecular weight but composed of different acyl substituents, e.g., 18:0a/18:2 from 18:1a/18:1. Unfortunately though, no preferential acyl fragmentation of the diacyl derivatives was observed from the *sn*-1 or *sn*-2 position under either FAB/MS or CAD FAB/MS/MS analysis. Thus exact positional assignment of the radyl substituents is not yet possible. However, during the CAD tandem MS studies of diacyl CMMC derivatives, all but one molecular species decomposed with greater loss of the free acid from the *sn*-1 and alkylketene from the *sn*-2 position. The species that did not follow this pattern was 16:0a/20:4. This anomaly may reflect a combined effect of the length and degree of unsaturation of the *sn*-2 acyl substituent (19). Further studies are required to clarify this observation. The acyl positional assignments, as listed in Table 3, are based on the assumption that the longest and most unsaturated acyl substituent is in the *sn*-2 position.

Exact assignment of the radyl substituents of the alkylacyl-CMMC species was possible. These derivatives yielded abundant MH^+ ions and a single fragment ion derived from loss of alkylketene from the *sn*-2 acyl group. As no fragment ions were observed from the alkyl substituent, the alkyl group was identified from the frament ion *E*, which retained the 1-*O*-alkyl group. The 2-*O*-acyl group was determined from the mass difference of the protonated molecular ion and the fragment ion *E*.

The usefulness of this method has been demonstrated in the analysis of molecular species of GPC from murine bone marrow-derived mast cells grown in normal media and in media supplemented with AA. As this is both a quantitative and qualitative method, not only are determinations of relative changes in GPC molecular species with fatty acid supplementation possible, but the absolute mole amounts of each molecular species can also be determined. After AA supplementation, 1-palmitoyl-2-arachidonoyl-GPC increased from 1.0 nmol/10^7 cells in unsupplemented media to 3.7 nmol/10^7 cells, and 1-hexadecyl-2-arachidonoyl-GPC increased from 0.9 nmol/10^7 cells to 2.3 nmol/10^7 cells. These increases occurred while the next most abundant arachidonoyl-containing species, 1-oleoyl-2-arachidonoyl-GPC and 1-octadecenyl-2-arachidonoyl-GPC, showed little or no change. Non-arachidonoyl-containing species are also affected. As *sn*-2 arachidonoyl species increase, diunsaturated species decrease and disaturated species increase.

These changes may reflect a homeostatic regulation in the balance of total saturated versus unsaturated radyl constituents.

This work was supported in part by a grant from the National Institutes of Health (HL34303).

Manuscript received 13 June 1991 and in revised form 29 October 1991.

REFERENCES

1. Ramesha, C. S., and W. C. Pickett. 1986. Platelet-activating factor and leukotriene biosynthesis is inhibited in polymorphonuclear leukocytes depleted of arachidonic acid. *J. Biol. Chem.* **261:** 7592–7595.
2. Chilton, F. H., and R. C. Murphy. 1986. Remodeling of arachidonate-containing phosphoglycerides within the human neutrophil. *J. Biol. Chem.* **261:** 7771–7777.
3. Chilton, F. H., J. M. Ellis, S. C. Olson, and R. L. Wykle. 1984. 1-O-Alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine. A common source of platelet-activating factor and arachidonate in human polymorphonuclear leukocytes. *J. Biol. Chem.* **259:** 12014–12019.
4. Mueller, H. W., J. T. O'Flaherty, D. G. Greene, M. P. Samuel, and R. L. Wykle. 1984. 1-*O*-Alkyl-linked glycerophospholipids of human neutrophils: distribution of arachidonate and other acyl residues in the ether-linked and diacyl species. *J. Lipid Res.* **25:** 383–388.
5. Mueller, H. W., J. T. O'Flaherty, and R. L. Wykle. 1984. The molecular species distribution of platelet-activating factor synthesized by rabbit and human neutrophils. *J. Biol. Chem.* **259:** 14554–14559.
6. Satouchi, K., M. Oda, K. Yasunaga, and K. Saito. 1985. Evidence for production of 1-acyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine concomitantly with platelet-activating factor. *Biochem. Biophys. Res. Commun.* **128:** 1409–1417.
7. Pinkard, R. N., E. M. Jackson, C. Hoppens, S. T. Weintraub, J. C. Ludwig, L. M. McManus, and G. E. Mott. 1984. Molecular heterogeneity of platelet-activating factor produced by stimulated human polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.* **122:** 325–332.
8. Sturk, A., M. C. L. Schaap, A. Prins, J. Wouter ten Cate, and H. van den Bosch. 1989. Synthesis of platelet-activating factor by human blood platelets and leukocytes. Evidence against selective utilization of cellular ether-linked phospholipids. *Biochim. Biophys. Acta.* **993:** 148–156.
9. Leslie, C. C., D. R. Voelker, J. Y. Channon, M. M. Wall, and P. T. Zelarney. 1988. Properties and purification of an arachidonoyl-hydrolyzing phospholipase A_2 from a macrophage cell line, RAW 264.7. *Biochim. Biophys. Acta.* **963:** 476–492.
10. Blank, M. L., M. Robinson, V. Fitzgerald, and F. Snyder. 1984. Novel quantitative method for determination of molecular species of phospholipids and diglycerides. *J. Chromatogr.* **298:** 473–482.
11. Kito, M., H. Takamura, H. Narita, and R. Urade. 1985. A sensitive method for quantitative analysis of phospholipid molecular species by high-performance liquid chromatography. *J. Biochem.* **98:** 327–331.
12. Batley, M., N. H. Packer, and J. W. Redomond. 1982. Molecular analysis of the phospholipids of *Escherichia Coli* K12. *Biochim. Biophys. Acta.* **710:** 400–405.
13. Nakagawa, Y., T. Sugiura, and K. Waku. 1985. The molecular species composition of diacyl-, alkylacyl- and alkenylacyl glycerophospholipids in rabbit alveolar macrophages. High amounts of 1-*O*-hexadecyl-2-arachidonyl molecular species in alkylacylglycerophosphocholine. *Biochim. Biophys. Acta.* **833:** 323–329.
14. Krüger, J., H. Rabe, G. Reichmann, and B. Rüstow. 1984. Separation and determination of diacylglycerols as their naphthylurethanes by high-performance liquid chromatography. *J. Chromatogr.* **307:** 387–392.
15. Rastegar, A., A. Pelletier, G. Duportail, L. Freysz, and C. Leray. 1990. Sensitive analysis of phospholipid molecular species by high-performance liquid chromatography using fluorescent naproxen derivatives of diacylglycerols. *J. Chromatogr.* **518:** 157–165.
16. Ramesha, C., W. C. Pickett, and D. V. K. Murthy. 1989. Sensitive method for the analysis of phospholipid subclasses and molecular species as 1-anthroyl derivatives of their diglycerides. *J. Chromatogr.* **491:** 37–48.
17. Jungalwala, F. B., J. E. Evans, and R. H. McCluer. 1984. Compositional and molecular species analysis of phospholipids by high performance liquid chromatography coupled with chemical ionization mass spectrometry. *J. Lipid Res.* **25:** 738–749.
18. Jensen, N. J., K. B. Tomer, and M. L. Gross. 1987. FAB MS/MS for phosphatidylinositol, -glycerol, -ethanolamine and other complex phospholipids. *Lipids.* **22:** 480–489.
19. Munster, H., and H. Budzikiewicz. 1988. Structural and mixture analysis of glycerophosphoric acid derivatives by fast atom bombardment tandem mass spectrometry. *Biol. Chem. Hoppe-Seyler.* **369:** 303–308.
20. Kayganich, K., and R. C. Murphy. 1991. Molecular species analysis of arachidonate containing glycerophosphocholines by tandem mass spectrometry. *J. Am. Chem. Soc. Mass Spectrom.* **2:** 45–54.
21. Haroldsen, P. E., and R. C. Murphy. 1987. Analysis of phospholipid molecular species in rat lung as dinitrobenzoate diglycerides by electron capture negative chemical ionization mass spectometry. *Biomed. Environ. Mass Spectrom.* **14:** 573–578.
22. Pierce, J. H., P. P. Di Fiore, S. A. Aaronson, M. Potter, J. Pumphrey, A. Scott, and J. N. Ihle. 1985. Neoplastic transformation of mast cells by Abelson-MuLV: abrogation of IL-3 dependence by a nonautocrine mechanism. *Cell.* **41:** 685–693.
23. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
24. Karlsson, K., D. Wiesler, M. Alasandro, and M. Novotny. 1985. 7-[(Chlorocarbonyl)methoxy]-4-methylcoumarin: a novel fluorescent reagent for the precolumn derivatization of hydroxy compounds in liquid chromatography. *Anal. Chem.* **57:** 229–234.
25. Yoshioka, S., S. Nakashima, Y. Okano, H. Hasegawa, A. Ichiyama, and Y. Nozawa. 1985. Phospholipid (diacyl, alkylacyl, alkenylacyl) and fatty acyl chain composition in murine mastocytoma cells. *J. Lipid Res.* **26:** 1134–1141.

26. Bettazzoli, L., J. A. Zirrolli, C. T. Reidhead, M. Shahgholi, and R. C. Murphy. 1990. Incorporation of arachidonic acid in glycerophospholipids of a murine bone marrow-derived mast cell. *Adv. Prostaglandin Thromboxane Leukotriene.* **20:** 71–78.

27. Lloyd, J. B. F. 1979. Fatty acid esters of 4-hydroxymethyl-7-coumarin: fluorescence characteristics and their variation in high -performance liquid chromatographic solvents. *J. Chromatogr.* **178:** 249–258.