Differential turnover of polyunsaturated fatty acids in plasmalogen and diacyl glycerophospholipids of isolated cardiac myocytes

Steven D. DaTorre* **and Michael H. Creer***,†

Cardiovascular Division, Department of Internal Medicine,* Washington University School of Medicine, St. Louis, MO, and Department of Pathology,† University of Arkansas for Medical Sciences, Little Rock, AR

Abstract To investigate the relative turnover of esterified polyunsaturated fatty acids in diacylglycerophospholipids and plasmalogens in isolated cardiac myocytes, we characterized the phospholipid composition and distribution of radiolabel in different phospholipid classes and in individual molecular species of diradyl choline (CGP) and ethanolamine (EGP) glycerophospholipids after incubation of isolated cardiac myocytes with [^{3}H]arachidonate or [^{14}C]linoleate. Plasmalogens in CGP (55%) and EGP (42%) quantitatively accounted for the total plasmalogen content (39%) of cardiac myocyte phospholipids. Plasmalogens comprised 86% and 51% of total arachidonylated CGP and EGP mass, respectively, and [^{3}H]arachidonate was primarily incorporated into plasmalogens in both CGP (65%) and EGP (61%) classes. The specificity activity of [^{3}H]arachidonylated diacyl-CGP was approximately 2- to 5-fold greater than that of [^{3}H]arachidonylated choline plasmalogen, whereas comparable specific activities were found in the [^{3}H]arachidonate-labeled ethanolamine plasmalogen and diacyl-EGP pools. Of the total linoleate-containing CGP and EGP mass, 54% and 57%, respectively, was esterified to plasmalogen molecular species. However, [^{14}C]linoleate was almost exclusively incorporated into diacyl-CGP (96%) and diacyl-EGP (86%). The specific activities of [^{14}C]linoleate-labeled diacyl-CGP and diacyl-EGP were 5- to 20-fold greater than that of the [^{14}C]linoleate-labeled plasmalogen pools. The differential incorporation of polyunsaturated fatty acids in plasmalogens and diacylglycerophospholipids demonstrates that the metabolism of the *sn*-2 fatty acyl moeity in these phospholipid subclasses is differentially regulated, possibly fulfilling separate and distinct physiologic roles. —**DaTorre, S. D., and M. H. Creer.** Differential turnover of polyunsaturated fatty acids in plasmalogen and diacyl glycerophospholipids of isolated cardiac myocytes. *J. Lipid Res.* 1991. **32:** 1159–1172.

Supplementary key words phospholipids • arachidonic acid • linoleic acid

Alterations in myocardial phospholipid metabolism play an important role in several physiologic and pathophysiologic processes in the heart. For example, during myocardial ischemia, hydrolysis of membrane phospholipids results in accumulation of free (unesterified) arachidonic acid (1), lysoglycerophospholipids (2), and alkenylacylglycerol (3). These amphiphilic lipid metabolites accumulate in ischemic myocardium as a consequence of the activation of phospholipases (4, 5) and/or inhibition of catabolism of these phospholipid-derived hydrolytic products (2). The accumulation of these compounds in the ischemic heart is temporally correlated with development of impaired cardiac function (6), disturbances in calcium homeostasis (6, 7), alterations in electrophysiologic properties that contribute to the development of ventricular arrhythmias (8), and progression to irreversible cell injury (9). These effects are likely to be mediated, at least in part, by changes in the biophysical properties of the membrane associated with alterations in membrane phospholipid composition and by modulation of integral membrane protein function by direct interaction of membrane proteins with these amphiphilic lipid metabolites (2, 10). Phospholipase-catalyzed hydrolysis of phospholipids in the sarcolemmal membrane is likely to be particularly important since the locally generated hydrolytic products would have direct access to those integral membrane proteins that regulate ion flux and participate in signal transduction.

The phospholipid composition of the sarcolemma and sarcoplasmic reticulum of the cardiac myocyte is unique in that plasmalogens comprise the predominant phospholipid constituents in both of these subcellular membrane compartments (11, 12). Although the sarcolemma and sarcoplasmic reticulum are comprised predominantly of plasmalogen molecular species, previous estimates of the

Abbreviations: CGP, diradyl choline glycerophospholipid; EGP, diradyl ethanolamine glycerophospholipid; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidylglycerol (cardiolipin); SPH, sphingomyelin; FAME, fatty acid methyl ester; DMA, dimethylacetal; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry; TLC, thin-layer chromatography.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

extent to which plasmalogens are selectively enriched in these membranes have been based exclusively on comparisons of the phospholipid composition of isolated membrane preparations with that of whole tissue. Such comparisons could be very misleading since the myocardium is also comprised of a variety of other cellular elements including endothelium, vascular smooth muscle, nerve, adipose, and connective tissue in addition to cardiac myocytes. Accordingly, one objective of the present study was to characterize the phospholipid composition of isolated adult canine cardiac myocytes.

The molecular composition of myocardial plasmalogen phospholipid species is characterized by a relatively high content of arachidonate esterified to the *sn*-2 position (11, 12). In addition, recent studies have demonstrated that canine myocardium contains a calcium-dependent phospholipase A_2 and a calcium-independent plasmalogen selective phospholipase A_2 (13). Despite these imporant observations, the functional significance of the high content of esterified arachidonate in plasmalogens and of separate pathways of hydrolysis of arachidonate from diacyl glycerophospholipids and plasmalogens is currently not known. In order to determine whether the specific enrichment of highly unsaturated fatty acids in plasmalogens fulfills a purely structural role or whether these moieties are metabolically active, we compared the relative turnover of esterified *sn*-2 fatty acids in individual diacyl and plasmalogen phospholipid molecular species after incubation of isolated cardiac myocytes with radiolabeled arachidonate and linoleate.

MATERIALS AND METHODS

Isolation of adult canine cardiac myocytes

Intact, viable myocytes were obtained from adult mongrel dogs of either sex using a perfusion technique modified from the method of Haworth and colleagues as previously described (14). This procedure yielded 15 to 40 million cells from approximately 10 to 20 g of left ventricular muscle. When the percentage of elongated viable myocytes fell below 70%, Percoll (Sigma) gradients were used to separate rounded, non-viable cells from intact rectangular myocytes, using a discontinuous Percoll gradient method adopted from Wittenberg and Robinson (15) and Heathers and colleagues (16). Elongated cells that sedimented to the interface were collected, pooled, and washed in Ca^{2+}-HEPES (500 μM Ca^{2+}) buffer. Final cell yields consisted of greater than 75% elongated myocytes when counted in a hemocytometer.

Labeling of adult canine cardiac myocytes

After washing, isolated canine cardiac myocytes were resuspended in 500 μM Ca^{2+}-HEPES buffer (8×10^5 cells/ml). Three 20-μl aliquots of the cell suspension were removed and the protein content was determined by a modification of the Lowry method as described by Markwell et al. (17). Two ml of Dulbecco's Modified Eagle Medium (GIBCO) containing 25 mM HEPES, 10% horse serum, and 500 μM $CaCl_2$ was then added to each well of a 6-well plastic tissue culture plate (Falcon). To each well, 0.8 μCi [^{3}H]arachidonic acid (76 Ci/mmol, New England Nuclear or 0.2 μCi [^{14}C]linoleic acid (0.95 Ci/mmol, New England Nuclear) in 10 μl ethanol was added and the plate was then placed in an incubator at 37°C under an atmosphere of 95% air/5% CO_2 for 1 h. After equilibration, 100 μl (80,000 cells) of the cell suspension was added to each well and the cells were incubated for 18 h. At the conclusion of the incubation period, the cells were removed, washed twice with 500 μM Ca^{2+}-HEPES buffer, resuspended in 600 μl of buffer, and extracted with chloroform and methanol as described below after addition of radiolabeled internal standards. The radiolabeled internal standards were obtained from New England Nuclear and are described in the legends of Figs. 4 and 5 and Tables 4 and 5. The cell extracts were subjected to gradient elution HPLC to isolate the major phospholipid classes and reverse phase HPLC to isolate individual phospholipid molecular species as described below.

Extraction, separation, and analysis of phospholipid classes

To characterize the phospholipid composition of isolated adult canine cardiac myocytes, 2–4 × 10^6 total cells (approximately 20–40 mg total cellular protein) were washed twice with Ca^{2+}-HEPES buffer and resuspended in a final volume of 2 ml of buffer. Three 100-μl aliquots were removed for assay of total protein. Cellular phospholipids were then extracted with chloroform and methanol by the method of Bligh and Dyer (18) at 0–4°C. The extract was evaporated to dryness under N_2 and the lipid residue was resuspended in 250 μl of chloroform–methanol 2:1 (v/v). Three 5-μl aliquots were removed for measurement of total lipid phosphorus (see below) and 50-μl aliquots were injected onto a Waters Associates HPLC system comprised of two Model 510 HPLC pumps, Model 680 automated gradient controller, Rheodyne injector, and Model 484 tunable absorbance detector interfaced to an IBM PC-AT computer using the Nelson Analytical 900 Series interface and 3000 series chromatography data system software. Phospholipids were separated into classes based on differences in polar headgroup composition using a gradient elution technique as previously described (19, 20) (System I). Flow rate was held constant at 1.5 ml/min throughout the separation and phospholipids eluting from the column were detected by monitoring UV absorbance at 203 nm. Fractions were collected corresponding to the following

Downloaded from www.jlr.org by guest, on June 18, 2012
ASBMB JOURNAL OF LIPID RESEARCH JLR

phospholipid classes (listed in order of elution): cardiolipin, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, and sphingomyelin. The isolated fractions were identified based on their co-elution with authenticated phospholipid standards and the fractions were found to be essentially homogenous in composition after TLC separation and visualization by I_2 staining of the corresponding HPLC column effluents on Silica Gel G plates using a solvent system comprised of chloroform–acetone–methanol–acetic acid–water 6:8:2:1:1 (v/v). Phospholipid classes were quantified by GLC analysis (see below) and by measurement of lipid phosphorus by the microphosphate assay method of Chen, Toribara, and Warner (21) after preliminary washing of the samples with 1.2 M Mg $(NO_3)_2$ in EtOH (22). The plasmalogen content of the choline glycerophospholipid (CGP) and ethanolamine glycerophospholipid (EGP) fractions was determined by quantification of the vinyl ether content based on a modification of the I_2 addition method of Gottfried and Rapport (23) as described by Kates (24). The alkyl ether content of CGP and EGP was determined by quantification of lipid phosphorus in the lysophospholipid fraction remaining after sequential, exhaustive base- and acid-catalyzed hydrolysis of the diradylphospholipids (11). The lysophosphatidylcholine and lysophosphatidylethanolamine fractions were isolated by HPLC as previously described (25).

The fatty acid/fatty aldehyde composition of glycerophospholipids was determined by GLC analysis of the fatty acid methyl ester (FAME) and dimethylacetal (DMA) derivatives produced after acid-catalyzed methanolysis (11). Identification of individual FAME species was established by comparison of their GLC retention times with commercially available standards (Alltech). Individual DMA species were identified by comparison of their GLC retention times with the DMA derivatives produced after acid-catalyzed methanolysis of lysoplasmenylcholine derived from bovine heart CGP. The synthesis and detailed characterization of the fatty aldehyde composition of lysoplasmenylcholine by gas chromatography–mass spectrometry and by reverse phase HPLC have been previously described (26). GLC quantification of individual FAME and DMA species was accomplished by comparison of the integrated flame-ionization detector response for each FAME and DMA species with the integrated response of the FAME species derived from arachidic acid which was added as an internal standard prior to derivatization. The observed integrated areas were corrected for differences in the detector response factor for individual FAME and DMA species which has previously been shown to be proportional to their molecular weights within experimental error (11). Quantification of phospholipid classes by GLC was accomplished as described by Kates (27).

Separation and quantification of individual choline and ethanolamine glycerophospholipid molecular species

Individual CGP and EGP molecular species were isolated by reverse phase HPLC (system II) and detected by monitoring UV absorbance at 203 nm as previously described (28). The molecular identity of individual molecular species was established by a combination of two or more of the following criteria: *1*) GLC characterization of the FAME and DMA derivatives produced after acid-catalyzed methanolysis of the phospholipid species recovered in column effluents, after extraction from the mobile phase with chloroform (26); *2*) comparison of absolute retention time, relative retention time, and order of elution of individual species with previously published results using the same chromatographic separation technique (12, 20, 28); and *3*) co-elution with specific radiolabeled phospholipid molecular species available commercially. Isolated fractions obtained by reverse phase HPLC were found to be comprised of a single, predominant phospholipid species by the demonstration of stoichiometric amounts ($\pm$ 20%) of DMA and FAME derivatives after acid methanolysis and GLC characterization.

Quantification of individual phospholipid molecular species was achieved by measurement of the integrated UV detector response (12) and by determination of lipid phosphorus in reverse phase HPLC column effluents. For lipid phosphorus determination, column effluents were taken to dryness under N_2, and electrically heated at 150°C for 2 h after addition of 0.4 ml 70% $HClO_4$. The samples were allowed to cool to room temperature and excess perchloric acid was neutralized by addition of 1 ml of 4.5 N KOH. The samples were centrifuged at 2000 *g* for 10 min to sediment the $KClO_4$ precipitate and 600 μl of the supernate was removed for assay of lipid phosphorus by the method of Itaya and Ui (29).

Fast atom bombardment mass spectrometry

Purified choline and ethanolamine glycerophospholipids derived from canine cardiac myocytes were dissolved in chloroform–methanol 1:1 (v/v) at a final concentration of 0.6 nmol/ml. Two-tenths μl of this solution was injected into a Rheodyne loop injector and samples were introduced through a flow probe into a VG Analytical ZAB-SE mass spectrometer using a Brownlee Labs Microgradient system to deliver 5% glycerol in water (v/v) at a flow rate of 3 μl/min. Ionization and desorption from the glycerol matrix were accomplished by Xenon fast atom bombardment with a beam energy of 8 keV and discharge current of 1 mA. Spectra were obtained from *m/z* 150 to *m/z* 850 using Analytical II-250J data system software. Mass calibration was accomplished based on peaks originating from glycerol. The spectra reported have been corrected

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH

for the contribution of $(glycerol)_n + 1$ peaks by subtraction of the FAB spectrum of glycerol.

RESULTS

Composition of the major phospholipid classes in isolated canine cardiac myocytes

Choline and ethanolamine glycerophospholipids comprised the major phospholipid classes in isolated canine cardiac myocytes (**Table 1**). Significant amounts of phosphatidylinositol, phosphatidylserine, diphosphatidylglycerol, and sphingomyelin were also present. Canine cardiac myocyte phospholipids contained 39 ± 3% (mean ± SE, n = 4) of vinyl ether functional groups. Alkenyl-ether phospholipids in the choline (55 ± 7%) and ethanolamine (42 ± 5%) glycerophospholipid classes, determined by I_2 addition, quantitatively accounted for the alkenyl-ether content of myocyte phospholipids. Diradyl choline and ethanolamine glycerophospholipids contained less than 2% of alkyl ether phospholipids. The total phospholipid content of the isolated cells was considerably lower than previously reported values for the phospholipid content of extracts derived from whole myocardium (160-180 nmol/mg protein) (5, 30). This difference could reflect the higher protein content (relative to phospholipid) of the isolated myocyte preparation.

Gas chromatographic characterization of the fatty acid and alkenyl ether composition of canine cardiac myocyte phospholipids

Both choline and ethanolamine glycerophospholipid derivatives were comprised of the dimethylacetals of palmitylaldehyde, stearylaldehyde, and oleylaldehyde and the fatty acid methyl esters of palmitic, stearic, oleic, linoleic, and arachidonic acid (**Fig. 1**). There was no evidence for the dimethylacetals of odd-chain length or branched-chain aldehydes or of fatty acid methyl esters containing other than 16, 18, or 20 carbon atoms in the aliphatic chain. However, we cannot exclude the presence of small amounts (<2%) of these derivatives. The saturated FAME and DMA derivatives of choline glycerophospholipids consisted predominantly of species with 16 carbon atoms whereas the saturated derivatives of ethanolamine glycerophospholipids consisted predominantly of species with 18 carbon atoms (**Table 2**). The unsaturated FAME derivatives of choline glycerophospholipids contained predominantly 18 carbon atoms with one or two double bonds while ethanolamine glycerophospholipids yielded the FAME derivative of arachidonic acid (20:4) as the predominant unsaturated species. Based on comparison of the relative amounts of 20:4 FAME in acid methanolysates with the relative mass of each phospholipid class given in Table 1, choline and ethanolamine glycerophospholipids comprised 26% and 68%, respectively, of the total arachidonylated phospholipid mass in the cardiac myocyte.

Phosphatidylinositol and phosphatidylserine acid methanolysates contained the FAME derivatives of stearic, linoleic, and arachidonic acid as the principal species (Table 2). The volatile derivatives produced from diphosphatidylglycerol were comprised almost exclusively of the fatty acid methyl ester of linoleic acid consistent with previous observations of the high content of esterified linoleate in this phospholipid class (31). There were no dimethylacetal derivatives identified in the phosphatidy-

TABLE 1. Phospholipid composition of isolated adult canine cardiac myocytes

Phospholipid Class	Microphosphate Assay[a]	GLC[a]	Total Phospholipid[b]	Plasmalogen[c]	Alkyl Ether[d]
	nmol phospholipid/mg protein			*mole %*	
CGP	47.5 ± 5.5	46.2 ± 5.3	41 ± 3%	55 ± 7%	1.5 ± 0.4%
EGP	43.6 ± 2.7	42.5 ± 3.1	38 ± 2%	42 ± 5%	0.6 ± 0.2%
PI	3.4 ± 0.8	2.5 ± 0.4	3 ± 1%		
PS	5.3 ± 1.3	4.3 ± 0.8	5 ± 1%		
DPG	2.9 ± 0.5	3.6 ± 0.5	3 ± 1%		
SPH	5.3 ± 1.6		5 ± 1%		

Values shown represent the mean ± SEM of separate measurements obtained using cells isolated from three or four different animals. Abbreviations used: CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidylglycerol (cardiolipin); SPH, sphingomyelin.

[a]Phospholipid classes were isolated by gradient elution HPLC (system 1) and quantified by microphosphate analysis and by GLC of the volatile fatty acid methyl ester and dimethylacetal derivatives as described under Materials and Methods.

[b]Values are expressed as a percentage of the total phospholipid phosphorus content (115 ± 9 nmol/mg protein, mean ± SEM, n = 4) in the chloroform-methanol extract of the isolated cell preparation.

[c]Plasmalogen content was determined by measurement of I_2 addition to activated olefins and expressed as a percentage of the total phosphorus content of the corresponding phospholipid class.

[d]Alkyl ether content was determined by quantitation of lipid phosphorus in the lysophosphoglyceride fraction remaining after sequential, exhaustive acid- and base-catalyzed hydrolysis and expressed as a percentage of the total phosphorus content of the corresponding phospholipid class.

Downloaded from www.jlr.org by guest, on June 18, 2012

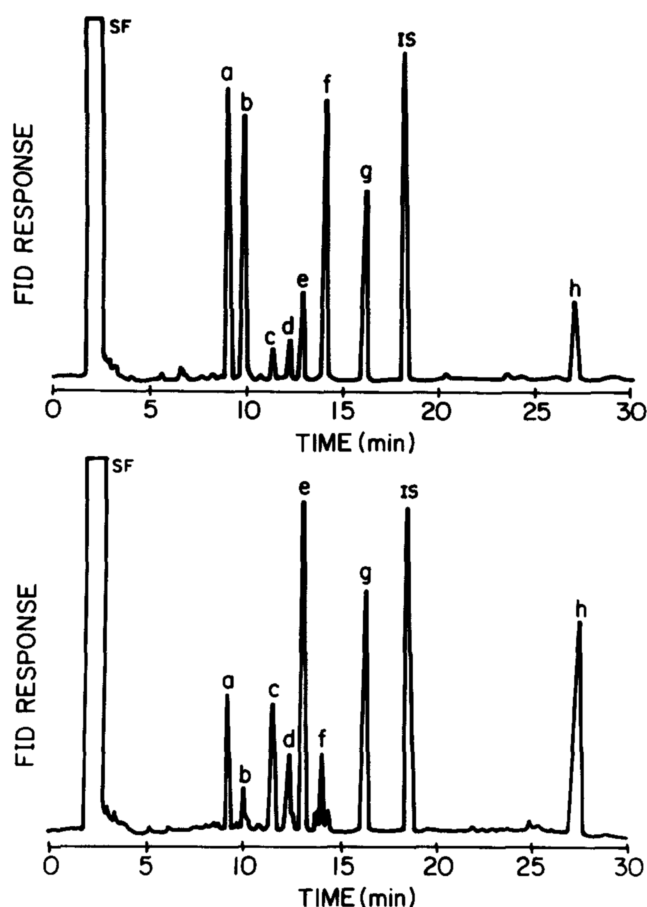

Fig. 1. GLC elution profiles of the volatile fatty acid methyl ester and dimethylacetal derivatives produced after acid-catalyzed methanolysis of choline glycerophospholipids (top) and ethanolamine glycerophospholipids (bottom) derived from isolated canine cardiac myocytes. The peaks identified by lower case letters correspond to those shown in Table 2. Individual peaks were identified and quantified by comparison of the integrated flame ionization detector (FID) response for each derivative to that of the methyl ester of arachidic acid which was added as an internal standard (IS) as described under Materials and Methods. SF refers to the solvent front.

linositol, phosphatidylserine, or diphosphatidylglycerol fractions.

Reverse phase HPLC separation and quantification of individual phospholipid molecular species

Since choline and ethanolamine glycerophospholipids contained both plasmalogen and diacyl phospholipids and together accounted for approximately 80% of the total glycerophospholipid mass in the cardiac myocyte, the distribution of individual molecular species within these two phospholipid classes was further characterized. The elution profiles for CGP and EGP molecular species after reverse phase HPLC are shown in **Fig. 2** and the corresponding molecular identities and relative mass contribution of each species are given in **Table 3**. The molecular identity of individual species was established by three independent criteria as described under Materials and Methods. To facilitate description of these results, a shorthand notation will be used to describe the composition of individual molecular species. The aliphatic groups are designated in the form (a:b, c:d) where a and b are the chain length and number of olefin functional groups for the aliphatic hydrocarbon chain at the *sn*-1 position and c:d corresponds to the same description for the *sn*-2 aliphatic chain. When referring to plasmalogen molecular species, the alkenyl ether functionality is not included in the designation of the total number of olefin groups. Diacyl glycerophospholipids are referred to by the abbreviation "Ptd" and plasmalogen glycerophospholipids by "Plas." Choline glycerophospholipids are represented by "Cho" and ethanolamine glycerophospholipids by "Eth." Thus, for example, 1-O-hexadec-1'-enyl-2-eicosa-5',8',11',14'-enoyl-*sn*-glycero-3-phosphocholine is simply abbreviated as (16:0, 20:4) PlasCho. This system of abbreviations is similar to that described by Dugan et al. (19).

TABLE 2. Composition of fatty acid methyl ester and dimethylacetal derivatives prepared from canine cardiac myocyte phospholipids

Phospholipid Class	Derivative[a] 16:0(D) (a)	16:0 (b)	18:0(D) (c)	18:1(D) (d)	18:0 (e)	18:1 (f)	18:2 (g)	20:4 (h)
	mole %							
CGP	20 ± 2	14 ± 2	3 ± 0.3	3 ± 0.4	8 ± 1	23 ± 3	17 ± 2	14 ± 2
EGP	5 ± 0.3	2 ± 0.2	9 ± 2	6 ± 1	23 ± 1	4 ± 1	12 ± 3	39 ± 2
PI	ND	4 ± 1	ND	ND	50 ± 5	4 ± 0.3	26 ± 5	18 ± 2
PS	ND	4 ± 2	ND	ND	38 ± 8	10 ± 5	33 ± 9	16 ± 5
DPG	ND	1 ± 0.3	ND	ND	1 ± 0.2	5 ± 0.3	92 ± 2	1 ± 0.2

Cardiac myocyte glycerophospholipids were subjected to acid-catalyzed methanolysis and the volatile derivatives were characterized by GLC as described under Materials and Methods. Abbreviations as described in the legend of Table 1.

[a]Mole % composition of each derivative was determined by comparing the integrated flame ionization detector response with that of the arachidic acid (20:0) internal standard after correction for differences in detector response factors for each derivative as described under Materials and Methods. (D) refers to dimethylacetal derivatives and the lower case letters correspond to the peak designations shown in Fig. 1. Values shown represent the mean ± SEM of separate measurements obtained using cells isolated from four different animals; ND, none detected.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

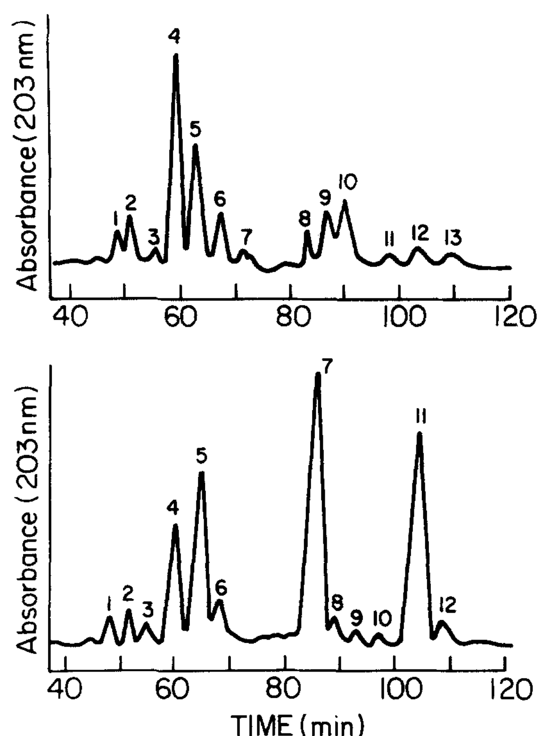

Fig. 2. Reverse phase HPLC elution profiles of choline glycerophospholipids (top) and ethanolamine glycerophospholipids (bottom) derived from isolated canine cardiac myocytes. Choline and ethanolamine glycerophospholipids (150-200 nmol) were isolated by gradient elution HPLC (system 1) and the individual molecular species were separated by reverse phase HPLC (system 2) and detected by monitoring UV absorption at 203 nm as described under Materials and Methods. The numbers above each peak correspond to the numbering scheme used to identify individual phospholipid molecular species given in Table 3.

The elution profile of choline glycerophospholipids from cardiac myocytes (Fig. 2) demonstrated predominant peaks at 58, 63, and 90 min (peaks 4, 5, and 10) representing plasmalogen molecular species (Table 3). The predominant peaks at 60, 65, 87, and 105 min (peaks 4, 5, 7, and 11) in the elution profile of ethanolamine glycerophospholipids (Fig. 2) represent both PtdEth and PlasEth species containing esterified arachidonic acid (Table 3). It is important to note that it is not possible to estimate the relative mass of each molecular species by direct inspection of the UV detector response due to the high molar absorptivity of the alkenyl ether functional group relative to that of other olefin groups.

Both PtdCho and PlasCho are predominantly comprised of species with 16 carbon atoms at the *sn*-1 position whereas both PtdEth and PlasEth are predominantly comprised of species with 18 carbon atoms at the *sn*-1 position (Table 3) in agreement with results obtained by GLC analysis (Table 2). In CGP, 32% of the total phospholipid mass is comprised of species containing esterified arachidonate with PlasCho species accounting for 86% of the total arachidonylated CGP mass. In EGP, 85% of the total EGP mass is comprised of arachidonylated species with 51% of the total esterified arachidonate in PlasEth species. In CGP and EGP, linoleate and arachidonate are esterified exclusively at the *sn*-2 position since, for plasmalogens, the 1-O-alk-1'-enyl linkage is located at the *sn*-1 position and the phosphodiester linkage is located at the *sn*-3 position (32). In addition, the retention characterisics and molecular composition analysis of individual diacyl CGP and EGP molecular species in canine cardiac myocytes were consistent with the presence of esterified linoleate and arachidonate only at the *sn*-2 position (28, 33).

Fast atom bombardment mass spectrometry (FAB-MS) of canine cardiac myocyte choline and ethanolamine glycerophospholipids

FAB-MS analyses of CGP and EGP are shown in **Fig. 3** and the molecular identities of corresponding mass ions are given in Table 3. Choline glycerophospholipids consisted predominantly of species with protonated molecular ions (MH^+) at a mass/charge (*m/z*) ratio of 742, 744, 758, and 760. As previously described (11), precise quantitative correlations of the relative amounts of PtdCho and PlasCho species based on comparison of the relative intensity of protonated molecular ions cannot be made due to differences in the relative rates of formation and decomposition of protonated molecular ions of PtdCho and PlasCho species in the complex ionization matrix of the FAB-MS ion source. For example, the more rapid loss of ketene from PlasCho species (resulting from cleavage of the O-acyl ester bond at the *sn*-2 position) results in the underrepresentation of protonated molecular ions corresponding to PlasCho species (11). Quantitative interpretation is further complicated by the presence of low-intensity sodiated parent ions (MNa^+) and of isobaric species (e.g., (18:1,18:1)PlasCho and (18:0,18:2)PlasCho). Nevertheless, since PlasCho species are underrepresented, the high relative abundance of ions of *m/z* 742, 744, 766, and 794 is in agreement with our previous findings of the relatively high content of PlasCho species.

FAB-MS of ethanolamine glycerophospholipids demonstrated prominent ions at *m/z* 768, 752, and 750. In addition to the facile loss of ketene from PlasEth species, PtdEth species undergo a more rapid loss of ethanolamine phosphate (11). The net effect of these two processes results in an overrepresentation of PlasEth in parent ion peaks. Accordingly, the high relative intensity of the protonated molecular ion at *m/z* 768 supports our previous findings of the high relative abundance of the (18:0,20:4)PtdEth species which accounts for 41% of the total ethanolamine glycerophospholipid mass and 48% of total arachidonylated ethanolamine glycerophospholipids (Table 3).

Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 3. Composition of choline and ethanolamine glycerophospholipid molecular species

Peak[a]	Composition[b]	$(MH)^+$	Relative %[c]	
			Microphosphate	Area Integration
		m/z		
Choline glycerophospholipid molecular species				
1	(16:0,20:4)PtdCho	782	1 ± 0.1%	2 ± 1%
2	(16:0,18:2)PtdCho	758	4 ± 2%	8 ± 4%
3	(18:1,18:2)PtdCho	784	1 ± 0.1%	2 ± 1%
4	(16:0,20:4)PlasCho	766	16 ± 4%	20 ± 5%
5	(16:0,18:2)PlasCho	742	16 ± 2%	16 ± 2%
6	(18:1,20:4)PlasCho	792	7 ± 0.4%	4 ± 2%
7	(16:0,18:1)PtdCho	760	9 ± 1%	12 ± 6%
8	(18:0,20:4)PtdCho	810	3 ± 1%	3 ± 1%
9	(18:0,18:2)PtdCho	786	6 ± 2%	9 ± 3%
10	(16:0,18:1)PlasCho	744	24 ± 2%	17 ± 4%
11	(18:1,18:1)PlasCho	770	4 ± 2%	2 ± 1%
12	(18:0,20:4)PlasCho	794	4 ± 1%	3 ± 1%
13	(18:0,18:2)PlasCho	770	3 ± 1%	1 ± 1%
Ethanolamine glycerophospholipid molecular species				
1	(16:0,20:4)PtdEth	740	1 ± 0.5%	1 ± 0.5%
2	(16:0,18:2)PtdEth	716	1 ± 0.8%	2 ± 1%
3	(18:1,18:2)PtdEth	742	2 ± 0.6%	2 ± 1%
4	(16:0,20:4)PlasEth	724	9 ± 1%	8 ± 2%
5	(18:1,20:4)PlasEth	750	16 ± 2%	12 ± 2%
6	(18:1,18:2)PlasEth	726	5 ± 1%	3 ± 1%
7	(18:0,20:4)PtdEth	768	38 ± 3%	44 ± 5%
8	(18:0,18:2)PtdEth	744	1 ± 1%	1 ± 1%
9	(16:0,18:1)PlasEth	702	2 ± 1%	1 ± 1%
10	(18:1,18:1)PlasEth	728	0.5 ± 0.3%	1 ± 0.6%
11	(18:0,20:4)PlasEth	752	21 ± 2%	20 ± 2%
12	(18:0,18:2)PlasEth	728	2 ± 1%	2 ± 1%

Individual phospholipid molecular species were isolated by reverse phase HPLC (system II) and identified as described under Materials and Methods.
[a]Peak designations correspond to those given in Fig. 2.
[b]The shorthand notation used to describe the composition of individual molecular species is discussed in detail under Results.
[c]The relative contribution (expressed as a percentage) for each molecular species to the total mass of phospholipid was determined by measurement of lipid phosphorus in corresponding column effluents and by comparison of the integrated UV detector response at 203 nm. Values shown represent the mean ± SEM of separate measurements obtained using cells isolated from four different animals. Comparison of results by microphosphate assay and area integration for each individual molecular species using a paired *t*-test did not demonstrate statistically significant differences (i.e, $P > 0.12$ for all comparisons).

Radiolabeling of isolated canine cardiac myocyte phospholipid classes

To investigate the turnover of the esterified *sn*-2 fatty acyl moeity in cardiac myocyte phospholipids, isolated cardiac myocytes were incubated for 18 h with $[^3H]$arachidonate and $[^{14}C]$linoleate as our previous results had demonstrated that these fatty acids were the predominant species present at the *sn*-2 position. To assess the effects of prolonged incubation on cell viability, at the conclusion of the incubation period, aliquots of the cell suspension were examined microscopically. In all reported experiments, there was less than a 10% reduction in the percentage of elongated, striated cardiac myocytes. In another series of experiments, cell viability was also assessed biochemically by measurement of CK release into the culture supernate at 4-h intervals. These studies demonstrated that total CK release into the supernatant represented less than 12% of the total cellular CK content at the end of the 18-h incubation. In addition, there were no significant alterations in total phospholipid content, in the relative mass of different phospholipid classes, or in the relative mass distribution of individual phospholipid molecular species based on comparison of FAB mass spectra of CGP and EGP before and after the incubation interval (data not shown).

The isolated cardiac myocytes incorporated substantial amounts of both ^3H-labeled arachidonate and ^{14}C-labeled linoleate into cardiac myocyte phospholipids (**Table 4**). The majority of $[^3H]$arachidonate was present in CGP and EGP glycerophospholipids which together comprised 89% of incorporated ^3H radioactivity. Although choline

JOURNAL OF LIPID RESEARCH ASBMB

Downloaded from www.jlr.org by guest, on June 18, 2012

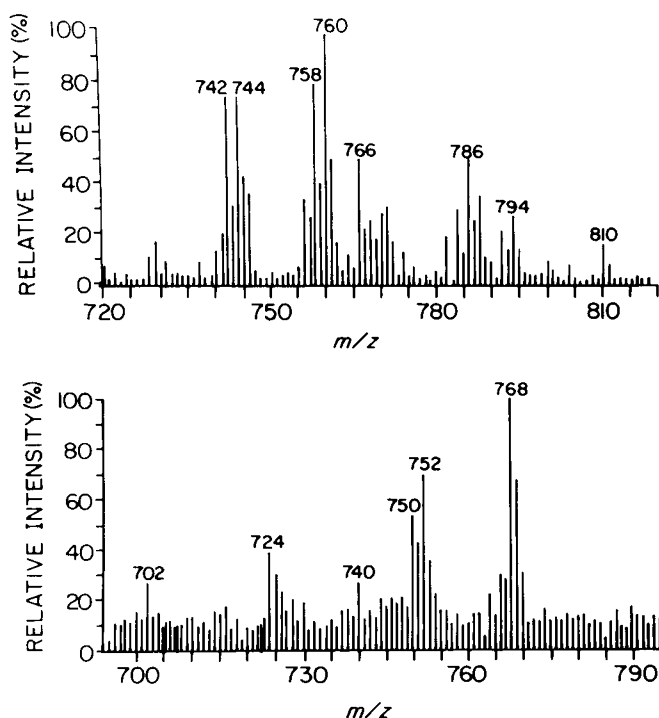

Fig. 3. Fast atom bombardment mass spectra of canine cardiac myocyte choline glycerophospholipids (top) and ethanolamine glycerophospholipids (bottom). The fast atom bombardment mass spectra were obtained as described under Materials and Methods. The mass/charge ratios of protonated molecular ions correspondng to individual phospholipid molecular species are given in Table 3.

and ethanolamine glycerophospholipids incorporated similar amounts of total [^{3}H]arachidonate, the mass of arachidonylated ethanolamine glycerophospholipid was approximately 2.5-fold greater than that of arachidonylated choline glycerophospholipids. Accordingly, the calculated specific activity of arachidonylated CGP was approximately 3-fold greater than that of arachidonylated EGP. Phosphatidylinositol accounted for only 2% of total arachidonylated phospholipid but represented 9% of total incorporated ^{3}H radioactivity and thus had the highest calculated specific activity of all phospholipid classes.

In cells labeled with [^{14}C]linoleate, CGP accounted for over 50% of incorporated ^{14}C radioactivity with the majority of remaining incorporated radioactivity approximately equally distributed in the EGP and PI classes. Again, marked differences in the calculated specific activities of the various phospholipid classes were noted with linoleate-containing PI species exhibiting a calculated specific activity that was substantially greater than that of CGP or EGP molecular species containing esterified linoleate. The amount of radiolabel incorporated into PS molecular species was $<3\%$ following incubation with either [^{3}H]arachidonate or [^{14}C]linoleate and the calculated specific activity was also low in comparison with other radiolabeled phospholipid classes. There was no evidence of significant incorporation of either [^{3}H]arachidonate or [^{14}C]linoleate into glycerophospholipid classes other than those shown in Table 4.

TABLE 4. Distribution of [^{3}H]arachidonic acid and [^{14}C]linoleic acid in isolated canine cardiac myocyte glycerophospholipids

Phospholipid Class	[^{3}H]Arachidonate-Labeled Glycerophospholipids			[^{14}C]Linoleate-Labeled Glycerophospholipids		
	dpm ^{3}H	Total ^{3}H dpm[a]	Calculated Specific Activity[b]	dpm ^{14}C	Total ^{14}C dpm[a]	Calculated Specific Activity[c]
	$\times$ *10^{-5}/mg protein*	%	*dpm $\times$ 10^{-4}/nmol*	$\times$ *10^{-4}/mg protein*	%	*dpm $\times$ 10^{-3}/nmol*
CGP	5.2 ± 0.4	50 ± 4	3.4 ± 0.3	8.7 ± 0.5	59 ± 3	5.5 ± 0.3
EGP	4.0 ± 0.6	39 ± 6	1.2 ± 0.2	2.6 ± 0.3	18 ± 2	2.4 ± 0.3
PI	1.0 ± 0.1	9 ± 1	8.0 ± 1.1	3.3 ± 0.4	22 ± 3	18.5 ± 2.4
PS	0.1 ± 0.04	1 ± 0.4	0.7 ± 0.2	0.3 ± 0.1	2 ± 1	0.7 ± 0.3

Isolated canine cardiac myocytes were labeled for 18 h with [^{3}H]arachidonic acid (0.4 µCi/ml, 40,000 cells/ml) or [^{14}C]linoleic acid (0.1 µCi/ml, 40,000 cells/ml) in Dulbecco's Modified Eagle Media containing 500 µM $CaCl_2$ and 25 mM HEPES at 37°C under an atmosphere of 95% air/5% CO_2. Unincorporated radioactivity was removed by washing the cells with Ca^{2+}/HEPES buffer and cellular phospholipids were extracted as described in Materials and Methods. Phospholipid classes were separated by gradient elution HPLC (system I) and the amount of incorporated label in each phospholipid class was determined by liquid scintillation counting. 1-Palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (5 × 10^4 dpm) was added to the cell suspension before extraction as an internal standard to correct for any small loss of [^{3}H]arachidonate-labeled phospholipid during the course of analysis and 1,2-dipalmitoyl-*sn*-glycero-3-phospho-[^{3}H]choline (5 × 10^4 dpm) was used as an internal standard for experiments using [^{14}C]linoleate-labeled cells. Abbreviations described in legend of Table 1. All values shown represent the mean ± SEM of separate measurements obtained using cells isolated from four or five different animals.

[a]Values are expressed as a percentage of the total amount of radioactivity incorporated into all phospholipid classes.

[b]The calculated specific activity was computed as the ratio of total ^{3}H radioactivity incorporated into each phospholipid class to the total mass of arachidonylated phospholipid in each phospholipid class.

[c]Specific activity was calculated as the ratio of total ^{14}C radioactivity to the total mass of linoleate-containing molecular species in each phospholipid class.

Downloaded from www.jlr.org by guest, on June 18, 2012

JOURNAL OF LIPID RESEARCH ASBMB

Distribution of radiolabel in individual choline and ethanolamine glycerophospholipid molecular species

The distribution of incorporated radioactivity in individual radiolabeled CGP and EGP molecular species is shown in **Fig. 4**. The identification of [^{3}H]arachidonate-labeled molecular species was based on the coelution of ^{3}H radioactivity measured in reverse phase HPLC column effluents with unlabeled arachidonylated phospholipid molecular species. In addition, we exploited the susceptibility of the alkenyl ether functional group to acid-catalyzed hydrolysis to further confirm the identification of plasmalogen molecular species and to provide further characterization of the composition of those minor peaks that could not be identified based on their coelution with unlabeled species of known composition. For these experiments, isolated [^{3}H]arachidonylated CGP or EGP were resuspended in 0.5 ml of 1 N HCl in methanol and 0.5 ml chloroform and incubated for 45 min at room temperature. The samples were then neutralized with 0.5 ml of 1 M Na_2CO_3, extracted twice with 1 ml chloroform, and unlabeled phospholipid was added to the sample as an internal reference prior to injection onto the reverse phase HPLC column as described in the legend of Fig. 4. The total ^{3}H radioactivity represented by each peak before and after acid treatment was compared after normalization based on the recovery of the ^{14}C-labeled internal standard. Molecular species were designated as acid-stable if the acid treatment did not result in greater than 15% loss of the expected ^{3}H radioactivity associated with the corresponding peak, and acid-labile if greater than 90% of the expected radioactivity was lost from the corresponding peak after acid treatment. Those peaks that were designated as acid-stable could represent alkylacyl or diacylglycerophospholipids.

In CGP (left panel of Fig. 4), [^{3}H]arachidonate was primarily incorporated into plasmalogen molecular species (peaks 4, "a", 6 and 12). However, significant amounts of ^{3}H radioactivity were also present in diacyl-CGP species (peaks 1 and 8). The distribution of [^{3}H]arachidonate in CGP molecular species is summarized in **Table 5**. The identification of [^{3}H]arachidonate-labeled CGP molecular species was also supported by the results of acid

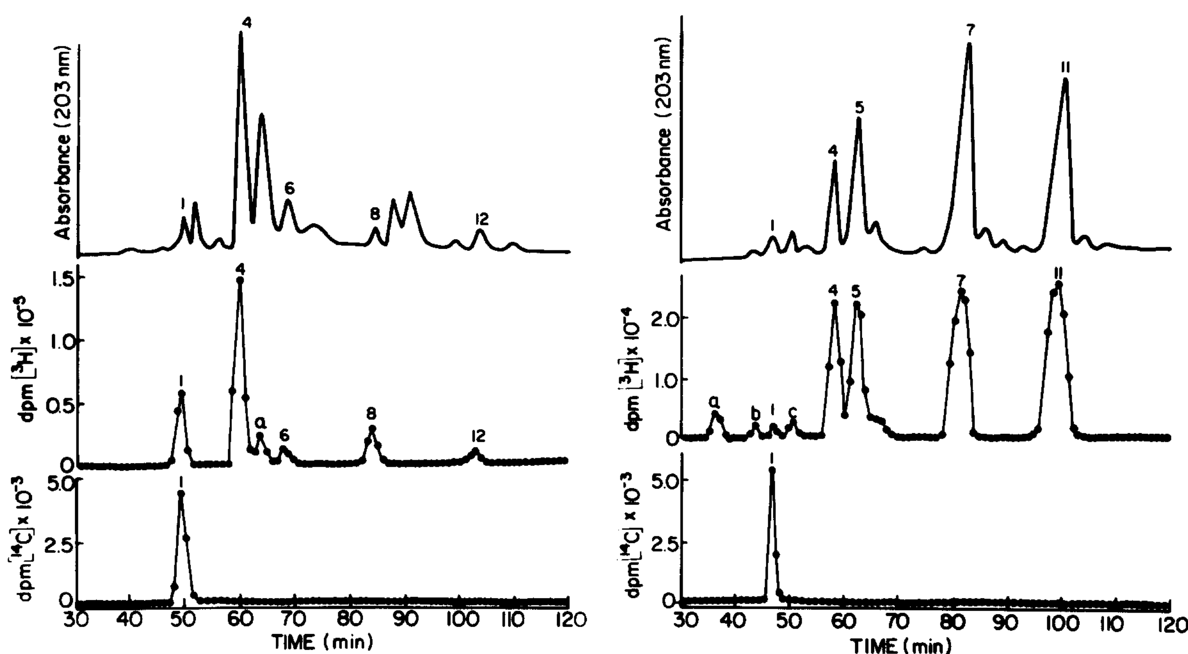

Fig. 4. Reverse phase HPLC elution profiles of [^{3}H]arachidonate-labeled choline (left panel) and ethanolamine (right panel) glycerophospholipid molecular species. Isolated canine cardiac myocytes were prelabeled with [^{3}H]arachidonic acid, extracted after addition of ^{14}C-labeled internal standards (vide infra) and choline and ethanolamine glycerophospholipids were isolated by gradient elution HPLC (system I) as described under Materials and Methods. To the isolated ^{3}H-labeled choline and ethanolamine glycerophospholipids, 150–200 nmol of unlabeled choline and ethanolamine glycerophospholipid derived from canine cardiac myocytes was added, respectively, as an internal reference prior to injection onto the reverse phase HPLC column. Left panel, top tracing: elution profile of canine cardiac myocyte choline glycerophospholipid molecular species. Left panel, middle tracing: elution profile of [^{3}H]arachidonate-labeled choline glycerophospholipid molecular species. Left panel, bottom tracing: elution profile of 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine internal standard. Right panel, top tracing: elution profile of canine cardiac myocyte ethanolamine glycerophospholipid molecular species. Right panel, middle tracing: elution profile of [^{3}H]arachidonate-labeled ethanolamine glycerophospholipid molecular species. Right panel, bottom tracing: elution profile of 1-palmitoyl-2-[^{14}C]arachidonyl-*sn*-glycero-3-phosphoethanolamine internal standard. Peaks identified by numbers represent arachidonylated glycerophospholipid molecular species and correspond to the numbering scheme presented in Table 3. The peaks identified by lower case letters represent arachidonylated species identified solely as acid-stable or acid-labile species as described under Results and in Table 5.

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org by guest, on June 18, 2012

TABLE 5. Distribution of [^{3}H]arachidonic acid and [^{14}C]linoleic acid in choline and ethanolamine glycerophospholipid molecular species

Peak[a]	Composition[b]	Total ^{3}H dpm[c]	Calculated Specific Activity[d]
			dpm × 10⁻⁴/nmol
[^{3}H]Arachidonate-labeled choline glycerophospholipid (CGP) species			
1	(16:0,20:4)PtdCho	21 ± 4	16.8
4	(16:0,20:4)PlasCho	45 ± 3	3.1
a	acid-labile CGP species	9 ± 1	
6	(18:1,20:4)PlasCho	6 ± 2	1.2
	+ acid-stable CGP species	3 ± 1	
8	(18:0,20:4)PtdCho	13 ± 3	4.9
12	(18:0,20:4)PlasCho	5 ± 2	1.1
[^{3}H]Arachidonate-labeled ethanolamine glycerophospholipid (EGP) species			
a	acid-stable EGP species	4 ± 1	
b	acid-labile EGP species	2 ± 0.1	
1	(16:0,20:4)PtdEth	2 ± 0.1	1.6
c	acid-stable EGP species	2 ± 0.3	
4	(16:0,20:4)PlasEth	14 ± 2	1.6
5	(18:1,20:4)PlasEth	19 ± 1	1.4
7	(18:0,20:4)PtdEth	32 ± 3	0.9
11	(18:0,20:4)PlasEth	26 ± 3	1.3

Peak	Composition	Total ^{14}C dpm	Calculated Specific Activity
		%	*dpm × 10⁻³/nmol*
[^{14}C]Linoleate-labeled choline glycerophospholipid (CGP) species			
a	acid-stable CGP species	8 ± 1	
2	(16:0,18:2)PtdCho	49 ± 2	15.1
3	(18:1,18:2)PtdCho	10 ± 1	13.1
5	(16:0,18:2)PlasCho	4 ± 2	0.6
9	(18:0,18:2)PtdCho	28 ± 3	5.9
[^{14}C]Linoleate-labeled ethanolamine glycerophospholipid (EGP) species			
a	acid-stable EGP species	11 ± 4	
2	(16:0,18:2)PtdEth	6 ± 1	1.1
3	(18:1,18:2)PtdEth	17 ± 2	2.1
b	acid-labile EGP species	6 ± 2	
6	(18:1,18:2)PlasEth	5 ± 1	0.3
8	(18:0,18:2)PtdEth	50 ± 6	14.9
12	(18:0,18:2)PlasEth	3 ± 1	0.4

Isolated canine cardiac myocytes were prelabeled with [^{3}H]arachidonic acid or [^{14}C]linoleic acid for 18 h, extracted after addition of radiolabeled internal standard, and the choline and ethanolamine glycerophospholipids were isolated as described under Materials and Methods and in the legend of Table 4. Individual choline and ethanolamine glycerophospholipid molecular species were resolved by reverse phase HPLC (system II) after addition of unlabeled phospholipid as an internal reference as described in the legends to Figs. 4 and 5.

[a]Peaks designated by numbers correspond to those shown in Table 3 and Figs. 4 and 5. Peaks designated by lower case letters were characterized as acid-stable or acid-labile molecular species as described under Results and correspond to the peaks shown in the elution profiles presented in Figs. 4 and 5.

[b]The shorthand notation used to describe the composition of individual phospholipid molecular species is discussed in detail under Results.

[c]Values are expressed as a percentage of the total radioactivity injected onto the reverse phase HPLC column after normalization based on the recovery of the internal standard. The ^{14}C-labeled internal standards that were employed for experiments using [^{3}H]arachidonate-labeled cells and the ^{3}H-labeled choline glycerophospholipid internal standard used for experiments using [^{14}C]linoleate-labeled cells are described in the legends of Figs. 4 and 5. Recovery correction for [^{14}C]linoleate-labeled ethanolamine glycerophospholipid species after reverse-phase HPLC was based on the integrated UV detector response for the (18:0,20:4) PtdEth species. The % total radioactivity values represent the mean ± SEM of separate measurements obtained using cells isolated from three or four different animals.

[d]The specific activity was computed as the ratio of the mean total dpm corresponding to each peak (after recovery correction) to the mass of each radiolabeled molecular species. The mass of individual phospholipid molecular species was calculated based on the total mass of choline or ethanolamine glycerophospholipid in the extract from the prelabeled cells and the relative contribution of each individual molecular species to the total choline or ethanolamine glycerophospholipid mass presented in Table 3. The specific activity of those peaks designated solely as acid-stable or acid-labile species was not determined.

Downloaded from www.jlr.org by guest, on June 18, 2012

treatment that demonstrated the acid-lability of the choline plasmalogen and acid-stability of the diacyl-CGP species. ^{3}H radioactivity associated with peak "a" in the elution profile of ^{3}H-labeled CGP species appeared to coelute with the (16:0,18:2)PlasCho species. GLC analysis of the reverse phase HPLC column effluent did not demonstrate significant amounts of the FAME derivative of arachidonic acid. This peak was therefore tentatively identified as an acid-labile plasmalogen molecular species. For peak 6, approximately 65% of the ^{3}H radioactivity was associated with an acid-labile species and the remainder was comprised of an acid-stable species. Based on retention characteristics, the acid-labile component was identified as (18:1,20:4) PlasCho and the calculated specific activity for this peak given in Table 5 was determined after correction for the contribution of the acid-stable component. Since plasmalogens comprised 85% of the total arachidonylated CGP but only 65% of the total [^{3}H]arachidonylated CGP, the specific activity of ^{3}H-labeled diacyl-CGP species was substantially greater than that of choline plasmalogen species (Table 5).

[^{3}H]Arachidonate-labeled EGP (right panel of Fig. 4) was predominantly comprised of plasmalogen molecular species (peaks 4, 5, and 11) but significant labeling of diacyl-EGP species (peaks 1 and 7) was also observed. These peak identifications were also confirmed by acid treatment. In addition to peaks 1 and 7 (diacyl-EGP species), minor amounts of radiolabel were also incorporated into two other acid-stable species (peaks "a" and "c") and into an acid-labile plasmalogen species (peak "b"). Plasmalogens accounted for 61% of the total [^{3}H]arachidonylated EGP (Table 5) and 51% of the total arachidonylated EGP mass (Table 3). Thus, in contrast to the results observed in the CGP class, the calculated specific activities of ^{3}H-labeled diacyl-EGP species and ethanolamine plasmalogen species were comparable in magnitude (Table 5).

The elution profiles of ^{14}C-labeled CGP and EGP molecular species produced after incubation of isolated cardiac myocytes with [^{14}C]linoleate are shown in **Fig. 5**. In CGP (left panel of Fig. 5), [^{14}C]linoleate was almost exclusively incorporated into diacyl-CGP species (peaks

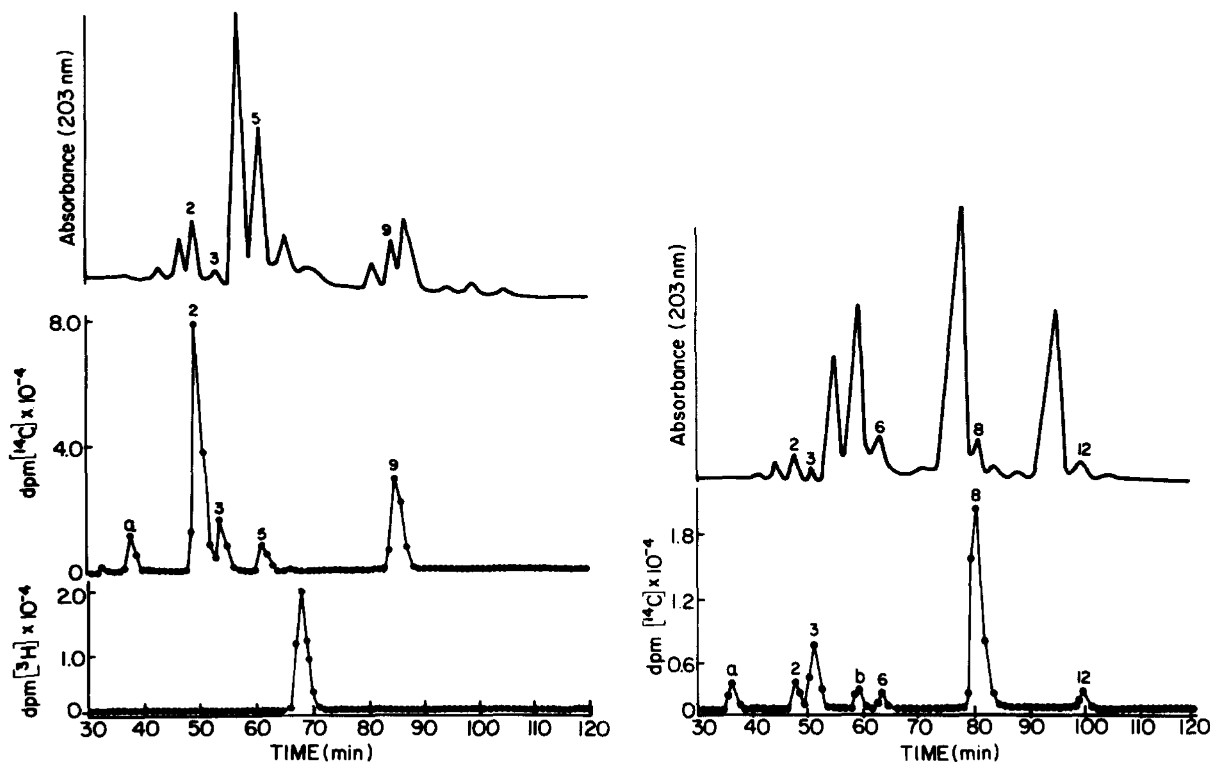

Fig. 5. Reverse phase HPLC elution profiles of [^{14}C]linoleate-labeled choline (left panel) and ethanolamine (right panel) glycerophospholipid molecular species. Isolated canine cardiac myocytes were prelabeled with [^{14}C]linoleic acid, extracted after addition of ^{3}H-labeled internal standard (vide infra) and choline and ethanolamine glycerophospholipids were isolated by gradient elution HPLC (system I) as described under Materials and Methods. To the isolated ^{14}C-labeled choline and ethanolamine glycerophospholipids, 150-200 nmol of unlabeled choline and ethanolamine glycerophospholipid derived from canine cardiac myocytes was added, respectively, as an internal reference prior to injection onto the reverse phase HPLC column. Left panel, top tracing: elution profile of canine cardiac myocyte choline glycerophospholipid molecular species. Left panel, middle tracing: elution profile of [^{14}C]linoleate-labeled choline glycerophospholipid molecular species. Left panel, bottom tracing: elution profile of 1,2-dipalmitoyl-*sn*-glycero-3-phospho-[^{3}H]choline internal standard. Right panel, upper tracing: elution profile of canine cardiac myocyte ethanolamine glycerophospholipid molecular species. Right panel, lower tracing: elution profile of [^{14}C]linoleate-labeled ethanolamine glycerophospholipid molecular species. Peaks identified by numbers represent linoleate-containing molecular species and correspond to the numbering scheme presented in Table 3. The peaks identified by lower case letters represent linoleate-containing species identified solely as acid-stable or acid-labile species as described under Results and in Table 5.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

2,3, and 9) with only minor amounts of incorporated label in choline plasmalogen (peak 5). The identification of these peaks was also confirmed by acid treatment as described above for the [^{3}H]arachidonate labeling experiments. [^{14}C]Linoleate-labeled CGP contained a minor contribution of ^{14}C radioactivity from an unidentified acid-stable species (peak "a"). Despite the fact that plasmalogens accounted for only 4% of total incorporated ^{14}C radioactivity in CGP (Table 5), 54% of the total linoleate-containing CGP mass was present in plasmalogen molecular species (Table 3). Consequently, the calculated specific activity of [^{14}C]linoleate-labeled diacyl CGP species was far greater than that of the ^{14}C-labeled choline plasmalogen pool (Table 5). A similar distribution of incorporated radioactivity was observed in [^{14}C]linoleate-labeled EGP (right panel of Fig. 5). Diacyl EGP species (peaks 2, 3, and 8) accounted for the overwhelming majority of incorporated ^{14}C radioactivity while only small amounts of [^{14}C]linoleate were incorporated into plasmalogen molecular species represented by peaks 6 and 12. The radiolabeled EGP species corresponding to peak "a" was identified as an acid-stable species and peak "b" was identified as an acid-labile (plasmalogen) species based on the results of acid treatment. The very low calculated specific activity of [^{14}C]linoleate-labeled ethanolamine plasmalogen species relative to diacyl EGP species (Table 5) is a reflection of the fact that only 14% of the total ^{14}C radioactivity was incorporated into plasmalogens but this phospholipid subclass comprised 57% of the total mass of linoleate-containing EGP (Table 3).

DISCUSSION

In the present study, the most surprising feature of the phospholipid composition of isolated canine cardiac myocytes was the observation that approximately 55-65% of the total mass of choline glycerophospholipid was comprised of plasmalogen molecular species. The high content of choline plasmalogen was confirmed by four independent analytical techniques. The plasmalogen content of canine cardiac myocyte choline glycerophospholipids was significantly greater than that seen in extracts derived from whole myocardium (33-45%) whereas the ethanolamine plasmalogen content of the isolated cardiac myocytes (42%) was similar to that previously reported in whole myocardial extracts (40-56%) derived from several different animal species (34, 35). The high content of choline plasmalogen relative to that of ethanolamine plasmalogen reflects the differential dilution of myocyte-derived choline and ethanolamine plasmalogen by phospholipids contributed from "non-myocyte" sources (e.g., endothelium and vascular smooth muscle) which contain significant amounts of ethanolamine plasmalogen but substantially lower amounts of choline plasmalogen (20, 35, 36). It is interesting to note that the plasmalogen content of canine cardiac myocyte choline glycerophospholipids was comparable to that seen in choline glycerophospholipids of isolated sarcolemma, mitochondria, and sarcoplasmic reticulum (51-57%) also prepared from canine myocardium (11, 12). In contrast, the ethanolamine plasmalogen content of isolated canine cardiac myocytes (42-47%) was considerably lower than that of canine myocardial sarcolemma (64%) and sarcoplasmic reticulum (73%) (11, 12). These results demonstrate that the distribution of choline plasmalogen in intracellular membrane compartments of the cardiac myocyte is widespread and relatively uniform, whereas ethanolamine plasmalogens appear to be selectively enriched in the sarcolemma and sarcoplasmic reticulum. These observations are likely to have considerable functional significance since accelerated phospholipid turnover during ischemia or following agonist-stimulated phospholipase activation may be localized to specific subcellular membrane compartments.

The isolated canine cardiac myocytes readily incorporated exogenous radiolabeled polyunsaturated fatty acids into endogenous phospholipid pools. The ^{3}H and ^{14}C radioactivity incorporated into phosphatidylinositol and phosphatidylserine was exclusively incorporated into acid-stable diradylglycerophospholipids with no evidence for incorporation of radioactivity into acid-labile plasmalogen species in these phospholipid classes. In choline and ethanolamine glycerophospholipids, [^{3}H]arachidonate was incorporated primarily into plasmalogen molecular species while [^{14}C]linoleate was distributed almost exclusively in diacyl glycerophospholipids. The marked differences that were noted in the calculated specific activity of radiolabeled plasmalogen and diacyl CGP and EGP species demonstrate that incorporation of radiolabeled polyunsaturated fatty acids into these phospholipids is not directly related to pool size. Accordingly, metabolism of the fatty acyl moiety esterfied to the *sn*-2 position of plasmalogen and diacyl CGP and EGP is differentially regulated in cardiac myocytes.

In any radiolabeling experiment, precise quantitative comparison of turnover rates based on measured specific radioactivities can be made only under conditions in which isotopic equillibrium is achieved (i.e., constant specific activity of a given phospholipid species in all phospholipid pools). However, in any radiolabeling experiment using whole cells, it is likely that incorporation of exogenous radiolabel is spatially and metabolically compartmentalized. For example, Miyazaki et al. (37) used electron microscopic autoradiographic techniques to define the subcellular distribution of [^{3}H]arachidonate incorporated into phospholipids of isolated rat cardiac myocytes and found marked differences in the specific activity (expressed as grains/μm^2 of membrane surface area)

Downloaded from www.jlr.org by guest, on June 18, 2012

of [^{3}H]arachidonate-labeled phospholipids present in different subcellular membrane compartments (spatial compartmentalization) at all time points examined. Substantial differences in the specific activity of individual phospholipid classes was also noted (metabolic compartmentalization). The spatial and metabolic compartmentalization of incorporated ^{3}H radioactivity was evident even under conditions in which the total amount of radioactivity in each phospholipid class had approached a steady-state condition. Similar results have also been obtained by other investigators in other types of cells (38, 39). The long incubation period (18 h) used for labeling the phospholipid pools of canine cardiac myocytes in the present study was selected to provide an approximation to steady-state conditions while minimizing the extent of irreversible cell injury. Although quantitative comparison of turnover rates is precluded by the spatial and metabolic compartmentalization of incorporated radioactivity, the results of the present study demonstrate that arachidonate esterified to plasmalogen molecular species represents a metabolically active pool that may play an important role as a precursor for the generation of biologically active eicosanoid metabolites. In contrast, the results of the linoleate labeling studies demonstrate that the relative turnover of linoleate-containing plasmalogen molecular species is very low, possibly reflecting the fact that these molecular species fulfill a predominantly structural role possibly serving to maintain a specific membrane microenvironment that may function to regulate the activity of integral membrane proteins. The high degree of selectivity for incorporation of [^{3}H]arachidonate into plasmalogens and particularly for the incorporation of [^{14}C]linoleate into diacyl phospholipids that we observed in canine cardiac myocytes should facilitate further investigation to assess the relative contribution of plasmalogen and diacyl phospholipid turnover to the pathophysiologic sequelae of myocardial ischemia.

The authors wish to thank Dr. Peter B. Corr for many helpful suggestions and stimulating discussions throughout the course of this study. FAB-MS analyses were carried out in the Washington University Mass Spectrometry Resource Facility which is supported by NIH grants RR-00954 and AM-20579. The expert technical advice and other assistance provided by Dr. Fong-Fu Hsu and Dr. William Sherman for the performance and interpretation of the FAB-MS analyses is also gratefully acknowledged. The authors also appreciate the technical assistance of Beverly Kekec and Evelyn Kanter and the preparation of the manuscript by Ava Ysaguirre.

Manuscript received 20 December 1990 and in revised form 10 April 1991.

REFERENCES

1. Chien, K. R., A. Han, A. Sen, L. M. Buja, and J. T. Willerson. 1984. Accumulation of unesterified arachidonic acid in ischemic myocardium. *Circ. Res.* **54:** 313-322.
2. Corr, P. B., R. W. Gross, and B. E. Sobel. 1984. Amphipathic metabolites and membrane dysfunction in ischemic myocardium. *Circ. Res.* **55:** 135-154.
3. Ford, D. A., and R. W. Gross. 1989. Differential accumulation of diacyl and plasmalogenic diglycerides during myocardial ischemia. *Circ. Res.* **64:** 173-177.
4. Gunn, M. D., A. Sen, A. Chang, J. T. Willerson, L. M. Buja, and K. R. Chien. 1985. Mechanisms of accumulation of arachidonic acid in cultured myocardial cells during ATP depletion. *Am. J. Physiol.* **249:** H1188-1194.
5. Otani, H., R. M. Engelman, J. A. Rousou, R. H. Breyer, and D. K. Das. 1986. Enhanced prostaglandin synthesis due to phospholipid breakdown in ischemic-reperfused myocardium. *J. Mol. Cell. Cardiol.* **18:** 953-961.
6. Burton, K. P., L. M. Buja, A. Sen, J. T. Willerson, and K. R. Chien. 1986. Accumulation of arachidonate in triacylglycerols and unesterified fatty acids during ischemia and reflow in the isolated rat heart. *Am. J. Pathol.* **124:** 238-245.
7. Borgers, M., and H. M. Piper. 1986. Calcium-shifts in anoxic cardiac myocytes. A cytochemical study. *J. Mol. Cell. Cardiol.* **18:** 439-448.
8. Corr, P. B., D. W. Snyder, M. E. Cain, W. A. Crafford, Jr., R. W. Gross, and B. E. Sobel. 1981. Electrophysiological effects of amphiphiles on canine Purkinje fibers. *Circ. Res.* **49:** 354-363.
9. Chien, K. R., R. G. Pfau, and J. L. Farber. 1979. Ischemic myocardial cell injury. Prevention by chlorpromazine of accelerated phospholipid degradation and associated membrane dysfunction. *Am. J. Pathol.* **97:** 505-522.
10. Fink, K. L., and R. W. Gross. 1984. Modulation of canine myocardial sarcolemmal membrane fluidity by amphiphilic compounds. *Circ. Res.* **55:** 585-594.
11. R. W. Gross. 1984. High plasmalogen and arachidonic acid content of canine myocardial sarcolemma: a fast atom bombardment mass spectroscopic and gas chromatography-mass spectroscopic characterization. *Biochemistry.* **23:** 158-165.
12. R. W. Gross. 1985. Identification of plasmalogen as the major phospholipid constituent of cardiac sarcoplasmic reticulum. *Biochemistry.* **24:** 1662-1668.
13. Wolf, R. A., and R. W. Gross. 1985. Identification of neutral active phospholipase C which hydrolyzes choline glycerophospholipids and plasmalogen selective phospholipase A_2 in canine myocardium. *J. Biol. Chem.* **260:** 7295-7303.
14. DaTorre, S. D., P. B. Corr, and M. H. Creer. 1990. A sensitive method for the quantification of the mass of inositol phosphates using gas chromatography-mass spectrometry. *J. Lipid Res.* **31:** 1925-1934.
15. Wittenberg, B. A., and T. F. Robinson. 1981. Oxygen requirements, morphology, cell coat and membrane permeability of Ca^{2+}-tolerant myocytes from hearts of adult rats. *Cell Tissue Res.* **216:** 231-251.
16. Heathers, G. P., K. A. Yamada, E. M. Kanter. and P. B. Corr. 1987. Long-chain acylcarnitines mediate the hypoxia-induced increase in α_1-adrenergic receptors on adult canine myocytes. *Circ. Res.* **61:** 735-746.
17. Markwell, M. A. K., S. H. Haas, N. E. Tolbert, and L. L. Bieber. 1981. Protein determination in membrane and lipoprotein samples: manual and automated procedures. *Methods Enzymol.* **72:** 296-303.
18. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *J. Biochem. Physiol.* **37:** 911-917.
19. Dugan, L. L., P. Demediuk, C. E. Pendley II, and L. A. Horrocks. 1986. Separation of phospholipids by high-

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

performance liquid chromatography: all major classes including ethanolamine and choline plasmalogens and most minor classes, including lysophosphatidylethanolamine. *J. Chromatogr.* **378:** 317–327.
20. Ford, D. A., and R. W. Gross. 1989. Plasmenylethanolamine is the major storage depot for arachidonic acid in rabbit vascular smooth muscle and is rapidly hydrolyzed after angiotensin II stimulation. *Proc. Natl. Acad. Sci. USA.* **86:** 3479–3483.
21. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. *Anal. Chem.* **28:** 1756–1758.
22. Creer, M. H., C. Pastor, P. B. Corr, R. W. Gross, and B. E. Sobel. 1985. Quantification of choline and ethanolamine phospholipids in rabbit myocardium. *Anal. Biochem.* **144:** 65–74.
23. Gottfried, E. L., and M. M. Rapport. 1962. The biochemistry of plasmalogens. I. Isolation and characterization of phosphatidal choline, a pure native plasmalogen. *J. Biol. Chem.* **237:** 329–333.
24. M. Kates. 1975. Techniques of Lipidology: Isolation, Analysis and Identification of Lipids. North Holland/Elsevier. New York, NY. 364–367.
25. Gross, R. W., and B. E. Sobel. 1980. Isocratic high-performance liquid chromatography separation of phosphoglycerides and lysophosphoglycerides *J. Chromatogr.* **197:** 79–85.
26. Creer, M. H., and R. W. Gross. 1985. Reversed-phase high-performance liquid chromatographic separation of molecular species of alkyl ether, vinyl ether, and monoacyl lysophospholipids. *J. Chromatogr.* **338:** 61–69.
27. M. Kates. 1986. Techniques of Lipidology: Isolation, Analysis and Identification of Lipids. Second ed. North Holland/Elsevier. New York, NY. 248–250.
28. Patton, G. M., J. M. Fasulo, and S. J. Robins. 1982. Separation of phospholipids and individual molecular species of phospholipids by high performance liquid chromatography. *J. Lipid Res.* **23:** 190–196.
29. Itaya, K., and M. Ui. 1966. A new micromethod for the colorimetric determination of inorganic phosphate. *Clin. Chim. Acta.* **14:** 361–366.
30. Gross, R. W., P. B. Corr, B. I. Lee, J. E. Saffitz, W. A. Crafford, Jr., and B. E. Sobel. 1982. Incorporation of radiolabeled lysophosphatidyl choline into canine Purkinje fibers and ventricular muscle. *Circ. Res.* **51:** 27–36.
31. K. Y. Hostetler. 1982. Polyglycerophospholipids: phosphatidylglycerol, diphosphatidylglycerol and bis(monoacylglycero)phosphate. *In* Phospholipids. J. N. Hawthorne and G. B. Ansell, editors. Elsevier Biomedical Press, New York, NY) 215–261.
32. D. J. Hanahan. 1972. Ether-linked lipids: chemistry and methods of measurement. *In* Ether Lipids: Chemistry and Biology. F. Snyder, editor. Academic Press, New York, NY. 25–50.
33. Creer, M. H., and R. W. Gross. 1985. Separation of isomeric lysophospholipids by reverse phase HPLC. *Lipids.* **20:** 922–928.
34. Horrocks, L. A., and M. Sharma. 1982. Plasmalogens and O-alkyl glycerophospholipids. *In* Phospholipids. J. N. Hawthorne and G. B. Ansell, editors. Elsevier Biomedical Press, New York, NY. 51–93.
35. Sugiura, T., and K. Waku. 1987. Composition of alkyl ether-linked phospholipids in mammalian tissues. *In* Platelet-Activating Factor and Related Lipid Mediators. F. Snyder, editor. Plenum Press, New York, NY. 55–85.
36. Blank, M. L., A. A. Spector, T. L. Kaduce, and F. Snyder. 1986. Composition and incorporation of [^{3}H]arachidonic acid into molecular species of phospholipid classes by cultured human endothelial cells. *Biochim. Biophys. Acta.* **877:** 211–215.
37. Miyazaki, Y., R. W. Gross, B. E. Sobel, and J. E. Saffitz. 1987. Biochemical and subcellular distribution of arachidonic acid in rat myocardium. *Am. J. Physiol* **253:** C846–853.
38. Laposata, M., C. M. Krueger, and J. E. Saffitz. 1987. Selective uptake of [^{3}H]arachidonic acid into the dense tubular system of human platelets. *Blood.* **70:** 832–837.
39. Neufeld, E. J., P. W. Majerus, C. M. Krueger, and J. E. Saffitz. 1985. Uptake and subcellular distribution of [^{3}H]arachidonic acid in murine fibrosarcoma cells measured by electron microscopic autoradiography. *J. Cell Biol.* **101:** 573–581.

Downloaded from www.jlr.org by guest, on June 18, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR