Identification of Plasmalogen as the Major Phospholipid Constituent of Cardiac Sarcoplasmic Reticulum†

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ABSTRACT: The phospholipid molecular species of canine myocardial sarcoplasmic reticulum were identified by fast atom bombardment mass spectrometry, reverse-phase high-performance liquid chromatography, and other conventional techniques. Cardiac sarcoplasmic reticulum contains 1.4 μmol of lipid P_i/mg of protein which is comprised of 53% plasmalogen. Cardiac sarcoplasmic reticulum ethanolamine glycerophospholipid contains 73% plasmalogen that is predominantly comprised of moieties with 18-carbon vinyl ethers at the *sn*-1 position and arachidonic acid at the *sn*-2 position. In contrast, canine skeletal muscle sarcoplasmic reticulum contains only 19% plasmalogen that is predominantly comprised of ethanolamine plasmalogen (78% of skeletal muscle sarcoplasmic reticulum ethanolamine glycerophospholipid) with arachidonic and docosatetraenoic acids at the *sn*-2 position. The possibility that tetraenoic ethanolamine plasmalogens in both cardiac and skeletal muscle sarcoplasmic reticulum facilitate calcium translocation by their propensity for adopting a hexagonal II conformation at physiologic temperatures is discussed.

Excitation–contraction coupling in myocardium is modulated by the intracellular concentration and distribution of calcium ions, which are regulated in part by the sarcoplasmic reticulum. Substantial evidence suggests that the interaction of sarcoplasmic reticulum membrane constituents with Ca^{2+}-ATPase modulates calcium translocation. For example, the rotational mobility of the Ca^{2+}-ATPase is modulated by its lipid environment and has been correlated with enzymic activity (Hidalgo et al., 1978). Although the Ca^{2+}-ATPase does not require specific phospholipids for enzymic activity (as long as they are in the liquid-crystal phase), the presence of phospholipid that assumes a hexagonal II phase is necessary for optimal rates of calcium translocation (Navarro et al., 1984). Both ^{31}P and ^{1}H NMR have suggested the presence of nonbilayer structures in sarcoplasmic reticulum vesicles (Davis & Inesi, 1971; Cullis & de Kruijff, 1979). The phase behavior of phospholipids is dependent on the relative steric bulk of the polar head group region compared to the aliphatic region (Cullis & de Kruijff, 1979), which is influenced by the chemical structure of the polar head group (Cullis & de Kruijff, 1979), the nature of the covalent linkage at the *sn*-1 carbon (Boggs et al., 1981; Seddon et al., 1983), and the degree of unsaturation of the fatty acyl chains (Cullis & de Kruijff, 1979). Since prior work with sarcoplasmic reticulum from skeletal muscle has demonstrated a marked sensitivity of calcium translocation to alterations in phospholipid constituents (Knowles & Racker, 1975), and since a detailed analysis of the phospholipid molecular species in cardiac sarcoplasmic reticulum has not been performed, fast atom bombardment mass spectroscopy, reverse-phase high-performance liquid chromatography (reverse-phase HPLC), and other conventional techniques were utilized to quantify the major phospholipid constituents of myocardial sarcoplasmic reticulum. The results demonstrate that the major phospholipid in canine myocardial sarcoplasmic reticulum is plasmalogen, that both skeletal muscle and cardiac sarcoplasmic reticulum ethanolamine glycerophospholipids are comprised predominantly of ethanolamine plasmalogens containing sterically bulky tetraenoic fatty acids at the *sn*-2 carbon, and that marked differences are present in choline glycerophospholipids from cardiac and skeletal muscle sarcoplasmic reticulum.

Materials and Methods

Preparation of Canine Sarcoplasmic Reticulum and Mitochondria. Mongrel dogs fed ad libitum were anesthetized with sodium pentothal (40 mg/kg), and myocardial homogenates were prepared as described previously (Gross, 1984). Sarcoplasmic reticulum from canine myocardium was prepared by the method of Jones et al. (1979) with the following modifications: (1) after homogenization in hypotonic buffer, both low-speed centrifugations were performed at $19700g_{max}$ for 20 min, and (2) after calcium loading, sarcoplasmic reticulum was isolated by centrifugation at $132000g_{max}$ for 60 min utilizing a discontinuous gradient of 0.25 M sucrose layered over 1.5 M sucrose containing 300 mM KCl, 50 mM PP_i, and 100 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris·HCl) (pH 7.2). Sarcoplasmic reticulum from canine skeletal muscle was prepared by an identical procedure after dissection of the gastrocnemius muscle. Mitochondria were isolated from canine myocardium by Potter–Elvehjem homogenization and centrifugation at $800g_{max}$ for 10 min, and the supernatant fraction was centrifuged at $8000g_{max}$ for 10 min. The mitochondrial fraction (pellet) was resuspended in 0.25 M sucrose and 10 mM histidine (pH 7.5) and used for subsequent analyses. Sarcolemma was prepared as described previously (Gross, 1984).

Phospholipid Extraction, Separation, and Quantification. Phospholipids from sarcoplasmic reticulum or mitochondria were extracted by the method of Bligh & Dyer (1959), filtered through 3-μm FALP Whatman filters, and evaporated to dryness under N_2. Phospholipids were dissolved in 100 μL of 2/1 (v/v) chloroform/methanol, and iterative 15-μL injections were made onto a Waters Associates HPLC apparatus. Phospholipids were initially separated into classes on a Partisil SCX 10/25 column by isocratic elution as described previously (Gross & Sobel, 1980). Choline glycerophospholipids were

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utilized directly, but due to small amounts of contamination in the ethanolamine glycerophospholipid fraction, further purification by thin-layer chromatography (TLC) on silica OF TLC plates (Analabs, Foxborough, MA) was performed as described previously (Gross, 1984). Phosphatidylinositol, phosphatidylserine, and diphosphatidylglycerol were separated by conventional two-dimensional (2-D) TLC by the method of Rouser et al. (1970). Phospholipid phosphorus was determined by the method of Chen (1956). Vinyl ether content was assayed by the I_2 addition method of Gottfried & Rapport (1962) as previously modified (Gross, 1984).

Phospholipid molecular species were separated with reverse-phase HPLC by isocratic elution with a mobile phase of methanol/acetonitrile/water (90.5/2.5/7) containing 20 mM choline chloride and a stationary phase of octadecyl silica (Patton et al., 1983). Each major peak was collected, internal standard [diarachidoyl(C20:0)-phosphatidylcholine] was added, and each peak was extracted by the method of Bligh & Dyer (1959) and quantified by capillary gas chromatography (capillary GC) after acid methanolysis (see below). The use of diarachidoylphosphatidylcholine for quantification of both plasmalogen and diacylphospholipid species was justified by the demonstration of identical partition coefficients into chloroform after Bligh and Dyer extraction for these two lipid classes and the demonstration of stoichiometric amounts of dimethyl acetals and fatty acid methyl esters (±2%) after acid methanolysis of homogeneous plasmalogen molecular species. Reverse-phase HPLC of equimolar amounts of plasmalogen and diacylphosphatidylcholines containing identical aliphatic chains demonstrated that ultraviolet absorption of the vinyl ether linkage was equivalent to 2.9 olefin linkages at 203 nm. Thus peaks from reverse-phase HPLC were quantified by integration of ultraviolet absorbance utilizing a Planix 7 integrator (Tanaya, Japan) and normalized by dividing each peak area by its number of double-bond equivalents (number of aliphatic double bonds + 2.9 for plasmalogens or the number of aliphatic double bonds for diacylphospholipids).

Methanolysis and Quantitative Gas Chromatographic Analysis. Methanolysis was performed as described previously (Gross, 1984). Capillary gas chromatography of fatty acid methyl esters and dimethyl acetals was performed with a Varian Associates 3700 gas chromatograph equipped with a Model 4270 data module. The injector and detector temperatures were 280 and 250 °C, respectively. Helium was used as the carrier gas at a flow rate of 2 cm^3/min with a 5/1 split ratio. Typically, 0.5-μL injections were made onto a 30-m capillary column coated with SP 2330 (Supelco, Bellefonte, PA). Fatty acid methyl esters and dimethyl acetals were eluted isothermally at 190 °C. Quantitation was accomplished with a flame ionization detector equipped with a capillary tip. Detector response was calibrated with a mixture of standards (Supelco) and was proportional to the molecular weight of each fatty acid or dimethyl acetal.

Miscellaneous Procedures. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis, marker enzyme analysis, alkyl ether analysis, gas chromatography–mass spectrometry, and fast atom bombardment mass spectrometry were performed as previously described (Gross & Sobel, 1983; Gross, 1983, 1984).

Results

Purity of the Sarcoplasmic Reticulum and Mitochondrial Fractions Isolated from Canine Myocardium. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified cardiac sarcoplasmic reticulum demonstrated a predominant band at 96 kDa, a less intense band at 55 kDa, and approximately 15 other minor protein bands (Figure 1). Oxalate-enhanced Ca^{2+},K^+-ATPase activity was utilized as a sarcoplasmic reticulum marker and had a specific activity of 97.8 ± 3.5 μmol of P_i mg^{-1} h^{-1} ($\bar{X}$ ± SE). Contamination by other myocardial subcellular membranes was minimal as estimated by oligomycin-inhibitable ATPase activity, NaN_3-inhibitable ATPase activity, and Na^+,K^+-ATPase activity (Table I). Thus, the protein electrophoretic profile, the absence of significant activities of marker enzymes from other subcellular fractions, and the high specific activity of Ca^{2+},K^+-ATPase demonstrate that these preparations were of a purity comparable with other highly purified preparations of myocardial sarcoplasmic reticulum reported in the literature (Jones et al., 1979; Chamberlain et al., 1983).

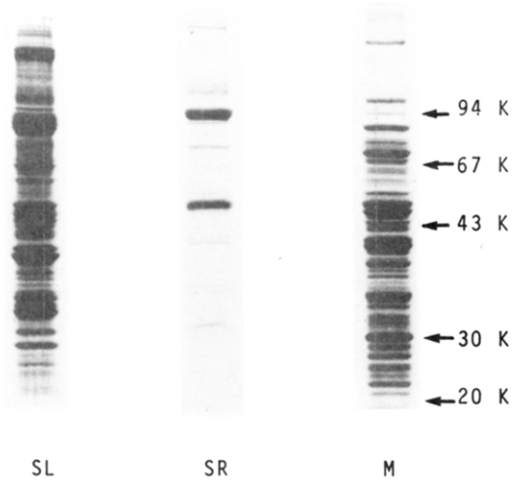

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of canine sarcolemma, sarcoplasmic reticulum, and mitochondria. Purified myocardial subcellular fractions (15–40 μg) were heated at 90 °C for 5 min with 100 mM 2-mercaptoethanol and 20% sodium dodecyl sulfate and electrophoresed for 8 h at 120 V on a 10% polyacrylamide gel. Proteins were visualized by staining with Coomassie blue. Numbers on the right correspond to molecular weight markers run on an adjacent lane. SL = sarcolemma; SR = sarcoplasmic reticulum; M = mitochondria.

Table I: Marker Enzyme Analysis of Cardiac Subcellular Fractions[a]

	sarcoplasmic reticulum	sarcolemma	mitochondria
Ca^{2+},K^+-ATPase (μmol mg^{-1} h^{-1})	98	3	2
Na^+,K^+-ATPase (μmol mg^{-1} h^{-1})	1	33	5
NaN_3-inhibitable ATPase (μmol mg^{-1} h^{-1})	<1	4	22
oligomycin-inhibitable ATPase (μmol mg^{-1} h^{-1})	<1	3	16
citrate synthetase (μmol mg^{-1} min^{-1})	<0.1	<0.1	1.7

[a] Data are the mean of at least three determinations. Standard errors for marker enzymes with substantial activity in a subcellular fraction were within 10% of the mean.

The mitochondrial fraction from canine myocardium contained a citrate synthetase activity of 1.7 μmol mg^{-1} min^{-1} and was enriched in several mitochondrial membrane markers including oligomycin-inhibitable ATPase and NaN_3-inhibitable ATPase (Table I) and contained 78 nmol of diphosphatidylglycerol/mg of protein (19% of mitochondrial phospholipid). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis demonstrated predominant protein bands at 50 kDa (doublet), 38 kDa (triplet), 33 kDa, and 30 kDa (Figure 1). The specific activity of sarcolemma or sarcoplasmic reticulum markers was minimal in the mitochondrial fraction (Table I).

Table II: Phospholipid Composition of Canine Sarcoplasmic Reticulum[a]

	nmol/mg of protein
choline glycerophospholipids	1027 ± 86
diacyl	(493)[b]
plasmalogen	(534)[b]
ethanolamine glycerophospholipids	316 ± 18
diacyl	(89)[b]
plasmalogen	(227)[b]
phosphatidylinositol	30 ± 2
sphingomyelin	26 ± 4
phosphatidylserine	16 ± 1
lysophosphatidylethanolamine	13 ± 3
lysophosphatidylcholine	6 ± 1

[a] Phospholipids were separated by HPLC or 2-D TLC and quantified by phosphate analysis as described under Materials and Methods. Results are the $\bar{X}$ ± SE of at least three determinations. [b] Plasmalogen and diacyl content was quantified by the difference between phosphate and I_2 addition as described under Materials and Methods.

Phospholipid Content and Constituents of Myocardial Sarcoplasmic Reticulum. Canine myocardial sarcoplasmic reticulum contained 1.4 μmol of lipid P_i/mg of protein. The major phospholipid constituents were choline (73%) and ethanolamine (22%) glycerophospholipids with small amounts of phosphatidylinositol, sphingomyelin, phosphatidylserine, and lysophospholipids also present (Table II). Diphosphatidylglycerol was not detectable in the purified sarcoplasmic reticulum preparation, demonstrating the absence of significant mitochondrial contamination. Sarcoplasmic reticulum choline glycerophospholipid contained 53 ± 3% plasmalogen, and ethanolamine glycerophospholipid contained 73 ± 4% plasmalogen as ascertained by the incorporation of I_2 into the vinyl ether linkage (Gottfried & Rapport, 1962). Alkaline methanolysis of choline or ethanolamine glycerophospholipids and subsequent acid-catalyzed methanolysis, separation of lysolipids by HPLC, and quantitation by phosphate analysis demonstrated that sarcoplasmic reticulum contained less than 2% alkyl ethers in both the choline and ethanolamine phospholipid fractions. Since choline and ethanolamine glycerophospholipid comprised the overwhelming majority of sarcoplasmic reticulum phospholipid, the molecular constituents of these phospholipid classes were quantified.

Fatty Acid and Vinyl Ether Composition of Canine Sarcoplasmic Reticulum Phospholipids. Acid-catalyzed methanolysis of sarcoplasmic reticulum choline and ethanolamine glycerophospholipids and subsequent capillary gas chromatography confirmed the high plasmalogen content of both the choline and ethanolamine glycerophospholipid fractions. Methanolysates of choline glycerophospholipids contained the dimethyl acetal of palmitic aldehyde (17%) and small amounts of the dimethyl acetals of stearic (3%) and oleic (4%) aldehydes and multiple other fatty acid methyl esters (Table III). Methanolysates of ethanolamine glycerophospholipids contained the dimethyl acetals of palmitic (11%), stearic (16%), and oleic (10%) aldehydes and were also remarkable for the predominance of arachidonic acid methyl ester (Table III). The complete fatty acid and vinyl ether profile of cardiac sarcoplasmic reticulum choline and ethanolamine glycerophospholipid fractions is given in Table III.

Mitochondrial choline glycerophospholipid methanolysates contained substantial amounts of the dimethyl acetal of palmitic aldehyde (Table III). Mitochondrial ethanolamine glycerophospholipid methanolysates were remarkable for the predominance of the fatty acid methyl esters of stearic and arachidonic acids (Table III). Acid methanolysates of the third major phospholipid class in the mitochondrial compartment, diphosphatidylglycerol, demonstrated a predominance of linoleic acid (82%) and smaller amounts of oleic (5%), palmitic (3%), and stearic (2%) acids. No dimethyl acetals derived from cardiolipin could be identified.

Positional Specificity of Fatty Acids and Vinyl Ethers in Choline and Ethanolamine Glycerophospholipids from Myocardial Sarcoplasmic Reticulum and Mitochondria. The regiospecific hydrolysis of phospholipids by phospholipase A_2 was utilized to determine the relative distribution of fatty acids and aldehydes at the *sn*-1 and *sn*-2 carbons. Lysolipids derived from choline glycerophospholipids after phospholipase A_2 treatment contained predominantly the dimethyl acetal of palmitic aldehyde after acid methanolysis (Table III). Free fatty acids released from choline glycerophospholipids by phospholipase A_2 treatment contained mostly unsaturated fatty acids although small amounts of saturated fatty acids were also identified (Table III).

Similarly, analysis of lysolipids isolated after phospholipase A_2 treatment of sarcoplasmic reticulum ethanolamine glycerophospholipids demonstrated a predominance of the dimethyl acetals of stearic, oleic, and palmitic aldehydes, as well as the fatty acid methyl ester of stearic acid (Table III). Analysis of fatty acids released from sarcoplasmic reticulum

Table III: Fatty Acid and Aldehyde Profiles of Native and Phospholipase-Treated Phospholipids from Canine Sarcoplasmic Reticulum and Mitochondria[a]

	16:0(D)	16:0(F)	18:0(D)	18:1(D)	18:0(F)	18:1(F)	18:2(F)	20:4(F)
sarcoplasmic reticulum								
CGP	17	14	3	4	8	23	14	16
PLA_2-CGP-FA	ND	5	ND	ND	4	38	26	25
PLA_2-CGP-LPC	54	13	8	7	12	4	1	1
EGP	11	3	16	10	8	4	5	42
PLA_2-EGP-FA	ND	4	ND	ND	6	11	10	70
PLA_2-EGP-LPE	17	2	38	26	17	ND	ND	ND
mitochondria								
CGP	19	15	3	4	6	22	16	14
PLA_2-CGP-FA	ND	7	ND	ND	3	32	28	30
PLA_2-CGP-LPC	38	22	11	12	12	2	2	1
EGP	4	2	10	5	24	3	10	42
PLA_2-EGP-FA	ND	5	ND	ND	3	9	5	77
PLA_2-EGP-LPE	6	4	18	12	56	3	ND	ND

[a] Sarcoplasmic reticulum or mitochondrial choline (CGP) or ethanolamine (EGP) glycerophospholipids were subjected to acid-catalyzed methanolysis and analyzed by capillary gas chromatography as described under Materials and Methods. Products of phospholipase-treated choline or ethanolamine glycerophospholipids were separated by HPLC and derivatized as described under Materials and Methods. Results are expressed in weight percent. PLA_2-XGP-FA and PLA_2-XGP-LPC(E) are the fatty acids (or aldehydes) and lysolipids identified after phospholipase treatment, purification, and derivatization. D refers to the dimethyl acetal of the given aldehyde and F to the fatty acid methyl ester. Trace amounts (<1%) of 16:1, 20:3, and 22:4 fatty acids were also identified. ND = not detected.

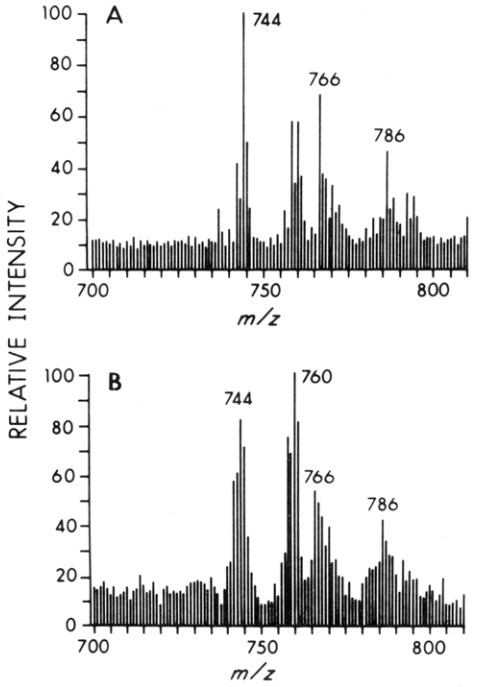

FIGURE 2: Fast atom bombardment mass spectrometry of sarcoplasmic reticulum and mitochondrial choline glycerophospholipids. Purified choline glycerophospholipids from sarcoplasmic reticulum (top panel) or mitochondria (bottom panel) were dissolved (approximately 300 μg of each) in 10 μL of 1/1 $CHCl_3$/MeOH, and 2 μL was mixed with 3 μL of glycerol on a copper probe. Fast atom bombardment mass spectrometry was performed as described.

ethanolamine glycerophospholipids by phospholipase A_2 demonstrated a predominance of arachidonic acid (Table III).

Phospholipase A_2 treatment of mitochondrial choline glycerophospholipids and subsequent acid methanolysis demonstrated large amounts of the dimethyl acetal of palmitic aldehyde and the methyl ester of palmitate derived from the *sn*-1 position (Table III). The *sn*-2 position of mitochondrial choline glycerophospholipids contained predominantly unsaturated fatty acids (Table III). Mitochondrial ethanolamine glycerophospholipids contained predominantly stearic acid at the *sn*-1 carbon and arachidonic acid at the *sn*-2 carbon (Table III).

Fast Atom Bombardment Mass Spectrometry of Canine Sarcoplasmic Reticulum and Mitochondrial Glycerophospholipids. Sarcoplasmic reticulum choline glycerophospholipids contained molecular species with major protonated parent ions $(MH)^+$ of *m/z* 744 and 766 (Figure 2) corresponding to molecular species with the vinyl ether of palmitate at the *sn*-1 carbon and oleate or arachidonate, respectively, at the *sn*-2 carbon. Other parent ions easily identified included species with *m/z* 758, 760, 786, and 810, whose molecular identity and relative abundances are given in Table IV. Ethanolamine glycerophospholipids were comprised predominantly of species with protonated parent ions at *m/z* 750 and 752 (Figure 3), corresponding to plasmalogens with arachidonic acid esterified to the *sn*-2 carbon and the vinyl ethers of oleic and stearic aldehydes, respectively, at the *sn*-1 carbon.

Fast atom bombardment mass spectrometry of mitochondrial choline glycerophospholipids demonstrated major protonated parent ions at *m/z* 760 and 758 (Figure 2), corresponding to diacylphospholipids with palmitate at the *sn*-1 carbon and oleate and linoleate, respectively, at the *sn*-2 carbon. Smaller amounts of molecular species with parent ions at *m/z* 744, 766, 786, and 810 were easily demonstrated (Figure 2). Fast atom bombardment mass spectrometry of

Table IV: Choline Glycerophospholipid Molecular Species in Myocardial Sarcoplasmic Reticulum[a]

peak	species (*sn*-1–*sn*-2)	*m/z*	%[b]	%[c]	%[d]
1	16:0–20:4	782	1.8	3.2	2.3
2	16:0–18:2	758	14.1	10.8	12.3
3	18:1–18:2	784	2.7	1.4	1.3
4	16:0–20:4 (P)	766	18.5	21.7	15.7
5	16:0–18:2 (P)	742	7.4	12.4	8.0
6	18:1–20:4 (P)	792	2.3	2.8	4.6
7	16:0–18:1	760	11.6	12.8	12.0
8	18:1–18:1	786	2.6	1.7	4.3
9	18:0–20:4	810	2.4	4.5	2.8
10	18:0–18:2	786	5.9	6.0	4.3
11	16:0–18:1 (P)	744	16.5	15.1	22.2
12	18:1–18:1 (P)	770	5.9	1.6	2.8
13	18:0–20:4 (P)	794	6.3	4.9	4.4
14	18:0–18:2 (P)	770	1.8	1.2	2.8

[a] Choline glycerophospholipid molecular species were determined by reverse-phase HPLC. Peak numbers correspond to those shown in the elution profile of Figure 4. P refers to plasmalogen molecular species. [b] Determined by addition of internal standards and capillary GC. [c] Determined by integration of HPLC ultraviolet absorbance. [d] Determined by fast atom bombardment mass spectrometry as described under Materials and Methods.

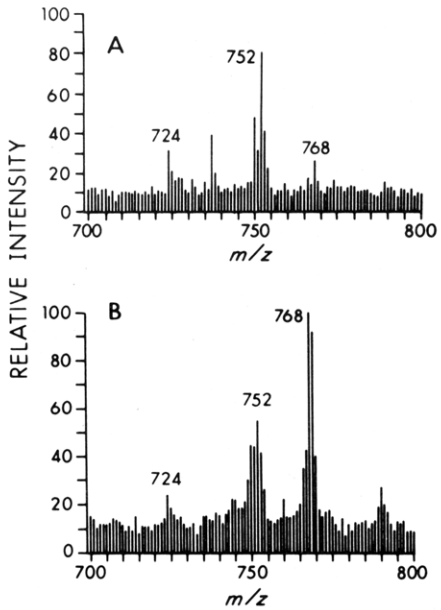

FIGURE 3: Fast atom bombardment mass spectrometry of sarcoplasmic reticulum or mitochondrial ethanolamine glycerophospholipids. Purified ethanolamine glycerophospholipids (approximately 300 μg) from sarcoplasmic reticulum (top panel) or mitochondria (bottom panel) were dissolved in 10 μL of 1/1 $CHCl_3$/MeOH, and 2 μL was mixed with 3 μL of glycerol on a copper probe. Fast atom bombardment mass spectrometry was performed as described. The peak at 737 (top panel) results from a glycerol polymer.

mitochondrial ethanolamine glycerophospholipids demonstrated a predominant peak at *m/z* 768 (Figure 3), corresponding to diacylphosphatidylethanolamine containing stearate at the *sn*-1 carbon and arachidonate at the *sn*-2 carbon. Diacylglycerophospholipid, but not plasmalogen ethanolamine glycerophospholipid, undergoes a facile loss of ethanolamine phosphate after ionization and desorption from the glycerol matrix (Gross, 1984). Thus the predominant peak in the mass spectrum of mitochondrial ethanolamine glycerophospholipid was at *m/z* 627 (768 − 141 → 627), which exceeded the parent ion intensity by a factor of 6. Thus, the relative intensities of the parent ions of ethanolamine glycerophospholipids do not accurately reflect their relative abundance due to differences in the rate of unimolecular decomposition of ionized plasmalogen and diacylphospholipids.

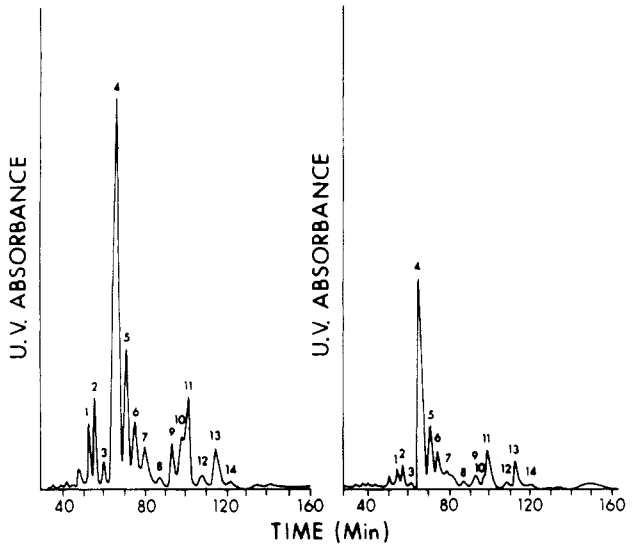

FIGURE 4: Reverse-phase HPLC of sarcoplasmic reticulum and mitochondrial choline glycerophospholipids. Choline glycerophospholipids (approximately 100–200 nmol) from the sarcoplasmic reticulum (left panel) or mitochondrial fractions (right panel) were purified by HPLC, injected onto an octadecyl silica column, and eluted isocratically with a mobile phase of methanol/acetonitrile/water (90.5/2.5/7) containing 20 mM choline chloride. Ultraviolet absorbance was monitored at 203 nm. The molecular identities of numbered peaks and their relative abundance are shown in Table IV.

Although the major parent ion peaks present in either choline or ethanolamine glycerophospholipids in a given sample were reproducibly identified, the relative intensities of peaks on replicate spectra of the same sample could differ by 15%. Furthermore, the presence of sodiated parent ions $(MNa)^+$ and differences in the kinetics of fragmentation lead to substantial errors in quantifying the relative abundance of phospholipid molecular species. Thus fast atom bombardment mass spectrometry of complex mixtures should be regarded as semiquantitative. To quantitatively characterize the molecular species present in canine myocardial sarcoplasmic reticulum, reverse-phase HPLC was utilized.

Reverse-Phase HPLC of Myocardial Sarcoplasmic Reticulum and Mitochondrial Glycerophospholipids. Reverse-phase HPLC of sarcoplasmic reticulum and mitochondrial choline and ethanolamine glycerophospholipids corroborated the phospholipid molecular species identified by fast atom bombardment mass spectrometry and gas chromatography. The molecular identities and amounts of diacyl and plasmalogen molecular species were established by collection of each major peak, addition of internal standard, derivatization, and quantification by capillary gas chromatography as described under Materials and Methods. The elution profile of myocardial sarcoplasmic reticulum choline glycerophospholipids is shown in Figure 4. The molecular identities of major peaks and their relative abundance determined from internal standards, integration of peak areas, and fast atom bombardment mass spectrometry are given in Table IV. Reverse-phase HPLC of myocardial mitochondrial choline glycerophospholipids demonstrated a similar elution profile (Figure 4).

Reverse-phase chromatography of ethanolamine glycerophospholipids from myocardial sarcoplasmic reticulum demonstrated predominant peaks at 85 (18:1–20:4 plasmalogen) and 138 min (18:0–20:4 plasmalogen) (Figure 5). The identities of other molecular species and their relative abundance based on internal standards, integration of peak areas, and fast atom bombardment mass spectrometry are given in Table V. Reverse-phase chromatography of ethanolamine glycerophospholipids from the mitochondrial fraction demonstrated a predominant peak at 112 min corresponding to 18:0–20:4 diacylphosphatidylethanolamine (Figure 5), in agreement with results from fast atom bombardment mass spectroscopy and capillary gas chromatography.

Table V: Ethanolamine Glycerophospholipid Molecular Species in Myocardial Sarcoplasmic Reticulum[a]

peak	species (*sn*-1–*sn*-2)	*m/z*	%[b]	%[c]	%[d]
1	16:0–20:4	740	1.2	3.3	ND
2	16:0–20:4 (P)	724	13.6	12.3	15.4
3	18:1–20:4 (P)	750	22.1	21.8	26.2
4	18:1–18:2 (P)	726	1.4	4.4	ND
5	18:0–20:4	768	16.2	17.0	10.1
6	18:0–20:4 (P)	752	45.5	41.2	48.3

[a] Ethanolamine glycerophospholipid molecular species were determined by reverse-phase HPLC. Peak numbers refer to the elution profile of phospholipids shown in Figure 5. P refers to plasmalogen molecular species. ND = not detected. [b] Determined by addition of internal standards and capillary GC. [c] Determined by integration of HPLC ultraviolet absorbance. [d] Determined by fast atom bombardment mass spectrometry as described under Materials and Methods.

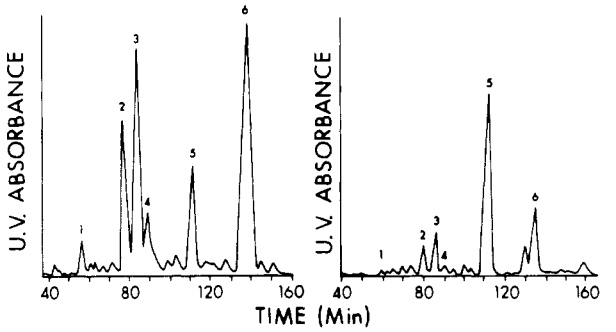

FIGURE 5: Reverse-phase HPLC of sarcoplasmic reticulum and mitochondrial ethanolamine glycerophospholipids. Ethanolamine glycerophospholipids (approximately 100–200 nmol) from the sarcoplasmic reticulum (left panel) or mitochondrial fractions (right panel) were purified by HPLC, injected onto an octadecyl silica column, and eluted isocratically with a mobile phase of methanol/acetonitrile/water (90.5/2.5/7) containing 20 mM choline chloride. Ultraviolet absorbance was monitored at 203 nm. The molecular identities of numbered peaks and their relative abundance are shown in Table V.

Quantification of Phospholipid Molecular Species Present in Sarcoplasmic Reticulum from Gastrocnemius Muscle. Since the phospholipid molecular constituents of canine myocardial sarcoplasmic reticulum differed substantially from the phospholipid constituents previously reported for rabbit (Van Winkle, 1983), rat (Marai & Kuksis, 1973), or human (Marai & Kuksis, 1973) skeletal muscle sarcoplasmic reticulum, the molecular species in canine myocardial and skeletal muscle sarcoplasmic reticulum were compared. Sarcoplasmic reticulum from gastrocnemius muscle had an oxalate-stimulated Ca^{2+},K^+-ATPase activity of 368 μmol of P_i mg^{-1} h^{-1}. Phospholipid molecular species present in skeletal muscle sarcoplasmic reticulum were quantified by reverse-phase HPLC after addition of internal standards and by integration of peak areas. In contrast to canine myocardial sarcoplasmic reticulum, gastrocnemius sarcoplasmic reticulum choline glycerophospholipid contained only small amounts (10%) of choline plasmalogen (Table VI). The predominant choline glycerophospholipid in skeletal muscle sarcoplasmic reticulum was 16:0–18:2 diacylphosphatidylcholine (Figure 6; Table VI). Ethanolamine glycerophospholipids from canine gastrocnemius sarcoplasmic reticulum contained 78% plasmalogen (determined by capillary gas chromatography) and were remarkable for a striking predominance of tetraenoic fatty acids including arachidonic and docosatetraenoic acids. The identity of both

Table VI: Choline Glycerophospholipid Molecular Species in Skeletal Muscle Sarcoplasmic Reticulum[a]

peak	species (*sn*-1–*sn*-2)	%[b]	%[c]
1	16:0–20:4	4.9	5.5
2	16:0–18:2	49.5	55.1
3	18:1–18:2	0.9	1.8
4	16:0–20:4 (P)	7.2	7.1
5	16:0–18:2 (P)	4.3	1.3
6	18:1–20:4 (P)	1.2	0.9
7	16:0–18:1	5.8	8.0
8	18:1–18:1	0.7	0.2
9	18:0–20:4	1.6	2.2
10	18:0–18:2	19.1	15.1
11	18:0–18:1	4.8	3.0

[a] Choline glycerophospholipid molecular species were determined by reverse-phase HPLC. Peak numbers refer to the elution profile of phospholipid molecular species shown in the left panel of Figure 6. P refers to plasmalogen molecular species. [b] Determined by addition of internal standards and capillary GC. [c] Determined by integration of HPLC ultraviolet absorbance as described under Materials and Methods.

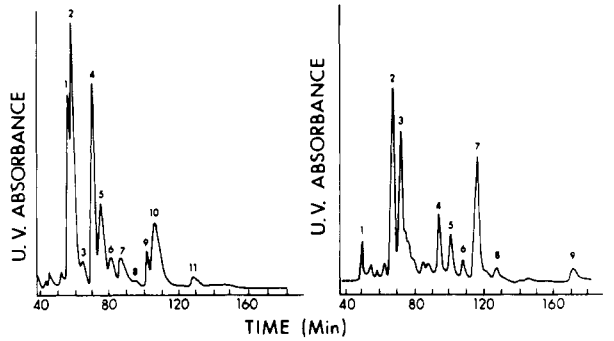

FIGURE 6: Reverse-phase HPLC of skeletal muscle sarcoplasmic reticulum choline and ethanolamine glycerophospholipids. Choline (left panel) or ethanolamine (right panel) glycerophospholipids (approximately 150 nmol) from skeletal muscle sarcoplasmic reticulum were separated by HPLC, injected onto an octadecyl silica column, and eluted isocratically as described under Materials and Methods. Ultraviolet absorbance was monitored at 203 nm. Major peaks were collected and quantified as described under Materials and Methods. The molecular identities of the numbered peaks are shown in Table VI (left panel) and Table VII (right panel).

arachidonic and docosatetraenoic acids was determined by comparison with standards on capillary gas chromatography and gas chromatography–mass spectrometry. Both tetraenoic fatty acids were predominantly present in plasmalogen and not diacylethanolamine glycerophospholipid. The phospholipid profiles of choline and ethanolamine glycerophospholipids in gastrocnemius sarcoplasmic reticulum are given in Tables VI and VII, respectively.

Discussion

The results of the present study demonstrate that plasmalogens and not diacylphospholipids comprise the majority of phospholipid present in canine myocardial sarcoplasmic reticulum. The results of four independent methods each demonstrate similar values of plasmalogen in the choline glycerophospholipid (52–60% plasmalogen) and ethanolamine glycerophospholipid (73–82% plasmalogen) fractions. Since choline and ethanolamine glycerophospholipids comprise over 90% of the phospholipid present in canine myocardial sarcoplasmic reticulum, the results unambiguously identify plasmalogens as the major phospholipid constituent of this subcellular fraction.

Phospholipid class heterogeneity is present in several myocardial membrane fractions, exemplified by the high content of diphosphatidylglycerol in mitochondria (Reddy et al., 1983), sphingomyelin in sarcolemma (Gross, 1984), and choline

Table VII: Ethanolamine Glycerophospholipid Molecular Species in Skeletal Muscle Sarcoplasmic Reticulum[a]

peak	species (*sn*-1–*sn*-2)	%[b]	%[c]
1	16:0–20:4	4.6	4.7
2	16:0–20:4 (P)	26.2	27.3
3	18:1–20:4 (P)	19.0	17.0
4	18:0–20:4	12.2	12.6
5	16:0–22:4 (P)	6.8	5.7
6	18:1–22:4 (P)	2.6	2.1
7	18:0–20:4 (P)	23.2	25.5
8	18:0–18:2 (P)	2.3	2.6
9	18:0–22:4 (P)	3.3	2.7

[a] Ethanolamine glycerophospholipid molecular species were determined by reverse-phase HPLC. Peak numbers refer to the elution profile of phospholipid molecular species shown in the right panel of Figure 6. P refers to plasmalogen molecular species. [b] Determined by addition of internal standards and capillary GC. [c] Determined by integration of HPLC ultraviolet absorbance as described under Materials and Methods.

glycerophospholipid in sarcoplasmic reticulum (Chamberlain, 1984). This study demonstrates that the phospholipid diversity in each major cardiac subcellular membrane fraction extends to the individual phospholipid molecular species present in each phospholipid class. For example, although the predominant ethanolamine glycerophospholipid in mitochondria is diacylphosphatidylethanolamine with arachidonic acid at the *sn*-2 carbon, the predominant ethanolamine glycerophospholipid in sarcoplasmic reticulum is 18:0–20:4 plasmalogen (Figure 3). Choline glycerophospholipids in sarcoplasmic reticulum, mitochondria, and sarcolemma (Gross, 1984) are similar although several subtle compositional differences between each are noted. Thus, although choline glycerophospholipid could distribute into each subcellular fraction with subsequent minor modifications by the sequential actions of phospholipase and coenzyme A:lysophosphatidylcholine acyltransferase, it is unlikely that the marked diversity in ethanolamine glycerophospholipids in the sarcoplasmic reticulum and mitochondrial compartments occurs by remodeling alone. The results suggest that specific mechanisms are operative that result in the preferential delivery of diacylethanolamine glycerophospholipid into the mitochondrial compartment and vinyl ether phospholipids into the sarcoplasmic reticulum and sarcolemmal compartments.

Although the phospholipid class distribution of skeletal muscle and cardiac muscle sarcoplasmic reticulum phospholipids has been reported to be similar (Chamberlain et al., 1983), the present study demonstrates a marked difference in both the plasmalogen content and individual molecular constituents present in skeletal and myocardial sarcoplasmic reticulum. Myocardial sarcoplasmic reticulum choline glycerophospholipid contained over 5 times the amount of plasmalogen compared to skeletal muscle sarcoplasmic reticulum. Furthermore, the predominant fatty acid in skeletal muscle sarcoplasmic reticulum choline glycerophospholipid was linoleic acid although oleic acid was prominent in myocardial sarcoplasmic reticulum choline glycerophospholipid. Both skeletal and myocardial sarcoplasmic reticulum ethanolamine glycerophospholipids contained similar amounts of plasmalogen (70–80%) containing tetraenoic fatty acids. However, myocardial sarcoplasmic reticulum contained only trace amounts (<1%) of docosatetraenoic fatty acid while skeletal muscle ethanolamine glycerophospholipids contained substantial amounts that were localized exclusively to plasmalogen molecular species. Taken together, the results demonstrate that canine sarcoplasmic reticulum from skeletal and cardiac muscle contains substantial differences in the relative abundance of different phospholipid molecular constituents.

This study demonstrates that reverse-phase HPLC of both choline and ethanolamine glycerophospholipids can resolve diacyl and plasmalogen molecular species containing identical aliphatic chains at the *sn*-1 and *sn*-2 positions. The presence of a vinyl ether linkage at the *sn*-1 carbon results in increased retention on octadecyl silica compared to corresponding diacyl compounds, which likely results from the decreased polarity of the vinyl ether linkage compared with the ester linkages found at the *sn*-1 position in diacylphospholipid molecular species. Ultraviolet absorption of phospholipids largely results from the $\pi \rightarrow \pi^*$ transition of olefins (λ_{max} = 165 nm), which is usually observed at 203 nm due to interference from the mobile phase and molecular oxygen at shorter wavelengths (Gross & Sobel, 1980). The nearly 3-fold higher extinction coefficient of vinyl ethers compared to olefins at 203 nm as well as the predominance of tetraenoic fatty acids in plasmalogens makes reverse-phase HPLC particularly well suited for analysis of plasmalogen molecular species. Although fast atom bombardment mass spectrometry was useful for identifying major parent ions, the presence of differential rates of fragmentation, sodiated phospholipid molecular species (Gross, 1984), ambiguities resulting from isobaric species, and the 15% variability in the relative intensity of peaks in replicate spectra demonstrate that quantitative analyses utilizing this technique alone should be interpreted with caution.

The effect of lipid composition on the function of calcium ATPase has been the subject of intense investigation. Evidence has been presented which suggests that calcium ATPase specifically requires phosphatidylethanolamine for maximum calcium transport activity (Knowles et al., 1975). However, the present study demonstrates that both skeletal muscle and myocardial sarcoplasmic reticulum contain only diminutive amounts of diacylphosphatidylethanolamine. Recently, the hypothesis has been advanced that maximum calcium translocation requires the presence of lipid that adopts a hexagonal II phase (Navarro et al., 1984). Ethanolamine plasmalogens containing oleic acid at the *sn*-2 position (but not alkyl ether or diacylethanolamine glycerophospholipids) undergo a lamellar to hexagonal phase transition at physiologic temperatures (Lohner et al., 1984). Thus the alkenyl ether bond effectively stabilizes the hexagonal II phase (Boggs et al., 1980; Lohner et al., 1984). Since increased steric bulk in the hydrophobic portion of a phospholipid molecule increases its propensity for adopting a hexagonal II phase (Cullis et al., 1979), the predominance of tetraenoic side chains at the *sn*-2 carbon in ethanolamine plasmalogens likely results in further stabilization of the hexagonal II phase.

Although dioleoylphosphatidylethanolamine has been utilized as the prototype of a lipid that assumes a hexagonal phase at physiologic temperatures and optimizes Ca^{2+} translocation with reconstituted Ca^{2+}-ATPase (Navarro et al., 1984), it is important to recognize that no diacylethanolamine glycerophospholipid molecular species with unsaturated fatty acyl chains at the *sn*-1 carbon could be demonstrated in either skeletal muscle or cardiac sarcoplasmic reticulum. Furthermore, since the lamellar to hexagonal phase transition temperature of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine is 69 °C (Lohner et al., 1984), it is unlikely that diacylethanolamine phospholipids are responsible for the observed lamellar to hexagonal II transition temperature of ethanolamine glycerophospholipids from skeletal muscle sarcoplasmic reticulum which occurs at −10 °C (Cullis et al., 1982). Finally, only small amounts of diacylethanolamine glycerophospholipids (5%) are present in skeletal muscle or cardiac sarcoplasmic reticulum. Taken together, these results suggest that the nonbilayer components previously observed in sarcoplasmic reticulum vesicles by ^{31}P NMR spectroscopy (Cullis & de Kruijff, 1979) likely arise from tetraenoic ethanolamine plasmalogens. The conservation of tetraenoic ethanolamine plasmalogens in both skeletal and cardiac muscle sarcoplasmic reticulum suggests a role for these moieties in calcium translocation, possibly due to their propensity for adopting a hexagonal II phase, but their ultimate physiologic importance in sarcoplasmic reticulum function and polymorphic phase behavior requires further investigation.

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