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High Plasmalogen and Arachidonic Acid Content of Canine Myocardial Sarcolemma: A Fast Atom Bombardment Mass Spectroscopic and Gas Chromatography–Mass Spectroscopic Characterization†

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ABSTRACT: Canine myocardial sarcolemma was purified, and its phospholipid constituents were determined by gas chromatography–mass spectrometry, fast atom bombardment mass spectrometry, and conventional techniques. Canine myocardial sarcolemma contained 2.7 μmol of lipid P_i/mg of protein which was comprised predominantly of choline glycerophospholipids (47%), ethanolamine glycerophospholipids (28%), and sphingomyelin (11%). Sarcolemmal phospholipids contained 40% plasmalogen which was quantitatively accounted for by choline (57% of choline glycerophospholipid) and ethanolamine (64% of ethanolamine glycerophospholipid) plasmalogens. Choline plasmalogens contained predominantly the vinyl ether of palmitic aldehyde though ethanolamine plasmalogens were composed predominantly of the vinyl ethers of stearic and oleic aldehydes. The majority of sarcolemmal ethanolamine glycerophospholipids (75%) contained arachidonic acid esterified to the *sn*-2 carbon. Sphingomyelin was composed predominantly of long-chain saturated fatty acids (stearic and arachidic) as well as substantial amounts (8%) of odd chain length saturated fatty acids. The possible functional role of these unusual phospholipid constituents is discussed.

Although it has been over 50 years since Feulgen's initial description of plasmalogens in biological tissues (Feulgen et al., 1924), their functional role has not been elucidated. The vinyl ether content of electrically active tissues such as brain (Scott et al., 1967; Wuthier, 1966; Freysz et al., 1968), peripheral nerve (Sheltawy & Dawson, 1966), or myocardium (Scott et al., 1967; Owens, 1966; Dawson et al., 1962) is 15–35% while other tissues such as liver (Scott et al., 1967; Dawson et al., 1962) and kidney (Scott et al., 1967) have a low content of plasmalogens (2–10%). Plasmalogens are distinguished from conventional diacyl phospholipids by the lack of an oxygen atom and the presence of two sp^2 carbons at the *sn*-1 position, which alter the molecular geometry and dynamics near the hydrophobic–hydrophilic interface (Paltauf, 1983). Furthermore, the *sn*-2 hydroxyl of plasmalogens is usually esterified to highly unsaturated fatty acids which would also contribute to altered molecular dynamics of plasmalogens in comparison with diacyl phospholipids in biologic membranes.

To identify the major phospholipid constituents of the electrically excitable membrane of myocardium, highly purified preparations of canine myocardial sarcolemma were analyzed with fast atom bombardment mass spectrometry, gas chromatography–mass spectrometry (GC–MS),[1] and conventional

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techniques. The present study demonstrates that the two major phospholipid classes of canine sarcolemma, choline and ethanolamine glycerophospholipids, contain 57 and 64% plasmalogen, respectively, that the *sn*-2 fatty acid of sarcolemmal ethanolamine glycerophospholipids is composed predominantly of arachidonic acid, and that sarcolemmal sphingomyelin contains substantial amounts of odd chain length fatty acids.

Materials and Methods

Preparation of Canine Sarcolemma. The sarcolemmal preparation utilized in this study was obtained with a modification of the method of Jones (Jones et al., 1980). Mongrel dogs fed ad libitum were anesthetized with sodium pentothal (40 mg/kg) and their hearts removed after a left thoracotomy. Atria, great vessels, and epicardial fat were removed, and ventricular muscle was minced into thin pieces (2 mm × 5 mm strips). Each of six 30-g aliquots of minced ventricular myocardium was placed into 100 mL of homogenization buffer [750 mM NaCl and 10 mM histidine, pH 7.5 (adjusted with 6 N HCl)] and homogenized at a setting of 6.5 for 5 s with a Polytron apparatus. The homogenates were centrifuged at $16000g_{max}$ for 20 min, and the supernatant was discarded. The pellets were resuspended in six 100-mL aliquots of the same buffer, Polytron homogenized, and centrifuged as before. The supernatant was discarded, the pellets were resuspended in six 100-mL aliquots of buffer [10 mM $NaHCO_3$ and 5 mM histidine (pH 7.5 adjusted with 6 N HCl)], homogenized with a Polytron apparatus at half-maximal speed for 5 s, and centrifuged at $16000g_{max}$ for 20 min. The supernatant was decanted, and the pellets were resuspended in buffer (10 mM $NaHCO_3$ and 5 mM histidine, pH 7.5). Sarcolemmal vesicles were formed by homogenizing the resuspended pellet at a setting of 6.5 with a Polytron apparatus for 3–30-s bursts each followed by a 3-min period for cooling. The homogenate was centrifuged at $16000g_{max}$ for 20 min. The supernatant fraction was removed and further centrifuged at $60000g_{max}$ for 40 min. The sarcolemmal vesicles were pelleted by this centrifugation, and the supernatant fraction was decanted and discarded. The sarcolemmal pellet was resuspended in 0.25 M sucrose and 10 mM histidine, pH 7.5, and centrifuged at $16000g_{max}$ for 15 min. The supernatant fraction from this centrifugation was layered over 0.55 M sucrose, 300 mM NaCl, 100 mM Tris-HCl, 50 mM sodium pyrophosphate, pH 7.1 and subsequently centrifuged at $170000g_{max}$ for 60 min. The sarcolemma formed a white flocctuant band at the 0.25–0.55 M sucrose interface and was aspirated with a Pasteur pipet. The sarcolemma was diluted into 4 volumes of ice-cold distilled water and centrifuged at $170000g_{max}$ for 30 min, and the pellet was resuspended in 0.25 M sucrose and 10 mM histidine buffer.

Phospholipid Extraction, Separation, and Analysis. Sarcolemmal phospholipids were extracted by the method of Bligh & Dyer (1959). The chloroform extract was filtered through 3-μm FALP Whatman filters and evaporated to dryness under N_2. All glassware was wrapped in aluminum foil to avoid exposure of the contents to light, and phospholipids were kept under N_2 at all times. Phospholipids were dissolved in 100 μL of 2/1 chloroform/methanol, and serial 15-μL injections were made onto a Waters Associates HPLC apparatus equipped with a U6K injector, a 6000A pump, and a variable wavelength UV detector (detection at 203 nm). Sarcolemmal phospholipids were separated with a Partisil SCX 10/25 microparticulate cation-exchange column (Whatman) as the stationary phase and a mobile phase of acetonitrile/methanol/H_2O (400/100/25) by isocratic elution (Gross & Sobel, 1980). Column eluates corresponding to ethanolamine and choline glycerophospholipids as well as sphingomyelin were collected, evaporated with N_2, and subsequently utilized for analysis. Choline glycerophospholipids and sphingomyelin were pure (verified by 2D TLC), but ethanolamine glycerophospholipids were contaminated by small amounts (<10%) of phosphatidylserine (Gross & Sobel, 1980) and therefore were further purified by thin-layer chromatography on silica OF TLC plates (Analabs, Foxboro, MA), with a solvent comprising chloroform/methanol/ammonium hydroxide (65/35/5). The band corresponding to phosphatidylethanolamine (R_f = 0.7) was scraped into a test tube, and ethanolamine glycerophospholipids were eluted by three 2-mL washes with 1/1 chloroform/methanol, filtered through Whatman No. 1 paper, and evaporated to dryness. Phosphatidylinositol, phosphatidylserine, and diphosphatidylglycerol were separated by conventional 2D TLC by the method of Rouser et al. (1970). Phospholipid phosphorus was determined by a micro phosphate assay previously described (Chen et al., 1956). Vinyl ether content was assayed by the I_2 addition method of Gottfried & Rapport (1962) with the following modification. A 30-min incubation time was utilized, and due to the low solubility of the ethanolamine glycerophospholipids in methanol/H_2O, the mixture was sonified every 5 min for 30 s in a Branson water bath sonicator.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Densitometry. Ten percent polyacrylamide gels were prepared according to the method of Laemmli (1970). Fifty micrograms of sarcolemmal protein was heated in 100 mM 2-mercaptoethanol (Bio-Rad, electrophoresis grade) and 20% sodium dodecyl sulfate (Bio-Rad, electrophoresis grade) for 5 min at 90 °C. The protein was loaded onto the stacking gel and electrophoresed for 2 h at 80 V and then for 8 h at 120 V. The gel was stained with Coomassie Blue (Laemmli, 1970), and scanning densitometry was performed with a Hoeffer (San Francisco, CA) scanning densitometer.

Marker Enzyme Analysis. Patent and latent (Na–K)ATPase activity was measured as described (Jones et al., 1980) in the absence or presence of 1 mg of alamethicin/mg of sarcolemmal protein (alamethicin was a gift of Dr. Grady and Upjohn Co., Kalamazoo, MI) in a medium containing 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, and 2.5 mM Tris–ATP in the absence or presence of 1 mM ouabain. The reaction was terminated with 8% silicotungstic acid, and inorganic phosphate was quantified by a colorimetric procedure previously described (Beller et al., 1976). Oligomycin-inhibitable ATPase activity was determined in a medium containing 5 mM $MgCl_2$, 1 mM EGTA, and 2.5 mM Tris–ATP in the presence or absence of 10 μM oligomycin. NaN_3-inhibitable ATPase activity was determined in a medium of 5 mM $MgCl_2$, 1 mM EGTA, and 2.5 mM Tris–ATP in the presence or absence of 5 mM NaN_3. Calcium-dependent ATPase activity was determined by the method of Jones (1979). Citrate synthetase was assayed by the method of Shepherd & Garland (1969). Lysophospholipase was assayed by the method of Gross (1983). Protein was determined by the Bio-Rad assay with bovine serum albumin as the standard.

[1] Abbreviations: CGP, choline glycerophospholipid; CK, creatine kinase; DMA, dimethyl acetal; EGP, ethanolamine glycerophospholipid; FAME, fatty acid methyl ester; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; SL, sarcolemma; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Methanolysis and Quantitative Gas Chromatographic Analysis. Purified sarcolemmal ethanolamine or choline glycerophospholipids (approximately 500 μg) were dissolved in 1 mL of 1 N anhydrous HCl/MeOH in a screw-top tube flushed with N_2 and heated at 90 °C for 90 min. The tubes were cooled in ice, and an excess of anhydrous sodium carbonate was added (pH checked with pH paper) followed by 1 volume of petroleum ether and 1 volume of H_2O. The mixture was vigorously vortexed, the layers were clarified by centrifugation, and the organic phase was placed into a test tube. The aqueous phase was reextracted twice more with 1 volume of petroleum ether. The combined petroleum ether extracts were evaporated to dryness with N_2 and utilized for gas chromatography within 24 h. Fatty acids in sphingomyelin were quantitatively methylated by heating approximately 500 μg of sphingomyelin with 1 mL of 12% BF_3/MeOH for 90 min at 90 °C (Morrison & Smith, 1964). A Hewlett-Packard Model 5790 gas chromatograph equipped with a Model 3390a integrator was utilized. A 10-ft glass column (2 mm i.d.) packed with 10% SP 2330-100/120 Chromosorb WAW (Supelco, Bellefonte, PA) was utilized with nitrogen carrier gas at a flow rate of 20 cm^3/min. Initial temperature (170 °C) was maintained for 7 min followed by a linear temperature program from 170 to 220 °C (4 °C/min). The integrated response of the flame ionization detector was calibrated with a mixture of standards (Supelco). The detector response factor (flame ionization detector) for fatty acid methyl esters and dimethyl acetals of selected chain lengths (C_{16-22}) was proportional to their molecular weights within experimental error.

Phospholipase A_2 Treatment of Sarcolemmal Phospholipids. Ethanolamine or choline glycerophospholipids (0.5–1 mg) purified as detailed above were incubated with 0.5 mg of snake venom phospholipase (*Naja naja*, Sigma, St. Louis, MO) for 90 min at 22 °C in 0.35 M sucrose, 20 mM Tris-HCl, and 10 mM $CaCl_2$ buffer, pH 7.2. Phosphoglycerides were suspended by sonification (initially for 1 min and subsequently for 30 s every 15 min) with a Branson water bath sonicator. Reaction products were extracted into chloroform (Bligh & Dyer, 1959), evaporated to dryness, and resuspended in 40 μL of 2/1 chloroform/methanol. Fatty acid, phosphoglycerides, and lysophosphoglycerides were separated by isocratic HPLC as previously described (Gross & Sobel, 1980). Fatty acids were esterified by treatment with 12% BF_3/MeOH (Morrison & Smith, 1964). The ester or vinyl ether linkage of the lysophosphoglyceride was derivitized by acid-catalyzed methanolysis as described above.

Determination of Alkyl Ether Content of Sarcolemmal Phospholipids. Approximately 1 mg of either choline or ethanolamine glycerophospholipid was dissolved in 1 mL of 0.5 N NaOH/MeOH and incubated at 22 °C for 60 min. The mixture was acidified with 6 N HCl to pH 1, incubated for 30 min at 22 °C and extracted into $CHCl_3$ (Bligh & Dyer, 1959), and the reaction products were separated by HPLC (Gross & Sobel, 1980). Column eluates corresponding to lysophosphoglycerides were evaporated, and phosphate was quantified (Chen, 1956).

Gas Chromatography–Mass Spectrometry. One- to three-microliter injections of the methanolysates were made onto a 4-ft glass column (2 mm i.d.) packed with 10% SP 2330 100/120 Chromosorb WAW with He as the carrier gas (25 cm^3/min). Initial temperature (150 °C) was maintained for 10 min. A linear temperature program from 150 to 170 °C (2 °C/min) followed by programming from 170 to 250 °C (4 °C/min) was utilized. Chemical ionization mass spectrometry with methane as reagent gas was performed with a Finnigan Model 3300 instrument.

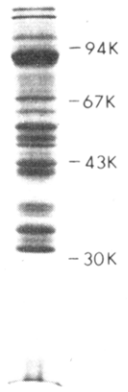

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of canine sarcolemma. Purified sarcolemmal protein (50 μg) was heated at 90 °C for 5 min with 100 mM 2-mercaptoethanol and 20% sodium dodecyl sulfate and electrophoresed for 8 h at 120 V on a 10% polyacrylamide gel. Proteins were visualized by staining with Coomassie Blue.

Fast Atom Bombardment Mass Spectrometry. Purified phospholipids (0.5–1 mg) were dissolved in 10 μL of 1/1 chloroform/methanol, and 2 μL was mixed with 3 μL of glycerol on a copper probe. Desorption and ionization from the glycerol matrix were accomplished with xenon fast atom bombardment from a Capillaritron ion gun (Phrasor Scientific Inc., Duarte, CA) mounted on a Finnigan 3300 mass spectrometer. The gun was operated at 10 kV and 20 μA with xenon. Spectra were obtained with the Washington University Mass Spectrometry Resource data system with a 90-ms dwell time at each m/z value in the region of interest. Pilot experiments with dipalmitoylphosphatidylethanolamine demonstrated that protonated molecular ions were distributed over a cluster of 5 mass units. To correct for background, stable isotope, and other contributions of neighboring species in the adjacent protonated molecular ion species, simultaneous equations were solved.

Results

Purity of the Sarcolemmal Preparations. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified canine sarcolemma demonstrated a predominant band at 93 kdalton with moderately intense bands present at 70, 55, 42, 40, 34, and 31 kdalton (Figure 1). The transsarcolemmal enzyme, (Na–K)ATPase, was utilized as a sarcolemmal marker and had a specific activity of 141 ± 13 μmol of P_i mg^{-1} h^{-1} ($\bar{X}$ ± SE) in the presence of 1 mg of alamethicin/mg of sarcolemmal protein (93-fold enrichment from homogenate). Basal activity of (Na–K)ATPase (33 ± 5 μmol of P_i mg^{-1} h^{-1}) was increased 4.3-fold by the addition of 1 mg of alamethicin/mg of sarcolemmal protein, demonstrating that approximately 75% of the vesicles were sealed and oriented in right-side-out configuration (Jones et al., 1980). Contamination by mitochondrial components was small as estimated by citrate synthetase activity (7% contamination), NaN_3-inhibitable ATPase activity (17% contamination), oligomycin-inhibitable ATPase activity (21% contamination), and diphosphatidylglycerol content (4% contamination). Purified sarcolemma preparations were minimally contaminated by sarcoplasmic reticulum as ascertained by calcium-dependent ATPase activity (3% contamination). The cytosolic markers CK and LDH were not detected in the purified sarcolemma preparation. The protein electrophoretic pattern, the specific activity of (Na–K)ATPase, the relative enrichment of sphingomyelin (see below), and the absence of substantial contamination by mitochondrial or

Table I: Phospholipid Composition of Canine Sarcolemma[a]

	nmol/mg of protein	%
choline glycerophospholipids	1255 ± 76	47
diacyl	516[b]	
plasmalogen	715[b]	
alkyl ether	24[c]	
ethanolamine glycerophospholipids	740 ± 37	28
diacyl	263[b]	
plasmalogen	474[b]	
alkyl ether	3[c]	
sphingomyelin	290 ± 12	11
phosphatidylinositol	167 ± 3	6
phosphatidylserine	130 ± 12	5
diphosphatidylglycerol	15 ± 5	0.6
lysophosphatidylcholine	24 ± 2	0.9
lysophosphatidylethanolamine	45 ± 2	1.7

[a] Phospholipids were separated by HPLC or 2D TLC and quantitated by PO_4 analysis as described under Materials and Methods. Results are reported as the $\bar{X}$ ± SE. [b] Diacyl phospholipids or plasmalogens were quantified by the difference between I_2 addition and PO_4 found in the choline or ethanolamine glycerophospholipid fraction after correction for the amount of alkyl ether phospholipid present. [c] Alkyl ether composition was determined by quantification of lysophosphoglyceride remaining after both acid- and base-catalyzed methanolysis.

sarcoplasmic reticulum markers demonstrate that these preparations were of a purity comparable with the best preparations thus far reported in the literature (Caroni & Carafoli, 1981; Jones et al., 1980).

Phospholipid Composition of Canine Sarcolemma. The predominant phospholipid classes present in canine sarcolemma were choline glycerophospholipids (47%) and ethanolamine glycerophospholipids (28%) accounting for 75% of total lipid extractable phosphate present in this preparation (Table I). A 4-fold enrichment of sphingomyelin from homogenate was present. Sarcolemma contained small amounts of phosphatidylinositol (6%) and phosphatidylserine (5%). Lysophosphatidylcholine and lysophosphatidylethanolamine were present in only minor amounts (Table I). Diphosphatidylglycerol, a phospholipid marker for mitochondrial inner membranes (Parsons et al., 1967), comprised only 0.6% of lipid-extractable phosphate. Since phospholipids containing choline and ethanolamine bases accounted for the majority (86%) of sarcolemmal phospholipids, the molecular constituents of these phospholipid classes were characterized.

Plasmalogen and Alkyl Ether Content of Canine Sarcolemma. Sarcolemmal phospholipids contained 40 ± 2% ($\bar{X}$ ± SE) vinyl ether linkages as ascertained by I_2 addition to activated olefins. Choline and ethanolamine glycerophospholipids contained the overwhelming majority of plasmalogens present in the sarcolemmal preparation (57 ± 2 and 64 ± 4% by I_2 addition, respectively) and quantitatively accounted for the vinyl ether content of canine sarcolemma. Vinyl ether linkages were not detected in sphingomyelin, phosphatidylinositol, or phosphatidylserine. The high plasmalogen content of choline and ethanolamine glycerophospholipids in canine sarcolemma was confirmed by three other independent methods (see below).

Alkaline methanolysis of choline and ethanolamine glycerophospholipids and subsequent acid-catalyzed methanolysis, lipid extraction, separation of lysolipids by HPLC, and quantitation by phosphate analysis demonstrated that sarcolemma contained only minor amounts of alkyl ether phospholipids (Table I).

Fatty Acid and Masked Aldehyde (Vinyl Ether) Composition of Canine Sarcolemmal Phospholipids. Acid-catalyzed

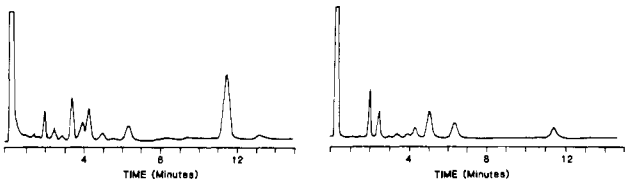

FIGURE 2: Gas chromatography of sarcolemmal ethanolamine and choline glycerophospholipids. Sarcolemmal ethanolamine (left panel) or choline (right panel) glycerophospholipids were subjected to acid-catalyzed methanolysis and analyzed by gas chromatography and mass spectrometry as described under Materials and Methods. Peaks corresponding to the 16:0 DMA (2.0 min), 16:0 FAME (2.5 min), 16:1 FAME (2.9 min), 18:0 DMA (3.4 min), 18:1 DMA (3.9), 18:0 FAME (4.3 min), 18:1 FAME (5.1 min), 18:2 FAME (6.3 min), 20:4 FAME (11.4 min), and 22:4 FAME (13.2 min) were identified by both comparisons with standards and mass spectrometry (DMA = dimethyl acetal; FAME = fatty acid methyl ester).

methanolysis of phospholipids separated by HPLC (and in the case of ethanolamine glycerophospholipids further purified by TLC) confirmed the high plasmalogen content of choline and ethanolamine glycerophospholipids. Sarcolemmal ethanolamine glycerophospholipid methanolysates contained the dimethyl acetal of oleic and stearic aldehydes and smaller amounts of the dimethyl acetal of palmitic aldehyde (Figure 2). Identification of peaks occurring at 2.0, 3.4, and 3.9 min as the dimethyl acetals of palmitic, stearic, and oleic aldehydes was accomplished by comparison of retention times with standards (obtained from acid-catalyzed methanolysis of lysophosphatidylcholine plasmalogen) and by characteristic mass spectra. Methane chemical ionization gas chromatography–mass spectrometry of these dimethyl acetals produced $(M - H)^+$ ions and high intensity fragment ions resulting from the facile additional loss of formaldehyde or methanol. The gas chromatographic coelution of these ion peaks (m/z 285, 255, and 253 for the dimethyl acetal of 16:0; m/z 313, 283, and 281 for the dimethyl acetal of 18:0; m/z 311, 281, and 279 for the dimethyl acetal of 18:1) in similar ratios further supported these assignments. No evidence for the dimethyl acetals of 16:1, 18:2, 20:0, or 20:4 fatty aldehydes in sarcolemma glycerophospholipids was obtained by gas chromatography–mass spectrometry, but the possible presence of small amounts of these components (<3%) cannot be excluded.

Methanolysis of sarcolemmal choline glycerophospholipids and subsequent gas chromatography demonstrated large amounts of palmitic aldehyde and smaller amounts of stearic and oleic aldehydes (Figure 2). These assignments were based as before on comparisons with standards and the cochromatography in similar ratios of major mass ions (described above).

Identification of fatty acid methyl esters was performed by both comparison of retention times with standards and identification of both the $(M - H)^+$ and $(MH)^+$ ions by gas chromatography–mass spectrometry. Sarcolemmal choline glycerophospholipid methanolysates contained 16:0, 18:0, 18:1, 18:2, and 20:4 fatty acid methyl esters (Table II). Trace amounts of 16:1 and 20:3 fatty acid methyl esters were also identified. In contrast, sarcolemmal ethanolamine glycerophospholipid methyl esters were composed predominantly of 20:4 fatty acid methyl esters. Small amounts of 16:0, 18:0, 18:1, 18:2, and 22:4 and trace amounts of 16:1 and 20:3 fatty acid methyl esters were identified by both comparisons with standards and by mass spectrometry.

Sphingomyelin isolated from canine sarcolemma exhibited an unusual fatty acid profile characterized by a predominance of saturated long-chain fatty acid methyl esters (16:0, 18:0, 20:0, and 22:0) (Table V). In addition, sarcolemmal sphingomyelin contained components with mass spectra and re-

Table II: Fatty Acid and Aldehyde Profiles of Native and Phospholipase-Treated Phospholipids of Canine Sarcolemma[a]

fraction	16:0(D)	16:0	16:1	18:0(D)	18:0	18:1(D)	18:1	18:2	20:4	22:4
SL	11	11	tr.	6	15	2	18	16	21	2
CGP	20	13	tr.	2	9	3	25	16	12	0
PLA_2-CGP-FA	0	6	tr.	0	1	0	42	30	21	0
PLA_2-CGP-LPC	48	21	2	4	17	3	3	1	0	0
EGP	6	3	1	14	12	9	2	9	42	2
PLA_2-EGP-FA	0	2	tr.	0	2	0	5	13	75	3
PLA_2-EGP-LPE	13	4	tr.	35	26	15	4	0	1	0

[a] Sarcolemma (SL), sarcolemmal choline (CGP), or ethanolamine (EGP) glycerophospholipids were subjected to acid-catalyzed methanolysis and analyzed by gas chromatography and gas chromatography–mass spectrometry as described under Materials and Methods. Products of phospholipase-treated choline or ethanolamine glycerophospholipids were separated by HPLC and derivitized as described under Materials and Methods. Results are expressed in weight percent. PLA_2-XGP-FA and PLA_2-XGP-LPC are the fatty acids (or aldehydes) and lysophosphoglycerides identified after phospholipase treatment, purification, and derivitization. (D) refers to the dimethyl acetal of the given aldehyde.

tention times consistent with saturated odd chain length fatty acid methyl esters (17:0, 19:0, 21:0, and 23:0) which were corroborated by fast atom bombardment mass spectrometry (see below). The possibility that molecular ions with masses identical with those of odd chain length fatty acid methyl esters were generated by hydroxylated even chain length fatty acid methyl esters with one unit of unsaturation was excluded by treatment of the methyl esters with *N,O*-bis(trimethylsilyl)-trifluoroacetamide/trimethylchlorosilane/pyridine (9/1/10 v/v/v) for 15 h and subsequent gas chromatography–mass spectrometry. Treated and untreated methyl esters from sphingomyelin displayed identical elution times and ion composition, demonstrating that saturated odd chain length fatty acid methyl esters were the source of these parent ions.

Positional Specificity of Choline and Ethanolamine Fatty Acids and Masked Aldehydes. To determine the positional specificity of fatty acids and aldehydes in choline and ethanolamine glycerophospholipids, the regiospecific cleavage by phospholipase A_2 (*Naja naja*) was utilized. Free fatty acids released from sarcolemmal ethanolamine glycerophospholipids by treatment with phospholipase A_2 were extracted, separated by HPLC, and esterified with BF_3/MeOH. Gas chromatography demonstrated that these native *sn*-2 fatty acids were composed predominantly of arachidonic acid with small amounts of oleic, linoleic, palmitic, and stearic acids also present (Table II). No dimethyl acetals were identified. Due to the small but unexpected amounts of saturated fatty acids released during these incubations, additional experiments were performed to exclude the possibility that *Naja naja* phospholipase A_2 contained lysophospholipase activity. Incubation of 1-[1-^{14}C]palmitoyllysophosphatidylcholine with phospholipase A_2 resulted in the liberation of [^{14}C]palmitic acid at a rate of 2% that of phospholipase activity under the reaction conditions employed. Thus, at least some of the saturated fatty acids released during the course of these incubations resulted from hydrolysis of lysophosphoglycerides. Phospholipase treatment of ethanolamine glycerophospholipids, extraction, separation of ethanolamine lysophosphoglycerides by HPLC, and subsequent acid-catalyzed methanolysis demonstrated a predominance of dimethyl acetals including the dimethyl acetals of stearic aldehyde, palmitic aldehyde, oleic aldehyde, and the fatty acid methyl esters of stearic and palmitic acid (Table II). Taken together, these results demonstrate that sarcolemmal ethanolamine glycerophospholipids are composed predominantly of vinyl ether linkages at the *sn*-1 carbon and of arachidonic acid at the *sn*-2 carbon.

Sarcolemmal choline glycerophospholipid hydrolysis by *Naja naja* phospholipase A_2 resulted in the liberation of predominantly unsaturated fatty acids which after purification by HPLC, evaporation, and esterification with BF_3/MeOH

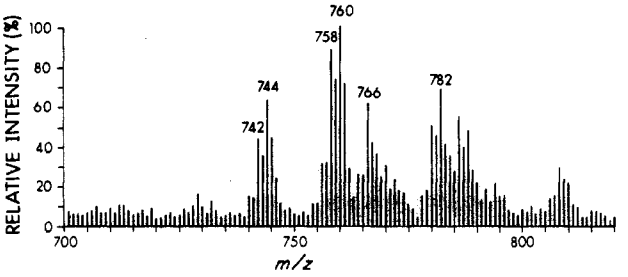

FIGURE 3: Fast atom bombardment mass spectrometry of sarcolemmal choline glycerophospholipids. Purified choline glycerophospholipids (750 μg) were dissolved in 10 μL of 1/1 $CHCl_3$/MeOH, and 2 μL was mixed with 3 μL of glycerol on a copper probe. Fast atom bombardment mass spectra were obtained as described under Materials and Methods.

were identified as the methyl esters of linoleic, oleic, arachidonic, stearic, and palmitic acid (Table II). Choline lysophosphoglycerides generated from phospholipase treatment of choline glycerophospholipids were purified by HPLC and subjected to acid-catalyzed methanolysis. Large amounts of the dimethyl acetal of palmitic aldehyde and the fatty acid methyl ester of palmitic and stearic acids were identified (Table II). Thus, the majority of sarcolemmal choline glycerophospholipids contain vinyl ether linkages at the *sn*-1 carbon and unsaturated fatty acids at the *sn*-2 carbon. These results demonstrate that choline and ethanolamine glycerophospholipids contain a high percentage of plasmalogens (similar to that determined with the I_2 addition assay) and that the *sn*-2 carbon of sarcolemmal phosphatides contains almost exclusively unsaturated fatty acids.

Fast Atom Bombardment Mass Spectrometry of Canine Sarcolemmal Glycerophospholipids. Sarcolemmal choline glycerophospholipids consisted predominantly of species with protonated parent ion $(MH)^+$ molecular masses of *m/z* 760 and 758 (Figure 3). In addition, other major species identified possessed protonated parent ion masses $(MH)^+$ of *m/z* 744, 766, and 782. Smaller amounts of parent ions with masses of *m/z* 742, 780, 786, 788, 792, 794, and 808 were easily identified. The major protonated molecular ions were assigned based on GC profiles of phospholipase-treated phosphoglycerides and the protonated molecular masses obtained by fast atom bombardment mass spectrometry (Table III). The high plasmalogen content of sarcolemmal choline glycerophospholipid is supported by the finding of parent ion peaks at *m/z* 742, 744, 766, 770, 772, 792, and 794 and the predominant fragment ion peak at *m/z* 480 mass units which resulted mainly from the loss of the 18:2 ketene from the 16:0–18:2 plasmalogen, the loss of the 18:1 ketene from the 16:0–18:1 plasmalogen, and the loss of the 20:4 ketene from

Table III: Sarcolemmal Choline Glycerophospholipid Composition Ascertained by Fast Atom Bombardment Mass Spectrometry and Phospholipase Treatment with Subsequent Gas Chromatography[a]

$(MH)^+$	FABMS (%)	assignment (*sn*-1-*sn*-2)	corrected (%)
m/z 742	5	16:0-18:2 (plasmalogen)	8
m/z 744	9	16:0-18:1 (plasmalogen)	15
m/z 758	11	16:0-18:2	7
m/z 760	16	16:0-18:1	11
m/z 766	10	16:0-20:4 (plasmalogen)	16
m/z 770	4	18:1-18:1 (plasmalogen)	6
m/z 772	4	18:0-18:1 (plasmalogen)	6
m/z 780	5	16:1-20:4	3
m/z 782	10	16:0-20:4	7
m/z 786	9	18:0-18:2	7
m/z 788	7	18:0-18:1	6
m/z 792	2	18:1-20:4 (plasmalogen)	3
m/z 794	3	18:0-20:4 (plasmalogen)	4
m/z 808	4	18:1-20:4	2

[a] Purified sarcolemmal choline glycerophospholipids were analyzed by fast atom bombardment mass spectrometry (FABMS), and ion peaks were quantified by solving simultaneous equations. Values on the right, "corrected (%)", refer to normalization for differences in ketene loss from the plasmalogen compared to the diacyl phospholipids as described under Results.

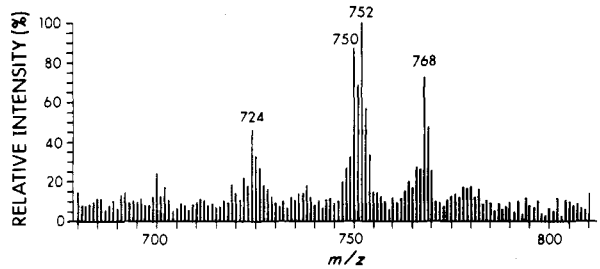

FIGURE 4: Fast atom bombardment mass spectrometry of sarcolemmal ethanolamine glycerophospholipids. Purified ethanolamine glycerophospholipids (500 μg) were dissolved in 10 μL of 1/1 $CHCl_3$/MeOH, and 2 μL was mixed with 3 μL of glycerol on a copper probe. Fast atom bombardment mass spectra were obtained as described under Materials and Methods.

the 16:0–20:4 plasmalogen. Since the relative intensity of an ion is dependent on its kinetics of formation and decomposition, the relative intensities of parent and fragment ions do not necessarily reflect molar ratios of phospholipid constituents present in a given sample. In particular, the fragment ion peak at *m/z* 480 mass units was 1.7 times as large as the fragment ion peak at *m/z* 496 mass units even though parent ion peaks corresponding to 16:0 *sn*-1 fatty esters were of higher intensity than the *sn*-1 16:0 vinyl ethers. Thus, the underrepresentation (37% vs. 57%) of plasmalogens by fast atom bombardment mass spectrometry is not unexpected. An attempt to partially correct for differences in fragmentation rates based on the relative intensities of fragment ion peaks (lysoplasmalogen/lysophosphatidylcholine) has been made (Table III). Results after this correction are in close agreement with the three quantitative techniques utilized in this study.

Fast atom bombardment mass spectrometry of sarcolemmal ethanolamine glycerophospholipids (Figure 4) demonstrated major ion peaks with protonated molecular ions at *m/z* 752 and 750 and smaller intensity ions at *m/z* 724 and 768. The assignments of these peaks were based upon GC–MS and profiles of phospholipase-treated sarcolemmal ethanolamine glycerophospholipids and are given in Table IV. Interestingly, ethanolamine in contrast to choline glycerophospholipids underwent a facile loss of ethanolamine phosphate particularly from the diacyl species, *m/z* (768 − 141 → 627). In agreement

Table IV: Sarcolemmal Ethanolamine Glycerophospholipid Composition Ascertained by Fast Atom Bombardment Mass Spectrometry and Phospholipase A_2 Treatment with Subsequent Gas Chromatography[a]

$(MH)^+$	FABMS (%)	assignment (*sn*-1-*sn*-2)
m/z 692	2	16:0-16:0
m/z 700	4	16:0-18:2 (plasmalogen)
m/z 702	2	16:0-18:1 (plasmalogen)
m/z 722	2	16:1-20:4 (plasmalogen)
m/z 724	13	16:0-20:4 (plasmalogen)
m/z 750	22	18:1-20:4 (plasmalogen)
m/z 752	27	18:0-20:4 (plasmalogen)
m/z 768	22	18:0-20:4
m/z 778	2	18:1-22:4 (plasmalogen)
m/z 780	2	18:0-22:4 (plasmalogen)

[a] Purified sarcolemmal ethanolamine glycerophospholipids were analyzed by fast atom bombardment mass spectrometry (FABMS), and ion peaks were quantified by solving simultaneous equations.

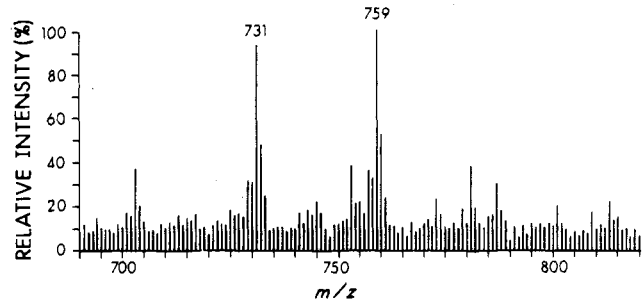

FIGURE 5: Fast atom bombardment mass spectrometry of sarcolemmal sphingomyelin. Purified sphingomyelin (300 μg) was dissolved in 4 μL of 2/1 $CHCl_3$/MeOH, and 2 μL was mixed with 3 μL of glycerol on a copper probe. Fast atom bombardment mass spectra were obtained as described under Materials and Methods.

with results obtained with choline glycerophospholipids the more facile loss of the 20:4 ketene from the ethanolamine plasmalogen compared to diacyl species was noted. These relative intensities have not been corrected because speculations would be necessary concerning the relative importance of two different fragmentation pathways in two separate species. The facile loss of phosphoethanolamine in diacyl but not plasmalogen ethanolamine glycerophospholipids may account for the overrepresentation of plasmalogens in parent ion peaks when compared with the other quantitative methods utilized in this study.

Fast atom bombardment mass spectrometry of sphingomyelin (Figure 5) demonstrated major peaks with molecular masses at *m/z* 731 and 759 and lower intensity ions at 703, 753, 757, 773, 781, and 787. The relative abundance of parent ion species in sarcolemmal sphingomyelin and fatty acid composition determined by both gas chromatography and mass spectrometry is shown in Table V. The parent ion peaks at 753, 781, and 809 corresponding to fatty acids with 3 units of unsaturation could not be corroborated by either gas chromatography or mass spectrometry and therefore most likely results from ionization of the $(MNa)^+$ species (Lehmann & Kessler, 1983). The low intensity of fragment ion peaks precluded corroboration of these assignments by analysis of fragmentation patterns.

Discussion

The results of the present study demonstrate the high plasmalogen content of sarcolemmal choline and ethanolamine glycerophospholipids. The high plasmalogen content of these phospholipid classes was documented by independent methods which gave similar results. Although myocardial homogenates

Table V: Sarcolemmal Sphingomyelin Composition Ascertained by Fast Atom Bombardment Mass Spectrometry and Gas Chromatography[a]

parent ion	FABMS (%)	assignment	GC (%)
m/z 701 $(MH)^+$	2	16:1	2
m/z 703 $(MH)^+$	8	16:0	12
m/z 717 $(MH)^+$	1	17:0	1
m/z 729 $(MH)^+$	5	18:1	5
m/z 731 $(MH)^+$	22	18:0	29
m/z 745 $(MH)^+$	3	19:0	2
m/z 753 $(MNa)^+$	7	18:0	*b*
m/z 757 $(MH)^+$	4	20:1	0
m/z 759 $(MH)^+$	22	20:0	28
m/z 773 $(MH)^+$	3	21:0	2
m/z 781 $(MNa)^+$	7	20:0	*b*
m/z 787 $(MH)^+$	5	22:0	5
m/z 801 $(MH)^+$	3	23:0	3
m/z 809 $(MNa)^+$	2	22:0	*b*
m/z 813 $(MH)^+$	4	24:1	6
m/z 815 $(MH)^+$	1	24:0	2

[a] Purified sarcolemmal sphingomyelin was subjected to fast atom bombardment mass spectrometry (FABMS), and ion peaks were quantitated by their relative intensities (data are expressed as a percent). Comparisons with gas chromatographic analysis (GC) were made (data are expressed as weight percent), and since no fatty acids corresponding to 20:3, 22:3, or 24:3 were found by GC, these ion peaks have been assigned to sodiated parent ions $(MNa)^+$. [b] Composition of the saturated fatty acid methyl ester was reported for the protonated parent ion.

have previously been demonstrated to contain 15–35% plasmalogens (Scott et al., 1967, Dawson et al., 1962), this investigation is the first to specifically identify plasmalogen as the major phospholipid constituent of sarcolemmal membranes.

Sarcolemmal ethanolamine glycerophospholipids are composed predominantly of moieties with vinyl ether linkages at the *sn*-1 carbon and esterified arachidonate at the *sn*-2 carbon. Myocytic metabolism of arachidonic acid through the cyclooxygenase pathway has been reported (Ahumada et al., 1980; Valhouny et al., 1979; Ellis et al., 1980; Bolton et al., 1980). Thus, sarcolemmal ethanolamine plasmalogens might represent a highly compartmentalized pool from which arachidonic acid could be released by plasmalogen-specific phospholipases with subsequent generation of bioactive metabolites via cyclooxygenase or lipoxygenase pathways. Recently, it has been demonstrated that inflammatory cells which invade infarcted regions of myocardium result in the production of prostaglandins (McCluskey et al., 1982). Thus, the interaction between inflammatory cells and myocytic sarcolemmal membranes (with which they have direct contact) may have physiological relevance. Alternatively, the role of arachidonate and the vinyl ether linkage could be related to a structural function in the sarcolemmal membrane. The presence of two sp^2 carbons at the *sn*-1 position and a tetraenoic fatty acyl side chain at the *sn*-2 position would likely result in markedly altered molecular dynamics in sarcolemmal membranes in comparison with conventional diacyl phosphatides.

Sarcolemmal choline plasmalogens differed from their ethanolamine counterparts with respect to the presence of oleic and linoleic acids esterified to the *sn*-2 carbon in contrast to the predominance of arachidonic acid at the *sn*-2 carbon of ethanolamine plasmalogen. The present study demonstrates a high degree of specificity in the fatty acid and masked aldehyde composition of phospholipid constituents in each major phospholipid class in canine sarcolemmal membranes. The results suggest that the synthesis and metabolism of these moieties is tightly regulated, presumably fulfilling specific functional requirements.

Sarcolemmal ethanolamine glycerophospholipids are composed predominantly of 20:4 fatty acid at the *sn*-2 carbon while choline glycerophospholipids were composed predominantly of 18:1 or 18:2 fatty acids at the *sn*-2 position. One factor contributing to these differences could be the greater degree of unsaturation present at the *sn*-2 side chain of ethanolamine phosphoglycerides which would result in a similar degree of penetration into the membrane interior of both phospholipid classes. The *sn*-1 fatty acyl side chain is usually of shorter chain length than the *sn*-2 acyl side chain. This could be attributed to the greater degree of unsaturation in the *sn*-2 compared to the *sn*-1 position and by the parallel course of the *sn*-2 side chain to the bilayer surface in its proximal portion (Pearson & Pasher, 1979).

The experiments utilizing phospholipase cleavage to determine the regiospecificity of the fatty acyl side chain demonstrated that the overwhelming majority of unsaturated fatty acids were present at the *sn*-2 position in both choline and ethanolamine sarcolemmal glycerophospholipids. In addition, it was demonstrated that commercially available *Naja naja* phospholipase exhibited lysophospholipase activity. Comparisons of the relative amounts of phospholipase to lysophospholipase activities suggest that some of the saturated fatty acids released by phospholipase treatment are the result of lysophospholipase activity. Since the 1-deacyl and 2-deacyl forms of lysophosphoglycerides are in equilibrium (van Deenen et al., 1963), lysophospholipase activity does not necessarily reflect hydrolysis at the *sn*-1 position but rather could reflect rates of isomerization of 1-acyllysophosphatidylcholine followed by regiospecific cleavage at the *sn*-2 carbon by phospholipase A_2.

The results demonstrate that substantial amounts of odd chain length fatty acids are present in sphingomyelin. The assignment of odd chain length fatty acids was made by mass spectrometry where both $(M - H)^+$ and $(MH)^+$ ion peaks were observed, a retention time by GC compatible with these assignments, the exclusion of hydroxy fatty acids in sarcolemmal sphingomyelin, and ion peaks corresponding to odd chain length fatty acid (17:0, 19:0, 21:0, and 23:0) in the fast atom bombardment mass spectra of sphingomyelin. No evidence for odd chain length fatty acids in other sarcolemmal phospholipid species examined has been obtained. Evidence supporting the presence of odd chain length fatty acids in sphingomyelin isolated from homogenates of embryonic chick heart has been presented previously (Wood, 1974).

The presence of sphingomyelin species corresponding to amides with 3 units of unsaturation (*m/z* 753, 781, and 809) in the mass spectra most likely results from sodiated sphingomyelins $(MNa)^+$ with saturated fatty acyl groups of 18-, 20-, and 22-carbon chain lengths, since fatty acid methyl esters with 3 units of unsaturation could not be identified by GC or GC–MS. Sodiated species of ethanolamine glycerophospholipids were not apparent (Figure 4). Sodiated species of choline glycerophospholipids were not prominent, evidenced by the relative ratio of peaks in the *m/z* cluster at 744 and 742 and the single peak at 766. The presence of small contributions to ion peaks at *m/z* 780 and 782 from sodiated species of choline glycerophospholipids cannot be excluded.

The high plasmalogen content and the presence of a highly specific fatty acid composition of each major phospholipid class present in canine sarcolemma suggest that sarcolemmal phospholipid composition is tightly regulated to fulfill specific functional roles. High vinyl ether content seems to be a ubiquitous characteristic of electrically active tissues and thus is likely involved in transmembrane ion movements either

indirectly by modulating the molecular dynamics of the membrane with subsequent effects on protein ion channels or directly by acting as an endogenous lipid ionophore. The identification of the high plasmalogen content of canine sarcolemma and the association of ethanolamine plasmalogens with arachidonic acid should facilitate the identification of the functional role of these moieties in sarcolemma and the mechanisms through which their metabolism is regulated.

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Registry No. Arachidonic acid, 506-32-1.

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