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Fatty acid profiles of plasmalogen choline and ethanolamine glycerophospholipids in pig and rat hearts ☆

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Abstract

The presence of relatively high concentrations of plasmalogen choline and ethanolamine in the heart of many animal species suggests a role of these ether-linked phospholipids in the pathophysiology of certain myocardial diseases. However, the fatty acid composition of myocardial plasmalogens in many species is not known. This study examined the fatty acid composition of the choline and ethanolamine glycerophospholipids in pig heart and compared the results with those in rat heart. Lipids were extracted from the heart biopsies obtained from pig and rat by standard techniques. Phosphoglycerides were separated by thin-layer chromatography followed by their derivatization into fatty acid methyl esters (FAMEs) and dimethyl acetals (DMAs). FAME and DMA samples were analyzed using gas chromatography–mass spectroscopy. Our results indicate striking differences in the fatty acid composition of both choline and ethanolamine glycerophosphates between rat heart and pig heart. Pig heart ethanolamine glycerophosphates are rich in linoleic acid (18:2) and arachidonic acid (20:4), but low in descosahexenoic (22:6) fatty acids while choline glycerophosphates are poor in both 20:4 and 22:6 fatty acids compared to those in rat hearts.

Keywords: Choline; Ethanolamine; Fatty acids; Glycerophospholipids; Pig heart; Plasmalogen; Rat heart

1. Introduction

Phospholipids are important constituents of the sarcolemma and sarcoplasmic reticulum of many electrically active tissues including heart. They are involved in the pathophysiology of cellular injury associated with several diseases of which ischemia reperfusion injury is probably the most important [1,2]. The reperfusion of ischemic myocardium potentiates the activation of several phospholipases [3,4], resulting in the loss of membrane phospholipids which is reflected in the accumulation of lysophosphoglycerides and free fatty acids, especially arachidonic acid [5]. Phospholipids are also susceptible to attack by free radicals which are generated during ischemia and reperfusion [6,7].

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In most biological tissues, including heart, two types of phospholipids are known to occur: diacyl and ether glycerolipids. In the latter group, the Sn-1 position is linked to the glycerol moiety by an ether linkage, whereas in the former group the linkage consists of an ester bond. When a Z-double bond occurs in the 1-alk-1-enyl moiety adjacent to the ether linkage the phosphatides are called plasmalogens (1-(1′-alkenyl)-2-acyl glycerophospholipids). Recently, these plasmalogens have been implicated in the pathogenesis of myocardial ischemia reperfusion injury [8,9].

In heart, high concentrations of choline and ethanolamine plasmalogens have been found to occur. For example, plasmalogens account for 57% and 64% of the total choline and ethanolamine phosphoglycerides, respectively, in the sarcolemmal membrane of dog heart [10]. In guinea pig heart, 39% of choline phosphoglycerides and 36% of ethanolamine phosphoglycerides in the mitochondria are present as plasmalogens [11]. Rat hearts comprise 1.8 mol% of plasmenylcholine and 20.4 mol% of plasmenylethanolamine [12]. Thus, it appears that the composition of the myocardial plasmalogens varies from one species to another. It also seems likely that the distribution and acyl composition of these plasmenyl glycerophospholipids should dictate the precise role of plasmalogens in ischemia reperfusion injury. However, the fatty acid composition of plasmalogens in the hearts of many species remains either unknown or only partially known. To fill this gap, we have determined the fatty acid profiles of choline and ethanolamine phosphoglycerides in two distinctly different species, rat and pig. Our data suggest that there are striking differences in the fatty acid composition of both choline and ethanolamine glycerophospholipids between pig and rat hearts.

2. Experimental

2.1. Preparation of heart biopsies

Sprague Dawley male rats of about 250 g body weight were anesthetized with intraperitoneal pentobarbital (8.5 mg per 100 g), the chests opened by thorasectomy, and blood flushed out of the hearts using Krebs Henseleit bicarbonate buffer. The hearts were quickly frozen in liquid nitrogen. The frozen tissues were stored at −70 °C until assayed for plasmalogens.

A pig about 20 kg in weight was placed on cardiopulmonary bypass, and a biopsy of about 0.5 g from the left ventricle was obtained using a pneumatic drill, and quickly frozen at liquid nitrogen temperature. The biopsies were stored at −70 °C until assayed.

2.2. Extraction, separation and derivatization of lipids

About 0.2 g of a biopsy from the left ventricle was homogenized in 20 volumes of ice-cold $CHCl_3$–CH_3OH (2:1. v/v) solution containing 0.005% butylated hydroxytoluene (BHT) using a Polytron homogenizer as described previously [13]. Diheptadecanoyl phosphatidylethanolamine and diarachidyl phosphatidylcholine were used as internal standards. Phospholipids were separated on silica gel K6 plates (Whatman, Clifton, NJ) using a mixture of chloroform–methanol–petroleum ether–acetic acid–boric acid (40:20:30:7:1.8, v/v/v/v/w) as a solvent system. The lipids on the silica gel plates were identified by cochromatography with authentic lipid standards after a brief exposure to iodine vapor, and the phosphoglycerides were scraped off into 0.5 ml of anhydrous CH_3OH containing 0.0005% BHT in a screwcap test tube. A 0.5 ml volume of 20% acetyl chloride in anhydrous CH_3OH was added, and the methanolyzate was neutralized with an excess of anhydrous Na_2CO_3. Fatty acid methyl esters (FAMEs) and dimethyl acetal (DMA) derivatives of phosphoglyceride acyl and alk-1-enyl groups were extracted by the addition of 2 ml of hexane and 0.6 ml of saturated NaCl aqueous solution. The derivatized products were extracted in hexane, evaporated to dryness under a stream of nitrogen, and reconstituted in 0.5 ml of hexane.

2.3. Gas chromatography (GC)

A 1 μl aliquot of the above hexane extract was injected into the injection port and run in a

splitless mode at 225 °C using a Hewlett Packard gas chromatograph model 5890A equipped with a model 7673A automatic injector and an SP 2330 polar phase capillary column (Supelco, Bellefonte, PA). The temperature program was as follows: 120 °C for 3 min, then increasing at 5 °C min^{-1} to 160 °C, then increasing at 10 °C min^{-1} to 250 °C. This temperature was maintained for a few minutes and then reduced to the initial temperature of 120 °C at 20 °C min^{-1}.

In order to confirm the DMA and FAME peaks, the remaining hexane extract, after the GC run, was spotted on an activated silica gel G thin-layer chromatography (TLC) plate and the chromatogram was developed with benzene. FAME ($R_f = 0.45$) and DMA ($R_f = 0.25$) were confirmed from the R_f values. The spots were scraped from the TLC plate, the derivatives were eluted from the silica gel with $CHCl_3$–CH_3OH (2:1, v/v), and were analyzed by GC under the same conditions as the original chromatography. The chromatograms were compared, and peaks identified as FAME or DMA.

2.4. *Gas chromatography–mass spectroscopy (GC–MS)*

The hexane extract was also subjected to GC–MS to confirm the identity of the derivatized peaks. A 1 µl aliquot of the hexane extract was injected into the injection port of a Hewlett-Packard model 5890A gas chromatograph with a polar phase Supelco SP-2330 (Bellefonte, PA) 30 m × 0.25 mm i.d. capillary column having a film thickness of 0.2 µm. The column was connected to a Finnigan MAT quadrupole mass spectrometer (Palo Alto, CA) via a heated transfer line. For data capture, the system was coupled to a Data General computer model DG 10 and a Printronix model MVP printer. The transfer line temperature was maintained at 250 °C while the head pressure on the inlet was maintained at 62 kPa (8 psi) using helium as carrier gas. A 4 µl sample was injected in each case. The injector temperature was 230 °C, and the injector was operated in the splitless mode. The temperature program consisted of a starting temperature of 140 °C which was held for 4 min, and then increased to 180 °C increments of 10 °C min^{-1}. The temperature was finally increased from 180 to 260 °C at a rate of 8 °C min^{-1}. The total run time was 14 min. The Finnigan MAT quadrupole mass spectrometer was used in combination with an INCOS 50B data system. The instrument was operated in the electron ionization mode: the ion source temperature was 180 °C, and the ionization energy was 70 eV.

3. Results

3.1. *Fatty acid composition of choline and ethanolamine glycerophosphates*

Representative gas chromatograms corresponding to the DMAs and FAMEs of glycerophospholipids derived from rat and pig hearts are shown in Figs. 1 and 2. In general (Table 1), ethanolamine glycerophosphates of the pig heart contained higher amounts of 16:0 DMA, 18:0 DMA, 18:1 DMA, 18:2 FAME and 20:4 FAME, but lower amounts of 16:0 FAME, 18:0 FAME, and 22:6 FAME compared to those of the rat heart. Choline glycerophosphate of the pig heart, on the other hand, contained higher amounts of 16:0 DMA, 16:0 FAME, 18:0 DMA, 18:1 DMA, 18:1 FAME, 18:2 FAME but lower amounts of 18:0 FAME, 20:4 FAME, and 22:6 FAME compared to those in the rat heart. The 18:0 DMA and 18:1 DMA derived from choline glycerophosphates were identified in the pig heart only. Differences in fatty acid composition between the choline and ethanolamine glycerophosphates were also noticed within the same species. For example, in the rat heart amounts of 16:0 DMA and 22:6 FAME were higher but amounts of 16:0 FAME, 18:1 FAME, 18:2 FAME and 20:4 FAME were lower in the ethanolamine fraction compared to those present in the choline glycerophosphates. Ethanolamine glycerophosphates of the pig heart contained lower amounts of 16:0 FAME, 18:1 FAME and 18:2 FAME, but higher amounts of 18:0 DMA, 18:1 DMA, 18:0 FAME, 20:4 FAME and 22:6 FAME as compared to those in choline glycerophosphates.

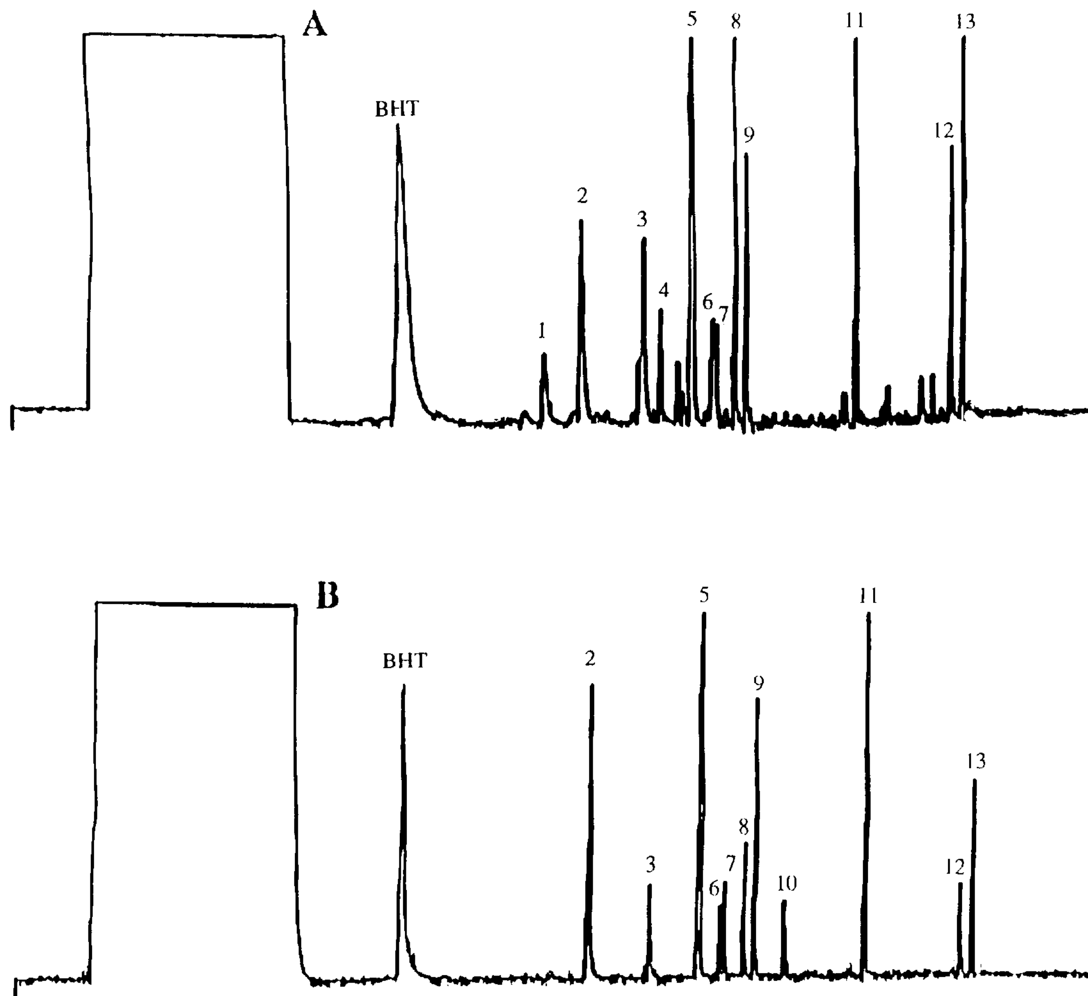

Fig. 1. A typical gas chromatograph of DMAs and FAMEs derived from rat myocardium. (A) Ethanolamine glycerophosphates and (B) choline glycerophosphates separated using a polar phase capillary column and a flame ionization detector. Peak 1, 16:0 DMA; peak 2, 16:0 methyl ester; peak 3, 18:0 DMA; peak 4, 18:1 DMA; peak 5, 18:0 methyl ester; peak 6, 18:1 methyl ester-d_7; peak 7, 18:1 methyl ester-d_9; peak 8, 19:0 methyl ester (internal standard used to check recoveries); peak 9, 18:2 methyl ester; peak 10, 20:0 methyl ester (internal standard); peak 11, 20:4 methyl ester; peak 12, unidentified fatty acid methyl ester; peak 13, 22:6 methyl ester.

3.2. *Mass spectra of fatty acid derivatives*

Mass spectra of the FAME and DMA are shown in Fig. 3. The molecular ion (M^+) peak of the FAME is distinct and characterized by the well-known McLafferty rearrangement and cleavage one bond removed from the carbonyl group. The methyl ester of aliphatic acids unbranched at the α-carbon gave a strong peak at m/z 74, usually base peaks, as can be seen in the 16:0 and 18:0 methyl esters. These methyl esters also produced an excellent diagnostic peak at $M^+ - 31$. Further, the cleavage at each C–C bond gave an alkyl ion (m/z 29, 43, 57, 71, . . . , etc.), and an oxygen-containing ion, $C_nH_{2n-1}O_2$. The peak m/z 87 representing the ion $(CH_2CH_2COOCH_3)^+$ was always more intense than its homologs, especially for saturated FAMEs. The mass spectra of DMAs were characterized by $(M - OCH_3)^+$ peaks and this cleavage was mediated by an oxy-

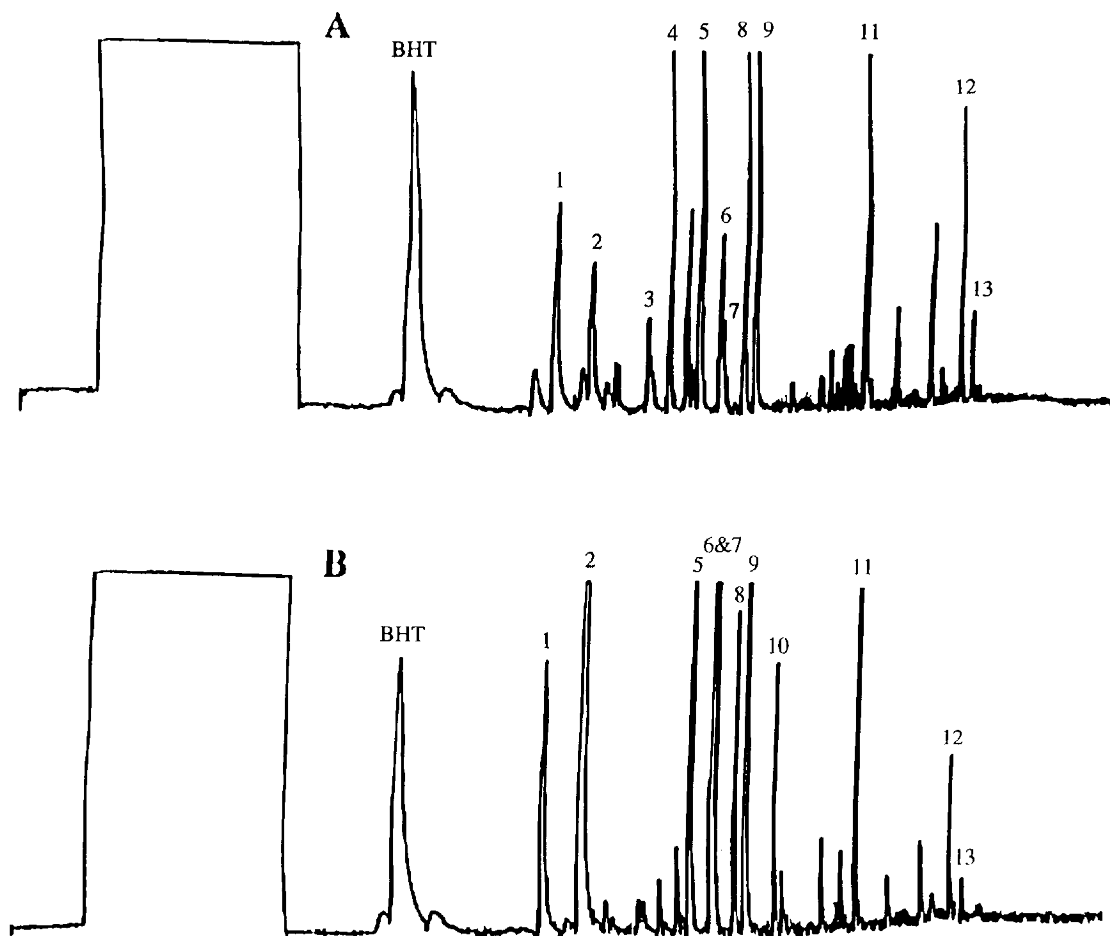

Fig. 2. A typical gas chromatograph of DMA and FAME derived from pig myocardium. (A) Ethanolamine glycerophosphates and (B) choline glycerophosphates separated using a polar phase capillary column and a flame ionization detector. Peak 1, 16:0 DMA; peak 2, 16:0 methyl ester; peak 3, 18:0 DMA; peak 4, 18:1 DMA; peak 5, 18:0 methyl ester; peak 6, 18:1 methyl ester-d_7; peak 7, 18:1 methyl ester-d_9; peak 8, 19:0 methyl ester (internal standard used to check recoveries); peak 9, 18:2 methyl ester; peak 10, 20:0 methyl ester (internal standard); peak 11, 20:4 methyl ester; peak 12, unidentified fatty acid methyl ester; peak 13, 22:6 methyl ester.

gen atom, and thus facile. The DMAs unbranched at the α-carbon produced a strong base peak at m/z 75, which is evident in the 16:0, 18:0 and 18:1 DMAs. Mass spectral analyses and comparison with authentic GLC standards confirmed the identity of the compounds analyzed by GC.

4. Discussion

The fact that accumulation of arachidonic acid is a distinct feature of myocardial ischemia reperfusion [14], and the plasmalogens contain a large amount of arachidonic acid in the Sn-2 position [15], make the plasmalogens a prime suspect in the pathophysiology of ischemia reperfusion injury. Moreover, the accumulation of lysoplasmenylethanolamine and not lysoplasmenylcholine suggests that plasmenyl ethanolamine molecular species are likely to serve as the principle storage for the arachidonic acid to be released during ischemia and reperfusion. A high concentration of arachidonic acid was also found in the plasmenylethanolamine content of rabbit aortic smooth muscle [16], and in guinea pig heart mitochondria and microsomes [11]. In dog heart, the

Table 1
Fatty acid methyl ester (FAME) and dimethylacetyl (DMA) derivatives from rat and pig myocardial choline and ethanolamine glycerophosphates

Species	Glycerophosphate (mol%)	16:0		18:0		18:1		18:2	20:4	22:6
		DMA	FAME	DMA	FAME	DMA	FAME	FAME	FAME	FAME
Rat	CPG	0.9 ± 0.1	21.5 ± 1.2	ND	26.4 ± 1.0	ND	8.2 ± 0.5	12.2 ± 1.3	21.2 ± 1.41	7.0 ± 0.6
	EPG	5.05 ± 0.3	10.4 ± 2.1	3.3 ± 0.5	28.0 ± 0.9	1.7 ± 0.3	5.4 ± 0.3	5.0 ± 0.5	14.1 ± 0.9	23.5 ± 1.0
Pig	CPG	12.0 ± 1.3	24.1 ± 1.0	1.1 ± 0.3	10.0 ± 1.3	1.5 ± 0.1	16.9 ± 1.3	23.7 ± 2.0	9.2 ± 0.7	0.5 ± 0.3
	EPG	12.0 ± 0.4	3.3 ± 0.4	10.9 ± 1.7	19.3 ± 1.5	5.3 ± 0.5	4.8 ± 0.4	12.2 ± 1.2	27.9 ± 0.8	1.5 ± 0.8

The results are expressed as the mean ± the standard error of the least six determinations. For each phosphoglyceride, the values do not add up to 100% because of minor species not being reported. Key: EPG, ethanolamine phosphoglycerides; CPG, choline phosphoglycerides; ND, not detected.

majority of sarcolemmal ethanolamine glycerophospholipids contained arachidonic acid (20:4) esterified to the Sn-2 position, while choline glycerophospholipids consisted of 18:1 or 18:2 fatty acids esterified at the Sn-2 position [17]. Both lysoplasmenylethanolamine and arachidonic acid are amphiphilic and potentially harmful products for the heart [18]. Arachidonic acid is also a substrate for cyclooxygenase and lipooxygenase and thus can lead to the formation of prostaglandins, thromboxanes and leukotrienes [14].

It has long been known that the fatty acid composition of plasmalogens in heart vary from one species to another [19]. The data from our results support these previous findings. Thus, pig heart ethanolamine glycerophospholipids are rich in 18:2 and 20:4 fatty acids, but low in 22:6 fatty acids compared to those present in the rat heart. Choline glycerophosphates of pig heart are also rich in 18:2, but poor in 20:4 and 22:6 fatty acids compared to those of rat heart. It seems likely that the majority of the 20:4 fatty acid in pig heart is derived from the plasmenyl ethanolamine fraction, whereas in the case of rat heart it may be derived from both the ethanolamine and the choline fractions. It is also interesting to note that the 22:6 fatty acid is present in the rat heart in a very high quantity, whereas pig heart contains only a relatively small amount of this fatty acid.

In this study, mass spectral analysis was used to confirm the identity of the FAME and DMA samples. The molecular ion peaks (M^+) of straight-chain fatty acid methyl esters are usually distinct as evident from the results of the 16:0, 18:0, 18:1, 18:2, 20:4, and 22:6 FAME samples. The most characterized peak is due to the familiar McLafferty rearrangement and cleavage one bond removed from the C=O group. Thus, a methyl ester of an aliphatic acid unbranched at the α-carbon gives a strong base peak at m/z 74, which is the case in both the 16:0 and 18:0 FAME samples. The ion R^+ ($R–C–OR_1 \rightarrow R^+–(C–OR_1)^+$) is prominent in the short-chain methyl esters, but diminishes rapidly with increasing chain length beyond C_6 FAMEs. The ion $R–C{=}O^+$ ($R–C–OR_1 \rightarrow R–C{=}O^+$) derived from FAMEs gave an excellent diagnostic peak at $M^- - 31$, which is present in most of our samples. The fragmentation pattern for methyl esters of straight-chain acids can be described in the same terms used for the pattern of the free acid. Cleavage at each C=C bond gives an alkyl ion (m/z 29, 43, 57, ...) and an oxygen-containing ion, $C_nH_{2n-1}O_2^+$ (m/z 59, 73, 87, ...). Thus, these hydrocarbons cluster at intervals of 14 mass units in the case of saturated FAMEs; in each cluster is a prominent peak at $C_nH_{2n-1}O_2$. The peak at m/z 87 representing the $(CH_2CH_2COOCH_3)^-$ ion is always more intense than its homologs.

DMAs were found to contain none to a very weak molecular ion peak (M^+), but with a prominent peak at $(M - OCH_3)^+$ or $(M - 31)^+$, which can be seen in the 16:0, 18:0, and 18:1 DMA samples. Cleavages occurring in the DMA samples are mediated by an oxygen atom, and are

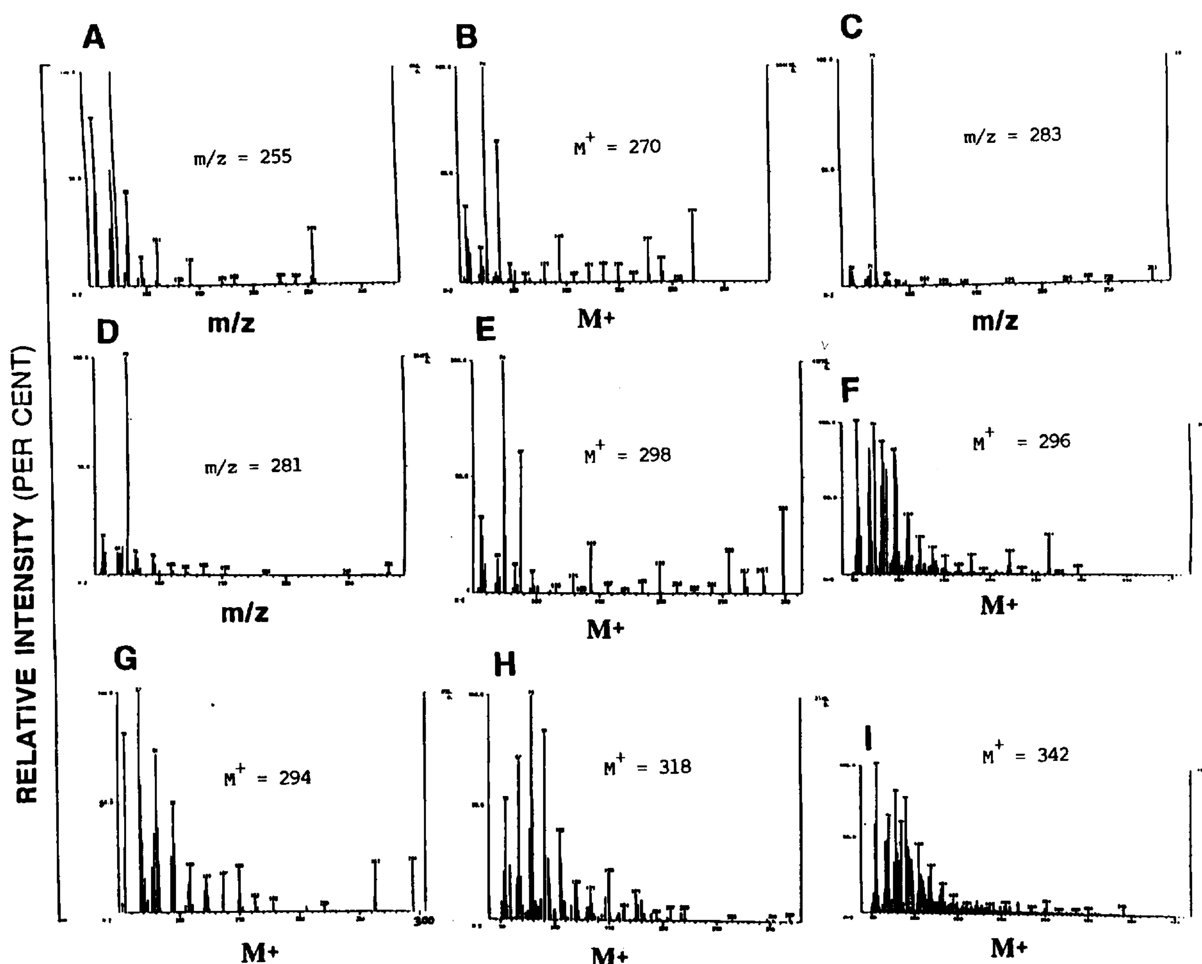

Fig. 3. Representative mass spectra for the DMAs and FAMEs of the designated peaks (shown as peak #) from Figs. 1 and 2. (A) 16:0 DMA (peak # 1); (B) 16:0 FAME (peak # 2); (C) 18:0 DMA (peak # 3); (D) 18:1 DMA (peak # 4); (E) 18:0 FAME (peak # 5); (F) 18:1 FAME (peak # 6); (G) 18:2 FAME (peak # 9); (H) 20:4 FAME (peak # 11); (I) 22:6 FAME (peak # 13).

thus facile; as usual elimination of the largest group is preferred and like aliphatic ethers, the first-formed oxygen-containing fragments can further decompose with hydrogen migration and olefin elimination. The major fragmentation pattern of the DMA samples matches these data. In addition, the mass spectral comparison of the FAME and DMA samples with authentic standards further confirms their presence in the isolated heart.

It should be noted, however, that although we analyzed some of the fatty acid compositions (i.e. 16:0, 18:0, 18:1) of plasmalogens, the results actually reflect a gross comparison of the phosphoglyceride acyl and alk-1-enyl moieties of heart between pig and rat. This study does not include the isolation of different membrane fractions and the characterization of 1,2-diester phosphoglyc-erides and 1-alk-1-enyl, 2-ester phosphoglycerides. Further studies using preparative TLC isolating the choline phosphoglyceride and ethanolamine phosphoglyceride fractions followed by hydrolyzing the polar head groups using phospholipase C, thereby liberating the diacylglycerols (DGs) (which can be readily acetylated), are required to obtain the specific fatty acid composition of the plasmalogens.

It is interesting to note that although the pig and rat myocardiums have comparable levels of choline phosphoglycerides, the choline: ethanolamine phosphoglycerides ratio is 0.77 in the rat heart as compared to 0.56 in the pig heart. Since the pig heart has 38% more ethanolamine phosphoglycerides in the plasmalogen form, the average volume of the ethanolamine phosphoglycerides of the pig heart may be larger than that in the rat heart, and therefore fewer ethanolamine phosphoglycerides may be necessary for proper membrane function.

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