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Invited Paper

Subcellular distribution, molecular dynamics and catabolism of plasmalogens in myocardium

Linda A. Scherrer and Richard W. Gross
Departments of Medicine and Chemistry, Washington University Medical School, St. Louis, Missouri 63110, USA

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Abstract

Recent studies have implicated accelerated sarcolemmal phospholipid catabolism as a mediator of the lethal sequelae of atherosclerotic heart disease. We have demonstrated that plasmalogens are the predominant phospholipid constituents of canine myocardium and that plasmalogens are hydrolyzed by a novel calcium independent plasmalogen selective phospholipase A_2. Since the activities of phospholipases are modulated by the molecular dynamics and interfacial characteristics of their phospholipid substrates, we compared the molecular dynamics of plasmenylcholine and phosphatidylcholine vesicles by electron spin resonance spectroscopy and deuterium magnetic resonance spectroscopy. Plasmenylcholine vesicles have separate and distinct molecular dynamics in comparisons to their phosphatidylcholine counterparts as ascertained by substantial decreases in the angular fluctuations and motional velocities of probes attached to their *sn*-2 aliphatic constituents. Furthermore, since free radical oxidation of myocardial lipid constituents occurs during myocardial ischemia and reperfusion, we demonstrated that $^1O_2$ mediated oxidation of plasmenylcholine resulted in the generation of several products which have chromatographic characteristics and molecular masses corresponding to 2-acyl lysophosphatide derivatives. Taken together, these studies underscore the biologic significance of the predominance of sarcolemmal plasmalogens present in mammalian myocardium and suggest that their catabolism by plasmalogen selective phospholipases and/or oxidative processes may contribute to the lethal sequelae of myocardial ischemia.

Introduction

The sequelae of atherosclerotic heart disease usually result from electrophysiologic dysfunction in regions of ischemic myocardium which predispose to ventricular dysrhythmias such as ventricular tachycardia or ventricular fibrillation. Since lipid metabolism is dramatically altered within seconds of myocardial ischemia, the hypothesis that ion channel and ion pump kinetics are modified during myocardial ischemia secondary to alterations in the chemical properties of sarcolemmal membrane lipid constituents [1] was received enthusiastically. During the last ten years, a substantial amount of experimental evidence has accrued which suggests that accumulation of amphiphilic lipids during myocardial ischemia is a primary determinant of electrophysiologic dysfunction manifest in ischemic myocardium (e.g. [2–6]).

Concomitant with myocardial ischemia, phospholipase A_2 is activated and fatty acid β-oxidation is inhibited (e.g. [3, 4, 7, 8]). The net result of these alterations is the accumulation of amphiphilic metabolites such as lysophosphoglycerides and long-

chain acylcarnitines in ischemic myocardium. Several lines of evidence suggest that the accumulation of amphiphilic metabolites is the biochemical progenitor of electrophysiologic dysfunction in ischemic zones including:

1) the temporal course of accumulation of long-chain acylcarnitines and lysophosphoglycerides parallels the electrophysiologic alterations present in ischemic zones [2];
2) both long-chain acylcarnitines and lysophosphoglycerides induce concentration-dependent decreases in maximum diastolic potential, action potential amplitude, $\dot{V}_{max}$ of phase 0 and action potential duration at concentrations comparable to those present in ischemic tissues [9–14];
3) the *in vitro* kinetics of enzymic mediators of amphiphile accumulation are entirely compatible with the temporal course and magnitude of amphiphile accumulation during ischemia [15–18];
4) both long-chain acylcarnitines and lysophosphoglycerides are present in sarcolemma in sufficient concentration at the time of electrophysiologic alterations to be primary mediators of electrophysiologic dysfunction [19, 20]; and
5) both long-chain acylcarnitines and lysophosphoglycerides are potent membrane perturbing agents which markedly alter the molecular dynamics and physical properties of the sarcolemmal membrane when they constitute only a small mol% of sarcolemmal phospholipid (i.e. 1–3 mol%) [21].

Thus, substantial evidence suggests that the accumulation of amphiphilic compounds contributes to the lethal sequelae of myocardial ischemia.

Since electrophysiologic alterations are likely mediated by alterations of chemical constituents in the sarcolemmal membrane and we have demonstrated that plasmalogen molecular species are the predominant phospholipid constituents of sarcolemma [22], it seems likely that the biochemical and biophysical alterations which are responsible for electrophysiologic dysfunction in ischemic zones likely involve plasmalogen molecular species. The purpose of this brief synopsis is to place the significance of plasmalogens into perspective and to integrate the amphiphile hypothesis with myocardial plasmalogen subcellular localization, catabolism, molecular dynamics and free radical oxidation.

Quantification of individual phospholipid molecular constituents in canine myocardium and their subcellular distribution

Since sarcolemma is the electrophysiologically active membrane in myocardium, our initial attention focused on the characterization of canine myocardial sarcolemmal phospholipid molecular constituents. Canine myocardial sarcolemma was purified by discontinuous sucrose density gradient centrifugation and possessed a Na-K ATPase activity of 141 μmol of $P_i\,mg^{-1}\,h^{-1}$. Sarcolemmal preparations were contaminated by small amounts of mitochondria (10–20%) and sarcoplasmic reticulum (3%) as assessed by marker enzyme analyses [22]. The predominant phospholipid classes present in canine myocardial sarcolemma were choline and ethanolamine glycerophospholipids which contained 47% and 28%, respectively, of total lipid extractable phosphate. Sarcolemma was significantly enriched in sphingomyelin (11%) and also contained small amounts of phosphatidylinositol (6%) and phosphatidylserine (5% [22]). Acid methanolysis and subsequent analysis by capillary gas chromatography demonstrated that canine myocardial sarcolemmal choline glycerophospholipids were comprised of 54% plasmenylcholine molecular species and that sarcolemmal ethanolamine glycerophospholipids were comprised of 67% plasmenylethanolamine molecular species [22]. The individual phospholipid molecular constituents in canine myocardial sarcolemmal choline and ethanolamine glycerophospholipids were determined by fast atom bombardment mass spectroscopy and reverse phase HPLC. Sarcolemmal choline glycerophospholipids consisted primarily of molecular species with protonated parent ions of m/z 742, 744, 758, 760, 766 and 782 corresponding to molecular species containing 16:0, 18:0, 18:1, 18:2 and 20:4 aliphatic constituents. Over 40% of plasmenylcholine molecular species contained arachi-

donic acid at the *sn*-2 position while only 28% of phosphatidylcholine molecular species contained arachidonic acid at the *sn*-2 position [22]. Remarkably, 86% of canine myocardial plasmenylethanolamine molecular species contained arachidonic acid at the *sn*-2 position (Fig. 1). Taken together, these studies demonstrate that the predominant phospholipid constituents present in canine myocardial sarcolemma were plasmenylcholine and plasmenylethanolamine and that the predominant fatty acid esterified to the *sn*-2 position of myocardial sarcolemmal ethanolamine glycerophospholipids was arachidonic acid.

To gain insight into the individual molecular constituents present in other canine myocardial subcellular membranes, additional experiments were performed. Canine myocardial sarcoplasmic reticulum and mitochondria were prepared by traditional methods and their purities, assessed by marker enzyme analysis, were greater than 95% [23]. Sarcoplasmic reticular ethanolamine glycerophospholipids were predominantly comprised of plasmenylethanolamine molecular species containing arachidonic acid at the *sn*-2 position. In contrast, the overwhelming majority of mitochondrial ethanolamine glycerophospholipids were comprised of phosphatidylethanolamine molecular species containing arachidonic acid at the *sn*-2 position [23]. All three subcellular membranes (sarcolemma, sarcoplasmic reticulum and mitochondria) contained similar amounts of each molecular class of choline glycerophospholipids (approximately 50% plasmenylcholine and 50% phosphatidylcholine) although modest differences in individual molecular species were present [23]. These results demonstrated that plasmenylethanolamines and phosphatidylethanolamines synthesized in myocardium are selectively directed to separate and distinct subcellular membrane compartments.

Identification and characterization of myocardial phospholipase A_2

Since the electrophysiologically active membrane of myocardium is comprised predominantly of plasmalogen molecular species, we sought to explore the hypothesis that a plasmalogen selective phospholipase was present in myocardium which preferentially hydrolyzed plasmalogen substrate. A first step in this endeavor was the synthesis of radiolabeled plasmalogens since these moieties are not commercially available. Radiolabeled plasmenylcholine was prepared by alkaline methanolysis of beef heart choline glycerophospholipids, reverse phase HPLC and subsequent acylation of purified lysoplasmenylcholine with radiolabeled fatty anhydride catalyzed by dimethylaminopyridine [24]. Having radiolabeled synthetic plasmalogens in hand, we were now prepared to characterize the hydrolysis of the predominant sarcolemmal phospholipid constituent by endogenous myocardial enzymes.

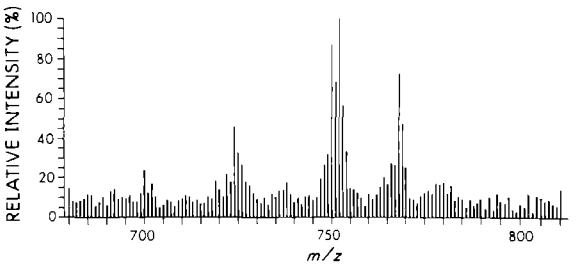

Fig. 1. Fast atom bombardment mass spectrometry of sarcolemmal ethanolamine glycerophospholipids. Purified ethanolamine glycerophospholipids (500 μg) were dissolved in 10 μl of 1/1 $CHCl_3$/MeOH, and 2 μl was mixed with 3 μl of glycerol on a copper probe. Fast atom bombardment mass spectra were obtained and the baseline was normalized. Peaks at m/z 724, 750 and 752 represent plasmenylethanolamine molecular species containing arachidonic acid at the *sn*-2 position while the peak at 768 corresponds to a phosphatidylethanolamine molecular species containing arachidonic acid at the *sn*-2 position.

Myocardial cytosolic and microsomal proteins were incubated with radiolabeled plasmenylcholine or phosphatidylcholine substrate, reaction products were separated by thin layer chromatography and quantified by scintillation spectrometry. The major detectable radiolabeled product present in these incubations was free fatty acid without detectable amounts of radiolabel present in diradyl glycerols, phosphatidic acid or lysophospholipid [18]. Although incubation of myocardial cytosol resulted in the preferential hydrolysis of plasmenylcholine, incubation of microsomal protein with phospholipid substrate resulted in the preferential

hydrolysis of phosphatidylcholine (Table 1). In each case, fatty acid release was not dependent upon the presence of free calcium since maximal enzymic activities were manifest in the presence of the calcium chelator, EGTA. Several conclusions based upon these results are possible. First, it seems clear that myocardium contains at least one calcium independent phospholipase A_2 activity. Second, the overwhelming majority of total measurable phospholipase A_2 activity present in myocardium is located in the cytosolic compartment. Third, myocardium contains at least one plasmalogen selective phospholipase A_2 activity.

To further characterize this novel enzymic activity, we partially purified myocardial phospholipase A_2 by tandem DEAE-Sephacel and hydroxylapatite chromatography [18]. This step resulted in the recovery of 81% of measurable phospholipase activity accompanied by a 2.8-fold increase in specific activity. Kinetic analysis of this preparation demonstrated that the maximum velocity for plasmenylcholine hydrolysis was 5 nmol/mg · h for plasmenylcholine but only 1 nmol/mg · h for phosphatidylcholine [18]. The partially purified phospholipase A_2 had an apparent K_m of 7 μM for plasmenylcholine and 3 μM for phosphatidylcholine. Both substrates had a pH optima between 6 and 7 and both were maximally active in the presence of EGTA. These results represented the first demonstration of a neutral active plasmalogen selective phospholipase A_2 in mammalian tissue. Thus, although myocardial phospholipase A_2 activity had previously been thought to be calcium dependent and located predominantly in the microsomal fraction (by analogy with other systems that had been extensively studied), these results demonstrated that the major measurable myocardial phospholipase A_2 activity is present in the cytosolic compartment and that it is calcium independent.

Table 1. Metabolism of plasmenylcholine and phosphatidylcholine catalyzed by myocardial cytosol and microsomes. Myocardial microsomes or cytosol were incubated with 2.5 μM [^{3}H] plasmenylcholine or [^{3}H] phosphatidylcholine (each radiolabeled at the sn-2-position with [^{3}H] oleic acid) in the presence of 1 mM EGTA or 1 mM $CaCl_2$. Fatty acid was extracted with butanol, separated by TLC, and quantified by scintillation spectrometry. Total phospholipase activity is expressed as mass hydrolyzed per gram wet weight of myocardium per h based on 110 mg of cytosolic protein/g wet weight and 5 mg of microsomal protein/g wet weight

	EGTA	Ca^{2+}
	nmol/gwet · h	
Cytosol		
Plasmenylcholine	91.3	13.2
Phosphatidylcholine	42.9	18.7
Microsomes		
Plasmenylcholine	2.5	1.5
Phosphatidylcholine	8.4	6.9

Molecular dynamics of plasmenylcholine and phosphatidylcholine vesicles

Since the predominant phospholipid constituents in canine myocardial sarcolemma were plasmalogen molecular species and we had partially purified the first calcium independent plasmalogen selective phospholipase A_2, we sought to compare and contrast the molecular dynamics of plasmenylcholine and phosphatidylcholine bilayers. These experiments were motivated by the fact that the molecular dynamics of biologic membranes are known to be primary determinants of transmembrane enzyme kinetics and that membrane molecular dynamics have dramatic effects on the activity of several phospholipases A_2. In initial experiments, we quantified the order parameter of 5 and 16-doxylstearate incorporated as substitutional impurities (1%) into vesicles comprised of plasmenylcholine or phosphatidylcholine. The order parameter(S) was substantially higher for both 5-doxylstearate (5DS) and 16-doxylstearate (16DS) in vesicles comprised of plasmenylcholine (e.g. S = 0.592 for 5DS and 0.107 for 16DS) in comparison to vesicles comprised of phosphatidylcholine (S = 0.487 for 5DS and 0.099 for 16DS) [25]. The motional velocity of plasmenylcholine and phosphatidylcholine vesicles was quantified by calculating the apparent rotational correlation times of spin-labeled plasmenylcholine and phosphatidylcholine vesicles. The apparent rotational correlation time of 16DS-plasmenylcholine in vesicles comprised of phosphatidylcholine was 0.86 ±

0.01ns while its apparent correlation time in plasmenylcholine vesicles was 0.89 ± 0.01ns [25]. Taken together, these results demonstrated that both the mean squared amplitude of motion (order parameter) as well as the motional velocity (inversely related to rotational correlation time) are significantly smaller in vesicles comprised of plasmenylcholine than vesicles comprised of phosphatidylcholine.

To gain further insight into the differences between the molecular dynamics present in plasmenylcholine and phosphatidylcholine vesicles, the ratio of the low-field to the mid-field peak height (H_1/H_0) in electron spin resonance spectra was compared in vesicles spin-labeled with 16-doxyl plasmenylcholine or 16-doxyl phosphatidylcholine. Although this ratio was less than unity for plasmenylcholine vesicles in the absence of cholesterol and decreased slowly with increasing mole percentages of cholesterol, it was greater than unity in vesicles comprised of phosphatidylcholine and decreased dramatically upon the addition of cholesterol (Fig. 2). No significant differences were present in this ratio when either of the two different spin-labels were employed. Accordingly, alterations in the H_1/H_0 ratio for plasmenylcholine vesicles in comparison to phosphatidylcholine vesicles likely reflect intrinsic alterations in the molecular conformation and dynamics of plasmenylcholine vesicles compared to phosphatidylcholine vesicles.

Since spin-labeled probes are potentially membrane perturbing agents, additional experiments were performed utilizing synthetically deuterated plasmenylcholine and phosphatidylcholine prepared in our laboratory. Typical ^2H NMR spectra of plasmenylcholine and phosphatidylcholine deuterated at the C-2 position of the *sn*-2 fatty acyl chain demonstrated substantial differences in the inner but not the outer splitting of the two Pake doublets (Fig. 3). Furthermore, the spectral intensity in the wings of the plasmenylcholine ^2H NMR spectra was also substantially greater than that present in the wings of spectra of phosphatidylcholine vesicles. Taken together, the results of these studies demonstrated that vesicles comprised of plasmenylcholine have substantially different molecular dynamics than phosphatidylcholine vesicles utilizing multiple probes of molecular motion employing independent techniques. It is tempting to speculate that such motional alterations underlie the predominance of plasmalogens in electrophysiologically active tissues since they confer unique dynamical properties to biologic membranes which could likely influence the conformation, dynamic motion and kinetics of transmembrane proteins solvated by plasmalogen molecular constituents. Furthermore, the observed alterations near the C-2 position of the *sn*-2 fatty acyl chain may render plasmalogens more susceptible to attack by phospholipases whose initial covalent interaction likely involves attack at the backside of the *sn*-2 carbonyl in phospholipids.

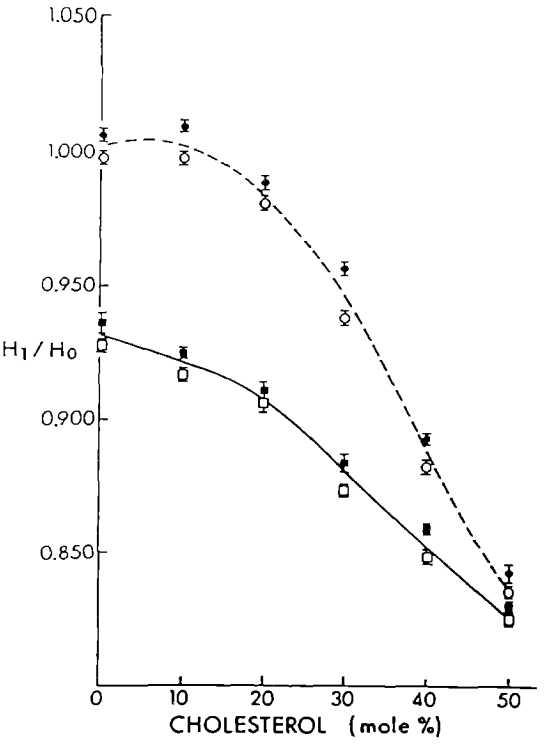

Fig. 2. Comparison of low-field to midfield peak height ratios of vesicles comprised of binary mixtures of phosphatidylcholine/cholesterol and plasmenylcholine/cholesterol. Spin-labeled phospholipid (■ and ●, 16DS phosphatidylcholine; □ and ○, 16DS plasmenylcholine) and unlabeled phospholipid (○ and ●, phosphatidylcholine; □ and ■, plasmenylcholine) with the indicated amounts of cholesterol were prepared. Electron spin resonance spectra were obtained at 38° C, and the relative ratios of the low-field to midfield peak height were calculated. Results are the x ± SE of at least six determinations.

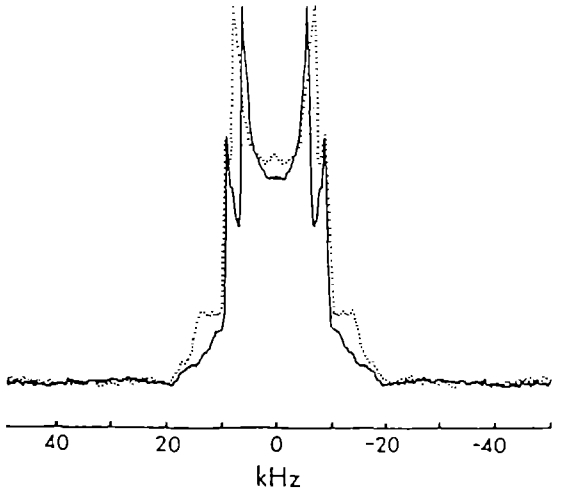

Fig. 3. Comparison of deuterium magnetic resonance spectra of specifically deuterated phosphatidylcholine and plasmenylcholine bilayers. Deuterium magnetic resonance spectra of deuterated plasmenylcholine (·····) and phosphatidylcholine (—), each deuterated at the C-2 position of the *sn*-2 acyl chain, were obtained at 30.7 MHz by utilizing a quadrupolar echo sequence with $\pi/2$ pulses of 4 μs duration and a delay of 40 μs between pulses at 41° C. Spectra of plasmenylcholine and phosphatidylcholine reproducibly demonstrated differences in the splitting of the inner pair of doublets and alterations in the spectral intensity of the wings of the observed resonances.

Free radical mediated oxidation of sarcolemmal phospholipids

Free radical mediated oxidation of endogenous lipid constituents has been implicated as an important mechanism of myocardial dysfunction in regions of ischemic myocardium (e.g. [26, 27]). Although attention has predominantly focused on free radical mediated oxidation of the polysunsaturated fatty acids present in myocardial phospholipids, it should be realized that the vinyl-ether linkage present in plasmenylcholine and plasmenylethanolamine is likely more susceptible to covalent alteration initiated by H radical abstraction due to the resultant oxygen stabilized resonance structures. Furthermore, $^1O_2$ is known to rapidly add to vinyl-ether linkages in model systems by 1–2 or by 1–3 addition [28]. Since $^1O_2$ is produced in biologic systems, we hypothesized that the generation of $^1O_2$ by invading white blood cells [29] or by metal catalyzed oxidation of superoxide [30] resulted in the nonenzymatic generation of 2-acyl lysophospholipids accompanied by deleterious effects on cell membrane physiologic function.

To explore this hypothesis, 2 mg of radiolabeled plasmenylcholine were irradiated under a 100W tungsten lamp for two hours in a solution containing 0.3 mg methylene blue (photosensitizer) in 7 ml of $CDCl_3$/MeOH, (6/1). Reaction products were extracted into $CHCl_3$, passed through a mini column comprised of Kieselgel 60, separated by TLC and quantified by scintillation spectrometry.

Irradiation of radiolabeled plasmenylcholine (1-O-Z-hexadecenyl-2-(9,10[^{3}H])-oleoyl-*sn*-glycero-3-phosphocholine) resulted in the appearance of two new radiolabeled products ($R_f = 0.32$, $R_f = 0.11$) accompanied by the disappearance of radiolabeled plasmenylcholine ($R_f = 0.4$) utilizing a solvent system comprised of $CHCl_3$/MeOH/NH_4OH (65/35/5). The appearance of these products was dependent on the presence of methylene blue and oxygen in the reaction mixture but did not occur in the dark and irradiation of radiolabeled phosphatidylcholine did not result in the appearance of these more polar products or the disappearance of starting material.

To identify the chemical structure of the more polar material ($R_f = 0.11$), 10 mg of unlabeled plasmenylcholine were irradiated and the polar reaction product ($R_f = 0.11$) was purified by preparative TLC, extracted and subjected to fast atom bombardment mass spectroscopy. Fast atom bombardment mass spectra demonstrated two clusters of m/z peaks 16 atomic mass units apart. Major peaks were present at m/z 520, 522, 536 and 538 (Fig. 4). Based on the known mechanisms of addition of $^1O_2$ to vinyl-ether linkages, we propose the structures shown in Fig. 5. These structures represent different oxidation states of the *sn*-1 carbon of 2-acyl lysophospholipid derivatives including the alcohol, aldehyde, acid and hydroperoxide. The assignment of the peak at m/z = 538 is tentative due to the known lability of lipid hydroperoxides and the fact that a m/z species of 538 could also be produced by incorporation of oxygen at other portions of the phospholipid molecule resulting in a species that contained radiolabeled oleic acid and

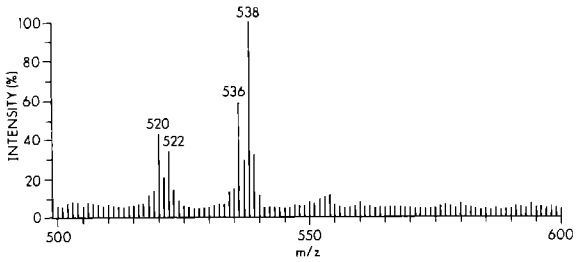

Fig. 4. Fast atom bombardment mass spectroscopy of the polar reaction product ($R_f = 0.11$) resulting from $^1O_2$ mediated oxidation of plasmenylcholine. Approximately 10 mg of unlabeled plasmenylcholine (containing oleic acid at the *sn*-2 position) were irradiated for 4 h utilizing a 100 W tungsten lamp. Reaction products were extracted into $CHCl_3$, separated by TLC and the region corresponding to a $R_f = 0.11$ was extracted with $CHCl_3$/MeOH and analyzed by fast atom bombardment mass spectroscopy. The relative intensities of each peak were corrected for background by baseline normalization.

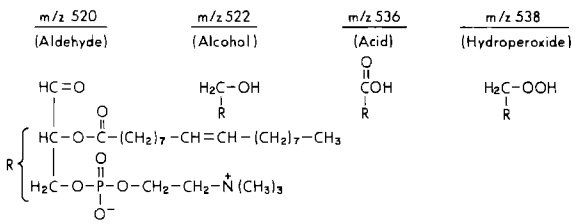

Fig. 5. Proposed structures of products resulting from $^1O_2$ mediated oxidation of plasmenylcholine.

would demonstrate similar chromatographic behavior. Since the compounds migrating with an $R_f = 0.11$ contained radiolabeled oleic acid, demonstrated similar chromatographic behavior as lysophospholipids and had m/z values compatible with different oxidation of the photolabile vinyl-ether linkage and the photo-sensitizer, the observed enhancement of cleavage of the vinyl-ether linkage seems reasonable. Examples of accelerated reaction velocities in membrane mimetic systems resulting from the close spatial opposition of two reactants are well-known [31]. Accordingly, it seems clear that $^1O_2$ can also mediate the cleavage of plasmenylcholine in biologically relevant systems (Fig. 6).

Taken together, these results demonstrate that the interaction of singlet oxygen in aqueous systems with bilayers comprised of plasmenylcholine

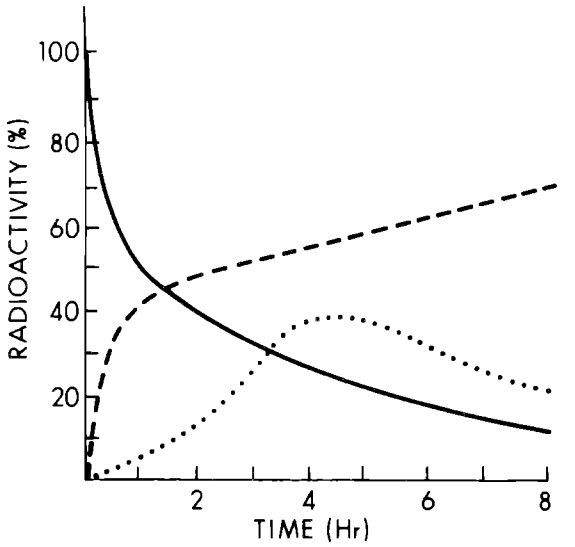

Fig. 6. Time course of $^1O_2$ mediated oxidation of plasmenylcholine vesicles in H_2O. Sonicates of radiolabeled plasmenylcholine and methylene blue were irradiated with a 100 W tungsten lamp during which time aliquots of the reaction mixture were extracted with chloroform, products were separated by TLC and the percentage of radioactivity present in plasmenylcholine ($R_f = 0.4$) (——), the less polar reaction product ($R_f = 0.32$) (– – –), or the more polar reaction product ($R_f = 0.11$) (·····) were quantified by scintillation spectrometry.

results in the cleavage of the *sn*-1 linked vinyl-ether and the production of 2-acyl lysophospholipids (or oxidation products thereof). Since $^1O_2$ may be produced in several biologic systems and since $^1O_2$ interacts with the predominant phospholipid constituent of sarcolemma, plasmenylcholine, resulting in the production of amphiphilic compounds (e.g. 2-acyl lysophospholipids), it seems likely that $^1O_2$ production would alter the dynamics and integrity of the sarcolemmal membrane by radical induced production of lysophospholipids. Accordingly, we propose that radical oxidation of the vinyl-ether linkage in sarcolemmal phospholipids may be a major target of oxidative processes during ischemia which potentially contributes to the sequelae of myocardial infarction.

Conclusions

The results of the studies presented in this brief

synopsis demonstrate that the majority of phospholipid constituents in the electrically active membrane of myocardium, sarcolemma, are plasmalogen molecular constituents. They further show that plasmalogens have separate and distinct molecular dynamics compared to their diacyl phospholipid counterparts which potentially facilitate the physiologic function of sarcolemmal transmembrane proteins such as ion channels or ion pumps. The major phospholipase A_2 activity in myocardium is present in the cytosolic fraction, is selective for plasmalogen substrate and is calcium independent. Selectivity for plasmenylcholine substrate may reflect alterations in the interfacial properties and physical characteristics of vesicles comprised of plasmalogen molecular constituents in comparisons to their diacyl phospholipid counterparts. Finally, the vinyl-ether linkage in sarcolemmal plasmalogens is a susceptible target for free radical oxidative processes that result in the generation of 2-acyl lysophospholipid derivatives which may contribute to the deleterious effects of radical mediated oxidation in regions of ischemic and reperfused myocardium.

Acknowledgements

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Address for offprints: R.W. Gross, Cardiovascular Biochemistry, Washington University School of Medicine, 660 South Euclid Avenue, Box 8020, St. Louis, MO 63110, USA