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PRODUCTION OF LYSOPHOSPHOLIPIDS AND FREE FATTY ACIDS BY A SARCOLEMMAL FRACTION FROM CANINE MYOCARDIUM

Kenneth Owens, David C. Pang and William B. Weglicki

Department of Biophysics, Medical College of Virginia
Virginia Commonwealth University, Richmond, Virginia 23298

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Summary: The potential for injury of myocardial sarcolemma by endogenous lipases was studied. The sarcolemmal fraction was incubated for 30 min under conditions found optimal for hydrolysis of exogenous phosphatidylethanolamine (5 mM calcium, pH 7.0, 37°C). Incubation of the sarcolemmal fraction increased significantly the level of total free fatty acids (14.1 to 31.1 nmoles/mg protein, $P < 0.001$); in addition, production of arachidonic acid was increased significantly ($P < 0.01$). Lysophosphatidylcholine was increased significantly ($P < 0.01$) but the content of lysophosphatidylethanolamine was unchanged. A large proportion of the above free fatty acids (10.2 nmoles/mg protein) was derived from the hydrolysis of triacylglycerols. These data demonstrate that the sarcolemmal fraction preferentially hydrolyses endogenous membrane phosphatidylcholine at neutral pH in the presence of calcium with the formation of lysophosphatidylcholine and free fatty acids including arachidonic acid.

INTRODUCTION

The susceptibility of the lipids of the sarcolemma of cardiac myocytes to attack by endogenous lipases represents a model of membrane injury which may be similar to myocardial ischemia. The aim of the present study was to determine the potential for injury of the sarcolemma by the accumulation of products of endogenous lipid hydrolysis, particularly lysophospholipids and FFA[1]. We have reported previously that enriched preparations of cardiac sarcolemma contain Ca^{2+}-stimulated PLAs active on exogenous phosphatidylethanolamine at neutral pH (1,2). PLA - catalyzed production of lysophospholipids and FFA by mitochondrial, microsomal (3) and lysosomal (4) fractions of the myocardium has been found using exogenous phospholipid substrates.

In the present study, the production of lysophospholipids and FFA was catalyzed in the presence of Ca^{2+} by endogenous, neutral-active lipases of a myocardial fraction enriched in sarcolemma.

Address correspondence to: Kenneth Owens, Ph.D., Department of Biophysics Box 694, MCV Station, Richmond, Virginia 23298

[1] Abbreviations: FFA; free long-chain fatty acids; Lyso PC, lysophosphatidylcholine; Lyso PE, lysophosphatidylethanolamine; PLAs, phospholipases A; TG, triacylglycerols.

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MATERIALS and METHODS

Preparation of Sarcolemmal Fraction: Dogs were anesthetized with pentobarbital (27 mg/kg) and ventilation was maintained by a Harvard respirator. The heart was excised and was cooled in ice-cold saline; the tissue samples were homogenized in sucrose (0.25 M) containing KCl (0.6 M) buffered with imidazole (10 mM, pH 7.0). A fraction enriched in sarcolemma was isolated by sucrose-gradient centrifugation (5). This fraction was found to band at a sucrose concentration of 48 to 52% (w/w). It showed a 7-fold increase over the homogenate in the activity of ouabain-sensitive ($Na^+ + K^+$)-stimulated, Mg^{2+} dependent ATPase (E.C. 3.6.1.3). It was diluted 3-fold and the pellet resulting from centrifugation (140,000 x g max for 30 min) was resuspended gently in sucrose (0.25 M) buffered with imidazole (20 mM, pH 7.0).

Incubation of the Sarcolemmal Fraction: Preliminary studies showed that this sarcolemmal fraction hydrolyzed 1-acyl 2-(1-^{14}C) linoleoyl-sn glycero-3-phosphatidylethanolamine optimally at 37°C in the presence of 5 mM calcium at pH 7.0 (2). These conditons of incubation were used for the examination of lipolysis of endogenous sarcolemmal lipids. The unincubated control suspension of sarcolemma (1 mg protein/mg) was kept on ice while all the other suspensions were incubated with rocking (80 strokes/min) for 30 min. The reaction was terminated by the addition of cold methanol.

Extraction of Lipids: The method of Folch, et al., (6) was used with the following modifications. In order to prevent differences in the extraction solubilities of lipids, 5 µmoles of calcium chloride were added to each ml of unincubated suspensions. Heneicosanoic acid was added as the internal fatty acid standard. Butylated hydroxytoluene (Ashland Oil Co., 0.1 mg/100 ml chloroform) was included as antioxidant. The mixture was held at 50°C for 5 min prior to being cooled to room temperature for washing with KCl (0.1 M). Lipid extracts were dried at room temperature under a stream of N_2, redissolved in chloroform and stored at -60°C prior to analysis of lipids. All analyses were corrected for reagent blanks.

The recovery factors for the Folch lipid extraction of 2-lysophospholipids were determined by the distribution of L-1-(1-^{14}C)-palmitoyl-lyso PC (New England Nuclear, Boston, MA) into the lower phase during the extraction of lipids from the sarcolemmal fraction; the mean recovery was 81.2 ± 0.1 (S.D.) %. The recovery of lyso PE was determined by two-dimensional TLC of lipids extracted from the sarcolemmal fraction, in the presence of added lyso PE from egg yolk (Sigma Chemical Co., St. Louis, MO); the mean recovery was 72.4 ± 7.4 (S.D.) %. The quantities of sarcolemmal lysophospholipids formed were corrected by these factors.

Lipid Analyses: Total phospholipid-P was determined (7) and membrane protein was assayed (8) with bovine serum albumin as the standard. Individual phospholipids were determined by two-dimensional TLC (9). Lipid samples were applied under an atmosphere of N_2; the plates were dried in vacuo for 30 min between developments. Mean recovery of phosphorus content of the spots corrected for equivalent blank areas was 97.3 ± 9.3 (S.D.) %.

Total lipid extracts were also separated by unidimensional TLC on silica gel GHR (Mallinkrodt, Darmstadt) using redistilled petroleum ether (60-80°C), diethylether, glacial acetic acid (80:20:1, by vol), containing BHT (0.1 mg/100 ml). Marker-lipids were visualized in iodine; unexposed areas containing phospholipids, FFA, TG, cholesterol esters and blank aeas were scraped into vials, sealed under N_2 and methylated at 100°C in boron trifluoride-methanol (15% w/v, Applied Science, State College, PA); an equal volume of ice-cold sodium hydroxide (5 N) was added prior to extraction into pentane

(10). The content of methyl esters and dimethylacetals was determined by GLC upon a glass column containing EGSS-X (10% w/w) on Gas Chrom P (Applied Science). Identities were confirmed by mass spectrometry.

All data represent results from 5 paired experiments. Differences between mean values (± S.D.) were tested for statistical significance ($P < 0.05$) by the paired Student's t-test.

RESULTS

The sarcolemmal enzymes catalyzed a two-fold increase in total FFA (control 14.1 vs incubated 31.1 nmoles/mg protein; $P < 0.01$). The significant increase in palmitic, stearic, and oleic acids (Fig. 1) inferred the activity of either phospholipase A_1 and/or TG lipase activity. In addition, due to the strict positional specificity of arachidonic acid for the 2-sn-position in glycerophospholipids, the significant increase in free arachidonic acid (Fig. 1) suggested endogenous PLA_2 activity. Lysophospholipase activity was not evident under these conditions since no loss of total

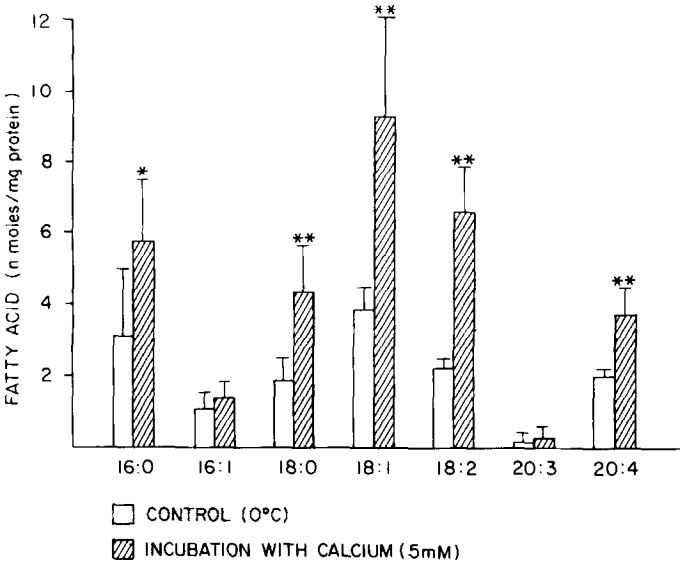

Figure 1 Content of free fatty acids of sarcolemmal fraction. 16:0 Palmitic; 16:1 palmitoleic; 18:0 stearic; 18:1 oleic; 18:2 linoleic; 20:3 eicosatrienoic; 20:4 arachidonic acids. Differences between incubated (37°, 5 mM Ca^{2+} for 30 min at pH 7.0) and unincubated control. *, **, and *** signify probability values between paired data by Student's t-test (N=5) of < 0.05, 0.01, and 0.001 respectively.

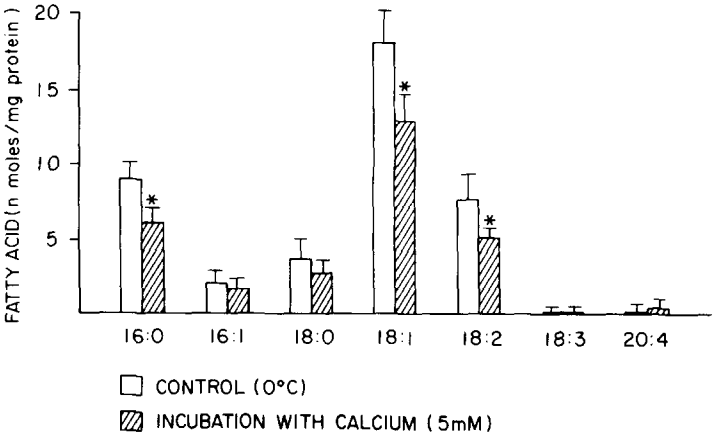

Figure 2 Content of esterified fatty acids of triacylglycerols of sarcolemmal fraction. 18:3 linolenic acid. Differences between incubated and unincubated control levels represent loss by endogenous lipases. Other details as in Figure 1.

phospholipid-P was found following incubation (control 453.9 ± 29.0 vs incubated 459.6 ± 16.2 nmoles phospholipid-P/mg protein).

An analysis of the TG content of the sarcolemmal fraction (control 13.7 ± 3.2 vs incubated 10.3 ± 2.5 nmoles TG/mg protein) indicated that this lipid was also a source of FFA, particularly palmitic, oleic and linoleic acids as shown in Fig. 2. However, the data indicate that lipolysis of TG could not contribute to the increase in arachidonic acid shown in Fig. 1. The cholesterol ester fraction was relatively small and no arachidonic acid was noted on analysis of the alkyl moieties (data not shown).

Table I lists the composition of phospholipids of control and incubated fractions; there was a significant increase in lyso PC. Interestingly, there was no increase in the content of lyso PE.

Table II presents an approximation of the stoichiometry of lipid changes during incubation with calcium. In the case of the formation of lyso PC (4.2 nmoles/mg protein), the sum of the free oleic, linoleic and arachidonic acids, which did not originate from the hydrolysis of TG was 4.8 (i.e., 11.6-6.8) nmoles/mg protein. Thus, under these conditions of incubation there was little evidence for substantial levels of lysophospholipase activity.

TABLE I

Content of Major Phospholipids of Sarcolemmal Fraction
Incubated Under Conditions of Optimal Endogenous Lipase Activity

Phospholipid	Control (0°)	Calcium Added (37°)	P
Phosphatidylcholine	177.8 ± 41.8	171.8 ± 26.6	NS
Lysophosphatidylcholine	3.1 ± 2.1	7.3 ± 2.4	<0.01
Phosphatidylethanolamine	131.5 ± 18.1	127.6 ± 12.6	NS
Lysophosphatidylethanolamine	5.1 ± 3.5	5.1 ± 1.3	NS
Phosphatidylinositol	20.3 ± 5.0	17.4 ± 3.7	NS
Phosphatidylserine	10.5 ± 6.6	6.7 ± 2.9	NS
Sphingomyelin	16.6 ± 8.9	14.9 ± 5.6	NS
Cardiolipin	53.8 ± 12.1	45.4 ± 10.3	NS

nmoles lipid-P/mg protein

Incubation for 30 min at pH 7.0 in the presence of added calcium (5 μmoles/ml).

TABLE II

Unsaturated Free Fatty Acids and Lysophospholipids
Produced by Endogenous Lipases

	Total Unsaturated FFA	Unsaturated FFA from Triacylglycerols	Unsaturated FFA from Phospholipids
Endogenous Lipases (Calcium Incubation)	11.6	6.8	4.8 (lyso PC-4.2 + lyso PE-O)

All values in nmoles/mg protein.

DISCUSSION

We have shown that a subcellular myocardial fraction enriched in sarcolemma had PLA activity which preferentially attacked endogenous phosphatidylcholine. We have characterized the specific changes in phospholipids and FFA including arachidonic acid, following digestion by endogenous lipases at neutral pH in the presence of 5 mM calcium. The results were interpreted as showing that the sarcolemmal preparation also possessed a neutral-active TG lipase.

No evidence of significant lysophospholipase activity was found. This may be associated with the minimal (< 1%) lysosomal marker enzyme activity in the sarcolemmal fraction (5) since a fraction enriched in lysosomes from rat

myocardium has been reported to hydrolyze lyso PE following incubation with labelled phosphatidylethanolamine (4). In a preliminary study (2), the canine sarcolemmal fraction hydrolyzed exogenous phosphatidylethanolamine maximally at pH 7.0 without the accumulation of lyso PE. This was in apparent contradiction to the present study in which there was no evidence of extensive hydrolysis of phosphatidylethanolamine. The accumulation of lyso PC and a similar but not significant loss of phosphatidylcholine may indicate some degree of substrate specificity. Thus, unlike the exogenous phosphatidylethanolamine this phospholipid in the membrane may be largely unavailable to the endogenous lipases. This deserves further study.

Endogenous lipases may produce local enrichment of FFA or lysophospholipids in the microenvironment of enzymes intrinsic to cell membranes such as $(Na^+ + K^+)$-ATPase (11) and adenylate and guanylate cyclases (12).

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