Isolation of a Human Myocardial Cytosolic Phospholipase A₂ Isoform

Fast Atom Bombardment Mass Spectroscopic and Reverse-Phase High Pressure Liquid Chromatography Identification of Choline and Ethanolamine Glycerophospholipid Substrates

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Abstract

Recent studies have demonstrated the existence of a novel family of calcium-independent plasmalogen-selective phospholipases A₂ in canine myocardium that have been implicated as enzymic mediators of ischemic membrane damage. We now report that human myocardium contains two functionally distinct isoforms of cytosolic calcium-independent phospholipase A₂. The major cytosolic phospholipase A₂ isoform preferentially hydrolyzes plasmalogen substrate, possesses a pH optimum of 7.0, and is chromatographically resolvable from a minor cytosolic calcium-independent phospholipase A₂ isoform that hydrolyzes plasmenylocholine and phosphatidylcholine substrates at similar rates and possesses a pH optimum of 8.5. The major cytosolic calcium-independent phospholipase A₂ isoform was identified as a 40-kD polypeptide after its 182,000-fold purification by sequential chromatographies to a final specific activity of 67 μmol/mg min. The purified 40-kD human myocardial phospholipase A₂ preferentially hydrolyzes plasmalogens containing arachidonic acid at the sn-2 position. Both reverse-phase HPLC and fast atom bombardment mass spectroscopic analysis of human myocardial ethanolamine and choline glycerophospholipids demonstrated that plasmenylethanolamine and plasmenylocholine molecular species containing arachidonic acid at the sn-2 position are prominent constituents of human myocardium. Collectively, these results identify and characterize the major human myocardial cytosolic calcium-independent phospholipase A₂ activity, demonstrate the presence of functionally distinct human myocardial cytosolic calcium-independent phospholipase A₂ isoforms, and document the abundance of arachidonoylated plasmalogens molecular species in human myocardium that serve as substrates. (J. Clin. Invest. 1993, 91:2513–2522.) Key words: arachidonic acid • myocardial ischemia • plasmalogens • phospholipase A₂ • phospholipids

Introduction

Phospholipases are critical proximal enzymic mediators of signal transduction processes in mammalian cells because they catalyze the regiospecific cleavage of the chemical precursors of biologically active lipid-derived second messengers (cf. references 1–4). The highly regulated and specific activation of intracellular phospholipases facilitates the appropriate adaptation of cellular function to external perturbations. However, the inappropriate activation of these signal transducers has been recognized as an important biochemical mechanism contributing to the pathophysiologic sequelae of several disease states including inflammation, atherosclerosis, and ischemic membrane dysfunction (cf. references 5–9). In myocardium, accelerated phospholipid catabolism resulting from the activation of phospholipases A₂ during ischemia has been implicated as the biochemical mechanism precipitating electrophysiologic dysfunction and myocytic cellular necrosis during myocardial infarction (e.g., references 10–15). Accordingly, recent attention has focused on the identification and characterization of enzymes mediating phospholipid catabolism in myocardium from a wide variety of mammalian species and the elucidation of the molecular identities of individual phospholipid constituents that serve as their substrates during an ischemic insult. Although substantial insights into some of the characteristics of enzymes mediating phospholipid catabolism in several species of mammalian myocardium have been made (13, 16–21), little information is presently available on the detailed chemical characteristics of human myocardial phospholipase(s) A₂. The multiplicity of different phospholipases A₂ present in human myocardium or the chemical identities of the individual phospholipid constituents present in human myocardium which serve as their lipid substrates.

Prior studies of the individual molecular species of phospholipids present in mammalian myocardium obtained from several species demonstrated the unanticipated finding that some subcellular membranes (e.g., sarclemma and sarcomplasmatic reticulum) were predominantly composed of plasmalogen molecular species containing arachidonic acid at the sn-2 position (22, 23). The potential physiologic and pathophysiologic significance of the high plasmalogen content present in these critical myocardial subcellular membrane compartments was underscored by the identification of a calcium-independent phospholipase A₂ in canine myocardium which selectively utilized plasmalogen substrate (17). It is now well established that myocardial plasmalogen content has substantial interspecies variation (22–27), and that a diverse array of phospholipases A₂ are present in mammalian tissues (13, 16, 17, 18, 20, 21). To delineate the individual molecular species of phospholipids present in human myocardium and to characterize the enzymes mediating their catalysis, human myocardial cytosolic phospholipase A₂ was purified from fresh human myocardium obtained from transplant recipients and detailed analyses of individual human myocardial phospholipid molecular species were performed. We now report the purification of the major human myocardial cytosolic calcium-independent phospholipase A₂ activity to apparent homogeneity, identify the presence...
of cytosolic calcium-independent phospholipase A₂ isoforms with distinct physical and kinetic characteristics, and demonstrate that the major observable cytosolic phospholipase A₂ isoform preferentially hydrolyzes plasmalogen and oleoyl anhydride yielding a stable diacylglycerol. Aminoacyl-ACP synthetase assay was employed to determine the relative efficiency of these isoforms as catalysts for linoleic acid synthesis. The activity of these enzymes was assayed using the branched-chain fatty acid release assay, which measures the rate of linoleic acid release from the enzyme-catalyzed hydrolysis of phosphatidylcholine. The results of these experiments demonstrated that the cytosolic calcium-independent phospholipase A₂ isoform had a significantly higher activity than the calcium-dependent isoform for the hydrolysis of phosphatidylcholine.

**Methods**

**Isolation of human myocardial cytosolic calcium-independent phospholipase A₂.** Human myocardial cytosolic calcium-independent phospholipase A₂ was isolated using a modification of the technique employed for the purification of canine cytosolic phospholipase A₂ (20, 28). Briefly, fresh human myocardium obtained from transplant recipients suffering from end-stage ischemic cardiomyopathy was placed in ice-cold isotonic saline solution. Left ventricular tissue was trimmed of epicardial fat and visible fibrotic lesions, weighed and placed in homogenization buffer (10 mM imidazole, 10 mM KCl, 0.25 M sucrose (grade I), pH 7.8) at 25°C wt/vol. All subsequent steps were performed at 4°C. Ventricular tissue was minced into small pieces with sharp surgical scissors and homogenized with three strokes of a loose-fitting Potter-Elvehjem apparatus (Fisher Scientific, St. Louis, MO) operated at 2,000 rpm. The cytosolic fraction was subsequently isolated by differential centrifugation as previously described (20). Briefly, crude myocardial homogenate was initially centrifuged at 10,000 ×g for 20 min and the upper layer of the supernatant containing visible lipid was gently removed by aspiration. The resultant supernatant was then centrifuged at 85,000 ×g for 60 min and the microsomal pellet was discarded.

Human myocardial cytosolic calcium-independent phospholipase A₂ was purified by sequential anion exchange, chromatofocusing, ATP-affinity, and Mono-Q chromatographies (20). Briefly, human myocardial cytosol was filtered through glass wool, dialyzed against 2 × 10 liter (8 h each) of buffer 1 (15 mM imidazole, 5 mM KPO₄, 10% glycerol, pH 7.8) and loaded onto a precolumn DEAE-Sepha cel column (5 × 5 cm, 2.5 ml/min). The column was washed with buffer 1 containing 1 mM DTT, and developed with a discontinuous step gradient of 0.2 M NaCl followed by 1 M NaCl in 10 mM imidazole, 10 mM KCl, 10% glycerol, 1 mM DTT, pH 8.0. Fractions containing the majority of phospholipase A₂ activity in the 0.2 M NaCl eluent were identified, pooled, and dialyzed against 20 liter of buffer 2 (10 mM imidazole, 10 mM KCl, 25% glycerol, 1 mM DTT, pH 8.0) for 15 h. The dialyzed DEAE-Sephasel 0.2 M NaCl eluent was next loaded onto a previously equilibrated PBE-94 (Pharmacia-LKB Biotechnology, Piscataway, NJ) chromatofocusing column (1.6 × 30 cm, 1.5 ml/min), which was developed with a pH gradient generated by application of buffer composed of 10% PB96, 5% PB74, 25% glycerol, 1 mM DTT, pH 5.4. Fractions containing the majority of phospholipase A₂ activity were immediately identified, pooled, and applied to a 1 × 1 cm N₅-{[6-aminoethyl] carbamoyl-methyl} ATP-agarose affinity column previously equilibrated in buffer 3 (10 mM imidazole, 25% glycerol, 1 mM DTT, pH 8.3). After loading, the affinity column was washed with buffer 3, buffer 3 containing 10 mM AMP (which removes the majority of bound proteins), buffer 3 alone (to remove UV absorbing AMP) and, finally, buffer 3 containing 1 mM ATP (which eluted human myocardial calcium-independent phospholipase A₂ in near quantitative yield). The active fractions from the ATP-agarose affinity column eluent were then applied to an HR5/5 Mono-Q column (Pharmacia-LKB Biotechnology) previously equilibrated in buffer 4 (20 mM imidazole, 25% glycerol, 1 mM DTT, pH 8.3) and phospholipase A₂ was eluted utilizing a discontinuous NaCl gradient (0–500 mM).
scraped into test tubes, extracted with chloroform/methanol (1/1, vol/ vol), dried under a stream of nitrogen gas and resuspended in 50 μl of acetone/isoamyl alcohol (85/15, vol/vol). Individual monobenzoate diradyl glycerol derivatives of human myocardial choline or ethanolamine glycerophospholipid molecular species were subsequently resolved by reverse-phase HPLC utilizing an octadecyl silica stationary phase and an acetone/isoamyl alcohol (85/15, vol/vol) mobile phase at a flow rate of 1 ml/min. Identification of each monobenzoate diradyl glycerol molecular species derived from human myocardial choline or ethanolamine glycerophospholipids was facilitated by collecting column eluents from each UV absorbance peak (231 nm), drying eluents under nitrogen and analyzing their acid methanolysis derivatives by capillary gas chromatography. The relative mass distribution of individual molecular species of straight-phase HPLC purified human myocardial choline or ethanolamine glycerophospholipids was also independently determined by fast atom bombardment mass spectrometry as previously described (22).

Sources of materials and miscellaneous procedures. Bolton-Hunter iodination, SDS-PAGE, 125I autoradiography, and protein determinations were performed as previously described (20). [3H]CH3I was obtained from Amersham Corp., Arlington Heights, IL. All other radiolabeled materials were purchased from Dupont-New England Nuclear, Boston, MA. Oleic and arachidonic acids were obtained from NuChek Prep, Inc. (Elysian, MN), while all other lipids were obtained from Avanti Polar Lipids (Alabaster, AL). DEAE-Sepharose, PBE-94, PB74, PB96, and Mono-Q columns were purchased from Pharmacia LKB Biotechnology, Inc. Nucleotides of the highest quality (i.e., vanadate-free where possible) were obtained from either Boehringer Mannheim Biochemicals, Indianapolis, IN or Sigma Chemical Co., St. Louis, MO. Most other reagents were obtained from either Aldrich Chemical Co., Milwaukee, WI or Sigma Chemical Co.

Results

Chromatographic resolution and kinetic characterization of human myocardial cytosolic phospholipase A2 isoforms. To characterize the polypeptide(s) mediating phospholipase A2 activity in human myocardial cytosol, the protein constituents in dialyzed cytosol were initially fractionated by anion exchange chromatography. The majority of phospholipase A2 activity was adsorbed to the stationary phase under the conditions employed and was eluted by application of buffer containing 200 mM NaCl. However, a second peak of phospholipase A2 activity required higher salt concentrations for elution (peak 2) (Fig. 1). Phospholipase A2 activity in peak 1 represented ~ 90% of recovered activity and was relatively stable while the enzymatic activity in peak 2 accounted for ~ 10% of eluted activity and was markedly labile under the conditions employed. No calcium-dependent phospholipase A2 activity in the cytosol or in column eluents was detectable under the conditions employed.

To determine if the polypeptide(s) catalyzing calcium-independent phospholipase A2 activity in peaks 1 and 2 (Fig. 1) possessed similar physical and kinetic characteristics, the substrate specificities, calcium requirements, and pH profiles of enzymatic activity in each peak were determined. Phospholipase A2 activity in peak 1 was calcium-independent, neutral active (pH optimum 7.0) and possessed a threefold selectivity for hydrolysis of 16:0,[1-3H]18:1 plasmalogenyl choline substrate over 16:0,[3H]18:1 phosphatidylycholine substrate (Fig. 2). Phospholipase A2 activity in peak 2 was also calcium-independent but, in sharp contrast, it possessed a basic pH optimum (pH 8.5) and hydrolyzed phosphatidylcholine and plasmalogenyl choline substrates at similar rates (Fig. 2). The regiospecificity of phospholipase A2 activities in peaks 1 and 2 was confirmed by demonstration of the concomitant release of sn-2 radiolabeled fatty acid and polar head group labeled lysylplasmenylcholine from 16:0,[3H]18:1 plasmalogenylcholine and 16:0, 18:1 ([3H]Me-cylcholine) plasmalogenylcholine, respectively.

Purification of the major human myocardial phospholipase A2 isoform. The polypeptide(s) catalyzing phospholipase A2 activity in peak 1 was purified to apparent homogeneity by sequential chromatofocusing, affinity, and Mono-Q chromatographies. The viability of the polypeptide catalyzing phospholipase A2 activity in peak 2 precluded further purification of the enzyme.

Figure 1. DEAE-Sepharose chromatography of human myocardial cytosolic calcium-independent phospholipases A2. Human myocardial cytosol was dialyzed and loaded onto a DEAE-Sepharose column, and phospholipase A2 activity was eluted with a discontinuous NaCl gradient as described in Methods. Aliquots of column eluents were assayed by quantifying radiolabeled fatty acid released (●) from 16:0,[3H]18:1 plasmalogenylcholine as described in Methods. (——) UV absorbance at 280 nm.

![Figure 1](image1)

Figure 2. Comparison of human myocardial cytosolic phospholipase A2 isoforms. Phospholipase A2 activity in peak 1 (200 mM NaCl eluent) or peak 2 (1 M NaCl eluent) was assessed at the indicated pH by quantifying radiolabeled fatty acid release from 16:0,[3H]18:1 plasmalogenylcholine or 16:0,[3H]18:1 phosphatidylcholine in buffer containing 200 mM K[PO4], 4 mM EGTA, 4 mM EDTA, pH 4.5–9.0, as described in Methods. Data are expressed as PK;: [3H]-fatty acid released from either 16:0,[3H]18:1 plasmalogenylcholine (●), or 16:0,[3H]18:1 phosphatidylcholine ( ○). PK2: [3H]-fatty acid released from either 16:0,[3H]18:1 plasmalogenylcholine ( ● ), or 16:0,[3H]18:1 phosphatidylcholine ( ▲ ). Data points represent the mean of duplicate determinations.

![Figure 2](image2)
this fraction in our hands. Phospholipase A₂ activity in peak 1 was focused into a major peak eluting at pH 6.9 during chromatofocusing which was reproducibly preceded by a smaller peak of activity eluting between pH 7.6–7.7 (Fig. 3). Further characterization of the pH optimum and choline glycerophospholipid subclass selectivity of the peak fraction from the major peak (pH 6.9) demonstrated the selective hydrolysis of vinyl ether containing choline glycerophospholipids at a pH optimum of 7.0. Interestingly, the earlier eluting peak preceding the elution of the major position of phospholipase A₂ activity hydrolyzed diacyl and vinyl ether containing choline glycerophospholipids at similar rates and possessed a pH optimum of 9 (similar to the behavior of peak 2 from the DEAE-Sephadex column).

Chromatofocusing fractions possessing the majority of phospholipase A₂ activity were pooled and subsequently loaded onto an ATP-agarose affinity matrix as described in Methods. Phospholipase A₂ activity selectively and quantitatively adsorbed to the ATP-affinity matrix while the majority of other proteins eluted in the void volume (Fig. 4). Application of buffer containing 10 mM AMP failed to elute substantive amounts of phospholipase A₂ activity even though the large majority of proteins which were adsorbed onto the affinity matrix were eluted (Fig. 4). Subsequent application of buffer containing 1 mM ATP resulted in recovery of 85% of applied phospholipase A₂ activity with a 120-fold purification of human myocardial cytosolic phospholipase A₂ activity achieved in a single step (Table I, Fig. 4). Human myocardial cytosolic calcium-independent phospholipase A₂ was subsequently purified to apparent homogeneity by application of the ATP-agarose eluent to an HR5/5 Mono-Q column with subsequent elution utilizing a shallow discontinuous NaCl gradient.
Table 1. Human Myocardial Cytosolic Calcium-independent Phospholipase A₂ Purification Table

<table>
<thead>
<tr>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>nmol/min</td>
<td>nmol/mg-min</td>
<td>-fold</td>
</tr>
<tr>
<td>Cytosol</td>
<td>963</td>
<td>356</td>
<td>0.37</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1*</td>
<td>247</td>
<td>271</td>
<td>1.10</td>
<td>3.00</td>
</tr>
<tr>
<td>Peak 2*</td>
<td>(66)</td>
<td>(36)</td>
<td>(0.54)</td>
<td>(1.5)</td>
</tr>
<tr>
<td>Chromatofocusing</td>
<td>7</td>
<td>189</td>
<td>27</td>
<td>73</td>
</tr>
<tr>
<td>ATP-agarose</td>
<td>0.05</td>
<td>160</td>
<td>3,200</td>
<td>8,650</td>
</tr>
<tr>
<td>Mono-Q</td>
<td>0.002</td>
<td>134</td>
<td>67,200</td>
<td>181,600</td>
</tr>
</tbody>
</table>

Human myocardial calcium-independent phospholipase A₂ was purified by sequential DEAE, chromatofocusing, ATP-agarose, and Mono-Q chromatographies. Phospholipase A₂ activity in human myocardial cytosol and column chromatographic fractions was assayed utilizing 16:0, [³H]18:1 plasmenylcholine substrate as described in Methods. The only radiolabeled product detected was [³H]oleic acid, without detectable radiolabeled lysophospholipid, diglyceride, monoglyceride, or phosphatidate.

* 200 mM NaCl eluent.

† Subsequent purification of cytosolic phospholipase A₂ activity utilized peak 1 from DEAE-Sepharose chromatography.

‡ 1 M NaCl eluent.

(Fig. 5). Collectively, this series of column chromatographic steps resulted in the 182,000-fold purification of the major human myocardial calcium-independent phospholipase A₂ isoform in 38% yield to a final specific activity of 67 µmol/mg-min (Table 1).

**SDS-PAGE and ¹²⁵I autoradiography.** The purity of the phospholipase A₂ preparation was analyzed by SDS-PAGE and ¹²⁵I autoradiography after Bolton-Hunter iodination (Fig. 6). An intense band at 40 kDa was observed in the most active fraction (fraction 13) from Mono-Q chromatography. Furthermore, the intensity of the 40-kDa band precisely correlated with the relative amounts of phospholipase A₂ activity found in each fraction of Mono-Q column chromatographic eluent. The high sensitivity and dynamic range of the visualization method employed (¹²⁵I autoradiography), the high specific activity of the purified polypeptide (67 µmol/mg-min), as well as the concordant appearance and disappearance of the 40-kDa band with phospholipase A₂ activity collectively demonstrate that the 40-kDa polypeptide catalyzes human myocardial phospholipase A₂ activity.

**Kinetic characterization of purified human myocardial phospholipase A₂.** To determine the phospholipid class selectivity of human myocardial calcium-independent phospholipase A₂, two types of kinetic experiments were performed. First, the initial velocity of phospholipase A₂ activity was determined as a function of substrate concentration utilizing either homogeneous phosphatidylcholine, phosphatidylinositol, or phosphatidylethanolamine containing radiolabeled arachidonic acid at the sn-2 position. Both phosphatidylcholine and phosphatidylinositol are present in the bilayer configuration at the assay temperature employed (i.e., 37°C), while phosphatidylethanolamine assumes an inverted hexagonal II phase.

**Figure 5.** Mono-Q chromatography of human myocardial cytolsis calcium-independent phospholipase A₂, Active fractions from ATP affinity chromatography were pooled and loaded onto an HR5/5 fast protein liquid chromatography-Mono-Q column, and phospholipase A₂ was eluted utilizing a nonlinear NaCl gradient as described in Methods. Phospholipase A₂ activity was assessed utilizing 16:0, [³H]18:1 plasmenylcholine substrate, and radiolabeled fatty acid release was quantified as described in Methods. (---) UV absorbance at 280 nm; (-----) NaCl concentration. Arrows identify the elution of UV absorbing nucleotides AMP, ADP, and ATP.

**Figure 6.** Elution of protein constituents from Mono-Q chromatography as revealed by ¹²⁵I autoradiography SDS-PAGE. Aliquots of the active fractions from fast protein liquid chromatography-Mono-Q chromatography were iodinated with Bolton-Hunter reagent, boiled for 1 min in the presence of 100 mM 2-mercaptoethanol and 10% SDS, loaded onto a polyacrylamide slab gel, electrophoresed, fixed, dried, and subsequently visualized by ¹²⁵I autoradiography. Fraction numbers on the x-axis correspond to fractions from the Mono-Q column shown in Fig. 5.
configuration at this temperature (35). Utilizing these homogeneous systems, and highly purified human myocardial cytosolic phospholipase A2 (Mono-Q eluent; 180,000-fold purified) phosphatidylyethanolamine was the preferred substrate, yielding an apparent $V_{\text{max}} = 194 \mu\text{mol/mg min}$ followed by phosphatidylcholine (apparent $V_{\text{max}} = 71 \mu\text{mol/mg min}$) with modest hydrolysis manifest utilizing the highly negatively charged phosphatidylinositol bilayer substrate (apparent $V_{\text{max}} = 8.4 \mu\text{mol/mg min}$). Furthermore, because the apparent affinity of purified phospholipase A2 for either choline or ethanolamine glycerophospholipid substrate is approximately an order of magnitude greater than that for the corresponding inositol glycerophospholipid (i.e., apparent $K_{\text{m}} = 4 \mu\text{M}$ for phosphatidylcholine or phosphatidylethanolamine vs. 34 $\mu\text{M}$ for phosphatidylinositol), the catalytic efficiency of the purified phospholipase A2 is over two orders of magnitude less utilizing phosphatidylinositol substrate in comparison to phosphatidylethanolamine or phosphatidylcholine.

In that the activities of phospholipases A2 are profoundly influenced by the interfacial characteristics of aggregate substrate, additional experiments were performed to compare hydrolysis of these individual classes of arachidonic acid containing phospholipids presented as substitutional impurities in vesicles comprised of binary mixtures of 10 mol% radiolabeled phosphatidylcholine, phosphatidylethanolamine, or phosphatidylethanolamine and 90 mol% unlabeled phosphatidylcholine (16:0, 20:4). The rank order of substrate selectivity in this system was phosphatidylcholine > phosphatidylethanolamine > phosphatidylinositol (Table II). Thus, these results demonstrate that phosphatidylinositol is a suitable substrate for human myocardial phospholipase A2 when presented in a physiologically relevant bilayer system possessing physiologically relevant surface charge and zwitterionic character (i.e., phosphatidylinositol was hydrolyzed at over one-half the rate observed for phosphatidylethanolamine in similar phosphatidylcholine matrices). The change in the relative rank order of substrate class selectivities (i.e., choline vs. ethanolamine glycerophospholipid) demonstrates that the physical state and interfacial characteristics of aggregate substrates are important determinants of the catalytic efficiency of human myocardial calcium-independent phospholipase A2.

To examine the subclass and molecular species specificities of the purified human myocardial phospholipase A2, additional experiments were performed utilizing phosphatidylethanolamine and plasmenylcholine substrates containing either radiolabeled oleic acid or arachidonic acid at the sn-2 position and enzyme purified through the ATP column chromatographic step (8,600-fold purified). Comparisons of the relative rates of hydrolysis demonstrated that human myocardial phospholipase A2 preferentially hydrolyzed vinyl ether containing choline glycerophospholipids (Fig. 7). Furthermore, plasmenylcholine substrate containing arachidonic acid at the sn-2 position was hydrolyzed over twofold more rapidly than plasmenylcholine substrate containing oleic acid at the sn-2 position. This contrasted with phosphatidylcholine molecular species containing either oleic or arachidonic acid at the sn-2 position which manifest similar rates of hydrolysis at each substrate concentration examined.

**Table II. Human Myocardial Cytosolic Calcium-independent Phospholipase A2 Class Specificity Assessed by Incorporating Radiolabeled Substrate as a Substitutional Impurity in a Phosphatidylcholine Matrix**

<table>
<thead>
<tr>
<th>sn-2 Radiolabeled glycerophospholipid</th>
<th>Fatty acid released (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>49</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>115</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>88</td>
</tr>
</tbody>
</table>

Fresh homogeneous myocardial cytosolic calcium-independent phospholipase A2 (18 mg of the Mono-Q eluent) was incubated with the indicated class of radiolabeled glycerophospholipid ([3H]arachidonic acid incorporated at the sn-2 position of each class) incorporated as a 10 mol% impurity within a 100 $\mu\text{M}$ bilayer matrix [bulk lipid concentration of phosphatidylcholine (16:0, 20:4)] for 60 s at 37°C. Released radiolabeled fatty acid was subsequently isolated after butanol extraction, separated by TLC, and quantified by scintillation spectrometry as described in Methods.

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*Figure 7. Lineweaver-Burk plot of the substrate concentration-velocity profiles of purified human myocardial cytosolic calcium-independent phospholipase A2. Purified human myocardial phospholipase A2 (ATP eluent 8,650-fold purified) was incubated with the indicated concentrations of sn-2 radiolabeled plasmenylcholine or phosphatidylcholine (containing either [3H]oleic or [3H]arachidonic acid at the sn-2 position as indicated) and reaction products were extracted with butanol, isolated by TLC, and quantified by scintillation spectrometry as described in Methods. Data points represent the mean of duplicate determinations. 16:0,[3H]18:1 phosphatidylcholine (○); 16:0,[3H]20:4 phosphatidylethanolamine (△); 16:0,[3H]18:1 plasmenylcholine (●); 16:0,[3H]20:4 plasmenylcholine (▲).*
Figure 8. Reverse-phase HPLC of monobenzoate derivatives of ethanolamine and choline glycerophospholipids in human ventricular tissue. Human ventricular ethanolamine and choline glycerophospholipids were purified by straight-phase HPLC, hydrolyzed by Bacillus cereus phospholipase C, and derivatized to their monobenzoate derivatives as described in Methods. Individual molecular species were separated by reverse-phase HPLC utilizing an octadecyl silica column employing isocratic elution with acetonitrile/isopropanol (85/15, vol/vol) as the mobile phase. (A) Peaks from reverse-phase HPLC analysis of monobenzoate derivatives of ethanolamine glycerophospholipids were identified as follows: 18:1-20:4 phosphatidylethanolamine (1), 16:0-20:4 phosphatidylethanolamine (2), 18:1-20:4 plasmenylethanolamine (3), 16:0-20:4 plasmenylethanolamine (4), 18:0-20:4 phosphatidylethanolamine (5), 18:0-18:2 phosphatidylethanolamine (6), and 18:0-20:4 plasmenylethanolamine (7). (B) The peaks of individual molecular species of the monobenzoate derivatives of choline glycerophospholipids were identified as follows: 16:0-20:4 phosphatidylcholine (1), 18:1-18:2 phosphatidylcholine (2), 16:0-18:2 phosphatidylcholine and 16:0-20:4 plasmenylcholine (3), 18:0-20:4 phosphatidylcholine (4), 16:0-18:2 plasmenylcholine (5), 16:0-18:1 phosphatidylcholine (6), 16:0-16:0 plasmenylcholine (7), 16:0-18:1 plasmenylcholine (8), 16:0-16:0 plasmenylcholine (9), and 18:0-18:1 phosphatidylcholine (10). Assignments of molecular species identities of derivatized ethanolamine and choline glycerophospholipids to each peak were determined by derivatizing eluents corresponding to each peak by acid methanolysis and subsequent analysis by capillary gas chromatography.

Reverse-phase HPLC of monobenzoate diradyl glycerol derivatives of human myocardial glycerophospholipids, as well as fast atom bombardment mass spectral analysis of underivatized ethanolamine glycerophospholipids, revealed that plasmenylethanolamine is the predominant molecular subclass of human myocardial ethanolamine glycerophospholipids (Figs. 8A and 9A; Table III). Plasmenylethanolamine molecular species predominantly contained either 16:0, 18:0 or 18:1 vinyl ether aliphatic constituents at the sn-1 position and arachidonic acid at the sn-2 position (Figs. 8A and 9A; Table III). The predominant phosphatidylethanolamine molecular species present in human myocardial ethanolamine glycerophospholipids contained stearic acid at the sn-1 position and arachidonic acid at the sn-2 position (Figs. 8A and 9A; Table III).

Reverse-phase HPLC of monobenzoate diradyl glycerol derivatives of human choline glycerophospholipids, as well as fast atom bombardment mass spectrometry of underivatized choline glycerophospholipids demonstrated that > 25% of the choline glycerophospholipids were composed of plasmalogen molecular species (Figs. 8B and 9B; Table III). Plasmenylcholine in human myocardium contained predominantly the vinyl

Figure 9. Fast atom bombardment mass spectrometry of human ventricular choline and ethanolamine glycerophospholipids. Straight-phase HPLC-purified (A) ethanolamine or (B) choline glycerophospholipids (~ 300 nmol) each were dissolved in 20 µl of chloroform/methanol (1/1, vol/vol) and 2 µl was subsequently mixed with ~ 3 µl of glycerol on a copper probe. Fast atom bombardment mass spectrometry was performed as described in Methods. Protonated molecular species of ethanolamine glycerophospholipids from ventricular tissue were identified as 16:0-20:4 plasmenylethanolamine (m/z = 724), 18:1-20:4 plasmenylethanolamine (m/z = 750), 18:0-20:4 plasmenylethanolamine (m/z = 752), and 18:0-20:4 phosphatidylethanolamine (m/z = 768). Protonated molecular species of choline glycerophospholipids were identified as 16:0-18:2 plasmenylcholine (m/z = 742), 16:0-18:1 plasmenylcholine (m/z = 744), 16:0-18:2 phosphatidylcholine (m/z = 758), 16:0-18:1 phosphatidylcholine (m/z = 760), 16:0-20:4 plasmenylcholine (m/z = 766) and 16:0-20:4 phosphatidylcholine (m/z = 782).
ether of palmitaldehyde at the sn-1 position with arachidonic > linoleic > oleic acid at the sn-2 position (Figs. 8 B and 9 B; Table III). Phosphatidylcholine molecular species in human myocardial choline glycerophospholipids contained palmitic acid at the sn-1 position and, conversely, arachidonic < linoleic < oleic acid at the sn-2 position (Figs. 8 B and 9 B; Table III). These results demonstrate the predominance of plasmalogens in the ethanolamine glycerophospholipid pool and the abundance of vinyl ether linkages in choline glycerophospholipid molecular species in human myocardium.

Discussion

The relationship of accelerated phospholipid catabolism during myocardial ischemia to the pathophysiologic sequelae of myocardial infarction has been an area of extensive investigation during the last decade. A multiplicity of experimental animal and cell culture models has been employed to study the potential relevance of the activation of phospholipases to the electrophysiologic dysfunction and myocytic cellular necrosis manifest during human myocardial infarction. During the course of these studies, a large diversity of mammalian phospholipases has been identified and an intriguing heterogeneity of individual phospholipid constituents in myocardium from many different species has been documented. Specifically, in several animal species (e.g., dog and rabbit) plasmalogens are the predominant phospholipid constituent of critical subcellular membranes (e.g., sarcolemma and sarcoplasmic reticulum) (22, 23), and plasmalogen-selective phospholipases A2 are the major measurable phospholipase activity in many tissues (13, 17, 20). Despite substantial evidence implicating the importance of plasmalogen catabolism during ischemic and reperfusion injury (13, 14, 36–38), the purification of human myocardial phospholipase A2 and the chemical identification of the individual molecular species of human myocardial phospholipids have not been forthcoming. Herein we demonstrate that the major isoform of human myocardial cytosolic calcium-independent phospholipase A2 activity is a 40-kD polypeptide (similar to that found in other mammalian species such as canine and rabbit myocardium) (13, 20) and that plasmalylcholine and plasmenylethanolamine molecular species enriched in arachidonic acid represent prominent chemical constituents of human myocardial phospholipids.

The purification strategy for this human myocardial phospholipase A2 exploited the previously identified affinity interaction between cytosolic calcium-independent phospholipase A2 and ATP (39) which facilitated the 182,000-fold purification of the human myocardial enzyme in 38% overall yield. During the course of the purification, evidence for the presence of functionally distinct isoforms of calcium-independent phospholipase A2 was uncovered. In contrast to previous experimental results in both canine and rabbit myocardium, human myocardium contained a calcium-independent phospholipase A2 activity which required high salt for elution from the anion exchange matrix and possessed distinct physical and kinetic properties. Although the major phospholipase A2 isoform selectively hydrolyzed plasmalogen substrate, possessed a pH optimum of 7.0, and was relatively stable, the minor isoform (eluting at high NaCl concentrations) hydrolyzed plasmenylcholine and phosphatidylcholine substrates at similar rates, possessed a pH optimum of 8.5 and was markedly labile. Although definitive identification of the presence of two separate phospholipase A2 gene products requires sequence determination and detailed chemical analyses of both isoforms, the present results identify the existence of two functionally distinct calcium-independent phospholipase A2 activities in human myocardium. The precise chemical differences underlying these functional alterations remain unknown at present.
Kinetic analyses of the class, subclass and molecular specificities of the purified human myocardial phospholipase A₂ in specific systems demonstrated that plasmenagens containing arachidonic acid at the sn-2 position are the preferred substrates. Accordingly, the molecular species distribution of phospholipids in human myocardium was analyzed by two independent methods to document the presence of plasmenagens and to identify the relative content of individual molecular species containing arachidonic acid. Substantial amounts of plasmalynolcholine and plasmenylethanolamine molecular species containing arachidonic acid were identified in human myocardium. These results represent the first identification of the individual molecular species distribution of phospholipids in human myocardium. The abundance of plasmenagal molecular species in human myocardium resembles that found in canine, rabbit and hamster myocardium and exceeds that present in rat or guinea pig myocardium (22–27).

**Conclusion.** These results represent the first reported isolation of calcium-independent phospholipase A₂ and its purification to apparent-homogeneity from intact human tissue. They further underscore the potential significance of calcium-independent phospholipases A₂ as the enzymic mediators of ischemia-induced phospholipolysis because this class of enzymes constitutes the overwhelming majority of measurable phospholipase activity in human myocardium. Accordingly, human myocardial cytosolic calcium-independent phospholipases A₂ represent an attractive target for pharmacologic attenuation of ischemic membrane dysfunction in compromised myocardium. Mechanism-based discrimination between calcium-dependent and calcium-independent phospholipases A₂ employing suicide inhibition has recently been reported (40). The development of isoform-specific calcium-independent phospholipase A₂ inhibitors which achieve their selectivity by exploiting the chemical determinants responsible for the distinct substrate specificities exhibited by calcium-independent phospholipase A₂ isoforms should facilitate the development of selective agents capable of attenuating membrane dysfunction during myocardial ischemia.

**Acknowledgments**

We would like to acknowledge the sustained efforts of Dr. Jeffrey Saf-sitz in the procurement of human ventricular myocardium for these studies. We also thank Rayna Hollenback for assistance in the preparation of this manuscript.

This research was supported by grant 34839 from the National Institutes of Health.

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