Purification and Characterization of Canine Myocardial Cytosolic Phospholipase A₂

A CALCIUM-INDEPENDENT PHOSPHOLIPASE WITH ABSOLUTE sn-2 REGIOSELECTIVITY FOR DIRADYL GLYCEROPHOSPHOLIPIDS

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Myocardial ischemia is associated with numerous biochemical alterations which collectively influence, and synergistically contribute to, the accumulation of amphiphilic metabolites in ischemic zones (e.g. Refs. 1-5). Concomitant with the onset of myocardial ischemia, phospholipase A₂ activity is augmented resulting in the release of unsaturated fatty acids and the accumulation of lysophospholipids (e.g. Refs. 6-8). Lysophospholipids are potent membrane perturbing metabolites which alter the dynamics of myocardial sarcolemmal membranes (9) and precipitate electrophysiologic alterations in vitro which are indistinguishable from those present during myocardial ischemia in vivo (10, 11). Accordingly, we and others have suggested that activation of phospholipase A₂ and the resultant accumulation of lysophospholipids is intimately related to the development of electrophysiologic dysfunction in ischemic myocardium.

Myocardial sarcolemma is predominantly comprised of plasmalogen molecular species (12, 13), and the sarcolemmal membrane is the primary target of accelerated phospholipid catabolism in myocytes subjected to simulated ischemia (14). In previous studies we demonstrated that the major measurable phospholipase A₂ activity in canine myocardium is calcium-independent and has direct physical access to the sarcolemmal membrane (15). Since accelerated sarcolemmal phospholipid catabolism has been implicated as the biochemical mechanism underlying electrophysiologic dysfunction and myocyte cell death during myocardial ischemia, the purification and characterization of this calcium-independent phospholipase A₂ is of obvious importance. We now report the 154,000-fold purification of canine myocardial cytosolic phospholipase A₂ to homogeneity and demonstrate that the purified enzyme has kinetic properties which make it the likely enzymatic mediator of accelerated sarcolemmal phospholipid catabolism during myocardial ischemia.

EXPERIMENTAL PROCEDURES

Purification of Canine Myocardial Cytosolic Phospholipase A₂—Mongrel dogs (25-35 kg) fed ad libitum were anesthetized with intravenous sodium pentothal (40 mg/kg). Following a left thoracotomy, the heart was removed and immediately placed in homogenization buffer (0.25 M sucrose, 10 mM imidazole, 10 mM KCl, 5 mM K[PO₄], pH 7.8) at 0 °C. Ventricular tissues were rapidly trimmed of fat, weighed, and placed in fresh ice-cold homogenization buffer (25% w/v). Myocardium was finely minced (0.2 × 0.4-cm pieces) and homogenized utilizing a loose-fitting Potter-Elvehjem homogenizer (3 strokes at 2,000 rpm) on ice. All further purification steps were performed at 4 °C. Nuclei, cellular debris, and mitochondria were removed by centrifugation at 10,000 × gₑₑₑ for 20 min, and the supernatant was subsequently centrifuged at 85,000 × gₑₑₑ for 60 min to separate the cytosolic and microsomal fractions.

The supernatant (cytosol) was initially filtered through glass wool, dialyzed twice (8 h/dialysis) against 10 liters of buffer 1 (15 mM imidazole, 5 mM K[PO₄], 10% glycerol, pH 7.8), and loaded onto a previously equilibrated DEAE-Sephaose column (5 × 7 cm, 3 ml/min). The column was subsequently washed with buffer 1 containing...
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1 nM DTT, and phospholipase A2 activity was eluted by application of a 100 mM NaCl stepwise gradient buffer consisting of 10 mM imidazole, 10 mM KCl, 10% glycerol, 1 mM DTT, pH 8.0. Active fractions were identified, pooled, dialyzed against 20 liters of buffer until no further activity was detected (10 mM imidazole, 10 mM KCl, 25% glycerol, 1 mM DTT, pH 8.0) and loaded onto a pre-equilibrated PBE-94 chromatofocusing column (1.6 x 30 cm, 1.8 ml/min). A shallow pH gradient was subsequently generated utilizing 10% PB96, 5% PB74, 25% glycerol, 1 mM DTT, pH 8.1. Active fractions from the chromatofocusing column were identified and immediately applied to a 1 x 1.5 cm N-[6-aminohexyl]carbamoylmethyl]ATP-agarose column previously equilibrated with buffer 3 (10 mM imidazole, 95% glycerol, 1 mM DTT, pH 8.3) (Sigma Lot No. 124F-78851 or Pharmacia LKB Biotechnology Inc. Lot No. AG5461101 gave the best yields) at 2 ml/min. The affinity column was extensively washed in buffer 3 containing 10 mM adenosine, and buffer 3 containing 10 mM AMP prior to further washing with buffer 3 alone (to remove uv absorbing AMP). Phospholipase A2 activity was quantitatively eluted by application of buffer 3 containing 1 mM ATP. The active fractions from ATP affinity chromatography were directly loaded onto an HR5/5 Mono Q column previously equilibrated with buffer 4 (20 mM imidazole, 25% glycerol, 1 mM DTT, pH 8.3), and myocardial phospholipase A2 activity was subsequently eluted as a single linearly migrating peak (0–450 mM NaCl). Active fractions were identified and immediately applied to a HPLC column (4 mm x 10 cm) previously equilibrated with buffer 5 (10 mM KPO4, 50% glycerol, 1 mM DTT, pH 7.4). Homogenous myocardial cytosolic phospholipase A2 was subsequently eluted utilizing a nonlinear KPO4 gradient (0–450 mM NaCl).

Preparation of Synthetic Phospholipids—Homogeneous 1-O-(Z)-hexadec-1'-enyl-GPC was obtained by alkaline methanolation of bovine heart choline glycerophospholipids, purified by silicic acid column chromatography, and was resolved into individual molecular species by isocratic reverse-phase HPLC as previously described (16). Synthesis of sn-2 radiolabeled plasmalogen was performed by dicyclohexylcarbodiimide-mediated esterification of radiolabeled fatty acid anhydride followed by its condensation on the sn-2 hydroxyl of 1-O-(Z)-hexadec-1'-enyl-GPC utilizing N,N-dimethyl-4-aminopyridine as catalyst (17). Each radiolabeled choline glycerophospholipid molecular species was initially purified by preparative thin layer chromatography (15) and subsequently purified by Partisil SCX-HPLC chromatography (18). Synthesis and purification of sn-2 radiolabeled lysophosphatidylcholine and alkyl-ether choline glycerophospholipid molecular species were performed similarly utilizing the appropriate radiolabeled fatty acid and lysophosphoglyceride as starting materials. Specific molecular species of unlabeled phosphatidylcholine, plasmalogen, or alkyl-ether choline glycerophospholipids were synthesized and purified similarly. To facilitate direct kinase assays between diacyl, vinyl ether, and alkyl-ether choline glycerophospholipid classes, radiolabeled molecular species of identical specific activities were synthesized by utilizing common preparations of freshly synthesized radiolabeled fatty acyl anhydride and the appropriate lysophospholipid subclass.

1 The abbreviations used are: DTT, dithiothreitol; DPPC, 1-palmitoyl-2-palmitoyl-sn-glycero-3-phosphocholine; DPPE, 1-palmitoyl-2-palmitoyl-sn-glycero-3-phosphoethanolamine; GPC, sn-glycero-3-phosphoglyceride; GPE, sn-glycerol-3-phosphate; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PA, phosphatic acid; PAP, 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphoethanolamine; PAPC, phosphatidylethanolamine; HPLC, high pressure liquid chromatography; PFLC, fast protein liquid chromatography; EGTA, ethylenebis(ethylenedinitrilotetraacetic acid); CHAPS, 3-[3-(cyclohexylpropyl)dimethylammonio]1-propanesulfonate acid.

Enzyme Assays—Phospholipase A2 activity in column chromatographic fractions was routinely assayed by incubating enzyme (5–50 μl) with 2 μM 1-O(Z)-hexadec-1'-enyl-2-[9,10-3H]octadec-9'-enoyl-GPC (introduced by ethanol injection (10 μl) in assay buffer (final conditions: 100 mM Tris, 4 mM EGTA, 5% glycerol, pH 7.0) at 37°C for 5 min in a final volume of 200 μl. Reactions were quenched by addition of 100 μl of butanol saturated思想, and the organic phase was separated by centrifugation. Released radiolabeled fatty acid was isolated by application of 25 μl of the butanol phase to channelled silica gel G plates, development in petroleum ether/ethyl ether/acetate acid (70:30:1), and subsequent quantification by scintillation spectrometry. Kinetic assays of phospholipase A2 activity were performed similarly except that incubations were performed for 1 min which resulted in linear reaction velocities with respect to both time and enzyme concentration for each substrate examined.

Hydrolysis of 1,2-dipalmitoyl-(N-methyl-[3H]G)PC and 1-[14C]palmitoyl-2-palmitoyl-GPE was assessed similarly except reactions were quenched with 200 μl of butanol and reaction products were separated by thin layer chromatography utilizing silica OF plates prepared with a solution of CHCl3/fucose/MeOH/CH3OH/H2O (68:2:2:1) (as previously described (19)).

Phospholipase A2 activity was assessed by incubating 100–150 μl of column eluents with 13 μM 1-[14C]palmitoyl lysophosphatidylcholine in assay buffer (final volume = 200 μl) for 5 min at 37°C. 1-[14C]palmitic acid was quantified as described above.

Purification of myocardial phospholipase A2—Purification of myocardial phospholipase A2 was achieved utilizing previously established techniques (22–24).

Sensitivity of Myocardial Phospholipase A2 Activity to Chemical Modification—The ATP affinity column eluate (5 μl) was incubated with either 1 mM dithiothreitol, 1 mM paraformaldehyde, or 10 μM phenylmethylsulfonyl fluoride, diazylated against buffer 3 and subsequently assayed as described above.

Thermal Denaturation—The purified protein was incubated at 37°C for 10 min at various concentrations of substrate (3 X K0). After an additional 1 min incubation, products were extracted with butanol, separated by thin layer chromatography and quantified as described above.

Ionization, Sodium Dodecyl Sulfate, Polyacrylamide Gel Electrophoresis, and Autoradiography of Myocardial Phospholipase A2—Aliquots of HPLC-hydroxyapatite active fractions (100 μl) were reacted with 250 μCi 105I-T-Bolton-Hunter reagent (specific activity = 4400 Ci/mmol) overnight at 0–4°C (32). Unbound inactivated reagent was removed during electrophoresis (unbound reagent precedes dye front) in 10% sodium dodecyl sulfate-polyacrylamide gels prepared by the Laemmli method (33). Gels were subsequently fixed (three changes) in H2O2/MeOH/AcOH (5:5:1) with packets of mixed bed resin in gauze to reduce the background intensity of autoradiographs of dried gels.

Protein Determinations—Protein content was determined utilizing a Bio-Rad protein assay kit (fourth through sixth steps) using bovine serum albumin as standard.

Sources of Materials—[14C]Palmitic acid, [14C]stearic acid, [14C]oleate, [14C]linoleic acid, [14C]linoieic acid, [14C]linolenic acid, [14C]palmitoyl LPC, [14C]palmitoyl-CoA, [14C]palmitoyl-2-palmitoyl-GPE, and [14C]palmitoyl-2-palmitoyl-GPE were purchased from Du Pont-New England Nuclear. [3H]Dipalmitoyl ethanolamine (specific activity = 20 Ci/mmol) was prepared by the method of Dr. D. A. Ford (Washington University). All other radiolabeled reagents were purchased from Amerham Corp. Bovine heart lecithin, DPPC, and palmitoyl LPC were purchased from Avanti Polar Lipids. PA and lys PA were purchased from Sigma Chemical Corp. Bovine serum albumin, glycerol, and butanol buffer reagents, and the following agarose matrices were obtained
from Sigma: d-ribose-5'-phosphate, AMP (N6-linkage), ADP (N6-linkage), ATP (N6, C-8, and ribose hydroxyl-linkages), GTP (ribose hydroxyl-linkage), and UTP (ribose hydroxyl-linkage). AG-CoA type 5, AG-ATP types 2-4, Blue Sepharose CL-6B, DEAE-Sephalcel, PB74, PB74, PB66, and Mono Q columns were purchased from Pharmacia LKB Biotechnology Inc. All HPLC columns were purchased from P. J. Cobert. Detergents and molecular weight standards were purchased from Pierce Chemical Co. Dicyclohexylcarbodiimide, N,N-dimethyl-4-aminopyridine, deoxycholate, and taurocholate were obtained from Aldrich. All other reagents were obtained from Fisher.

RESULTS

Characterization of Crude Myocardial Cytosolic Phospholipase A₂ Activity—As previously demonstrated (15), the major measurable phospholipase A₂ activity in canine myocardium was present in the cytosolic fraction and manifest maximal enzymic activity in the presence of the calcium chelator EGTA. No calcium-independent hydrolysis of plasmenylcholine substrate could be detected in homogenates of whole blood or plasma. The release of fatty acid from the sn-2 position of plasmalogen substrate by the cytosolic enzyme was catalyzed by phospholipase A₂ since inclusion of an excess of lysophospholipid, diacylglycerol, 1-O-alkyl-1'-enyl-2-acylsn-glycerol or phosphatidic acid did not significantly attenuate the rate of fatty acid release from radiolabeled plasmenylcholine substrate. Kinetic analyses of the cytosolic fraction utilizing several synthetic sn-2 radio-labeled diacyl, alkyl-acyl, and vinyl-ether choline glycerophospholipid molecular species (Table I) confirm and extend our previous report (15) that the major phospholipase A₂ activity in myocardium selectively hydrolyzes ether-linked choline glycerophospholipids. Furthermore, the present results indicate that cytosol contains a calcium-independent phospholipase A₂ activity which preferentially hydrolyzes choline glycerophospholipids containing arachidonic acid at the sn-2 position.

Purification of Canine Myocardial Cytosolic Calcium-independent Phospholipase A₂—To characterize the polypeptide(s) responsible for the observed calcium-independent phospholipase A₂ activity, canine myocardial cytosolic phospholipase A₂ was purified to homogeneity by sequential anion chromatography, and HPLC-hydroxylapatite chromatographies. First, dialyzed cytosol was applied to a DEAE-Sephalcel column, and phospholipase A₂ activity was quantitatively and selectively absorbed (over 99% of other proteins present in the load eluted in the void volume which was devoid of phospholipase activity). The specificity of the interaction between myocardial phospholipase A₂ and the ATP matrix was further exploited through utilization of sequential washes of the affinity matrix with 10 mM adenosine and 10 mM AMP (which removed the majority of bound protein but did not elute substantive phospholipase A₂ activity). Enzyme activity was quantitatively eluted from the ATP-agarose matrix with 1 mM ATP (Fig. 2). Use of this nucleotide affinity matrix resulted in a 150-fold purification of myocardial phospholipase A₂ in quantitative yield accompanied by a 50-fold reduction in volume. Thus, this 3-day procedure results in a 52,000-fold purification of myocardial phospholipase A₂ activity in 86% yield which is moderately stable when stored at 0–4°C (t1/2 = 5–7 d).

TABLE I
Choline glycerophospholipid subclass specificity of myocardial cytosolic phospholipase A₂ activity

| Subclass         | Molecular species | Vmax   | K0    |
|------------------|-------------------|--------|-------|
|                  | sn-1   | sn-2   | pmol/min·μM | μM    |
| Phosphatidylcholine | 16:0 | 18:1   | 0.5   | 18    |
| Plasmenylcholine   | 16:0 | 18:1   | 1.5   | 16    |
| Phosphatidylcholine | 16:0 | 20:4   | 1.1   | 7     |
| Alkyl-ether choline| 16:0 | 20:4   | 1.5   | 9     |
| Glycerophospholipid| 16:0 | 20:4   | 4.6   | 8     |

Myocardial cytosol was incubated with 1–100 μM radiolabeled phospholipid in the presence of 4 mM EGTA and fatty acid was extracted with butanol, separated by thin layer chromatography, and quantified by scintillation spectrometry as described under "Experimental Procedures." All substrates were examined at a minimum of five concentrations each in duplicate from multiple preparations.
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FIG. 2. ATP affinity chromatography of myocardial phospholipase A2. Active fractions from chromatofocusing were immediately applied to a previously equilibrated ATP-agarose column. After loading, the column was washed with equilibration buffer containing 10 mM adenosine, buffer containing 10 mM AMP, and buffer alone for the indicated volumes. Phospholipase activity was eluted with buffer containing 1 mM ATP as described under "Experimental Procedures." Aliquots of column eluates were incubated with 1-O-(Z)-hexadec-1'-enyl-2-[9,10-3H]octade-9'-enoyl-GPC and released radiolabeled fatty acid (O) was quantified as described under "Experimental Procedures."

FIG. 3. FPLC-anion exchange chromatography of myocardial phospholipase A2. The active fractions from ATP affinity chromatography were loaded onto a previously equilibrated HR5/5 Mono Q column, and phospholipase A2 was eluted utilizing a nonlinear NaCl gradient as described under "Experimental Procedures." Phospholipase A2 activity was assayed utilizing 1-O-(Z)-hexadec-1'-enyl-2-[9,10-3H]octade-9'-enoyl-GPC as substrate and fatty acid release (O) was quantified as described under "Experimental Procedures." Lysophospholipase and palmitoyl-CoA hydrolase activities were assayed by quantifying fatty acid release from 1-[l-3Cl]palmitoyl lysophosphatidylcholine (O), or [l-3Cl]palmitoyl-CoA (O), respectively, as described under "Experimental Procedures." Approximately five times the substrate concentration and 20 times the amount of enzyme were used for assays of lysophospholipase and palmitoyl-CoA hydrolase activities in comparison with phospholipase A2 assays as described under "Experimental Procedures." 

Phospholipase A2 was further purified by application of the ATP-agarose eluate onto an FPLC-Mono Q anion exchange column which was subsequently eluted utilizing a shallow discontinuous NaCl gradient (Fig. 3). Mono Q active fractions were directly loaded onto an HPLC-hydroxylapatite column, and phospholipase A2 activity was eluted with a nonlinear K[PO4] gradient as described under "Experimental Procedures." Since the purified enzyme was extremely labile (t1/2 = 30 min at 4 °C), assays of enzymic activity following hydroxylapatite chromatography were performed directly after elution of each fraction. Collectively, this series of column chromatographic steps resulted in a 154,000-fold purification of canine myocardial cytosolic phospholipase A2 to a specific activity of 227 μmol/mg min with an overall yield of 19% (Table II).

Purity of Myocardial Phospholipase A2 after Column Chromatography—To assess the purity of myocardial phospholipase A2 after sequential column chromatographies, the active fractions from the hydroxylapatite column were iodinated with Bolton-Hunter reagent, separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and protein was visualized by autoradiography. Only a single intense band at 40 kDa was observed in the most active fraction (Fig. 5).

FIG. 4. HPLC-hydroxylapatite chromatography of myocardial phospholipase A2. The active fractions from Mono Q chromatography were immediately loaded onto a previously equilibrated HPLC hydroxylapatite column, and phospholipase A2 activity was eluted with a nonlinear K[PO4] gradient as described under "Experimental Procedures." Lysophospholipase (O) and palmitoyl-CoA hydrolase (O) activities were assayed as described under "Experimental Procedures" with over five times the substrate concentration and 20 times the amount of enzyme in comparison to phospholipase A2 assays (A).

FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of myocardial cytosolic phospholipase A2. Aliquots of the active fractions from HPLC-hydroxylapatite chromatography were iodinated, boiled for 3 min in the presence of 100 mM 2-mercaptoethanol and 10% SDS, loaded onto a 10% polyacrylamide slab gel, electrophoresed, fixed, dried, and subsequently visualized by autoradiography as described under "Experimental Procedures." Fraction numbers on the x axis correspond to fractions from the hydroxylapatite column shown in Fig. 4.
Furthermore, the relative intensity of the 40-kDa band precisely paralleled the elution profile of phospholipase A₂ activity during hydroxylapatite chromatography (compare Figs. 4 and 5). In multiple preparations, the 40-kDa polypeptide was the only protein whose intensity paralleled enzymic activity (n > 10) and was the only band visualized after autoradiography of the most active hydroxylapatite fraction in three other independent preparations. Attempts to recover any phospholipase A₂ activity from multiple acrylamide-based gel electrophoresis systems utilizing either pulverized, extracted, or electroeluted gel slices have failed. In fact, incubation of enzyme with even minute amounts of polymerized acrylamide results in complete and unrecoverable loss of all enzymic activity.

Characterization of Myocardial Phospholipase A₂ Binding to Nucleotide Affinity Matrices—The specificity of the interaction responsible for the adsorption of calcium-independent phospholipase A₂ to immobilized nucleotide affinity matrices was examined to gain insights into the chemical interactions contributing to the association of ATP with this phospholipase. Of the three ATP resins tested (see "Experimental Procedures"), coupling via the N⁰-aminogroup provided the highest yield. Attachment through the C-8 or the ribose hydroxyl groups resulted in recovery of 60–80% of loaded enzymic activity in the ATP wash with the majority of remaining activity present in the void volume. Other matrices such as GTP-agarose, UTP-agarose, ADP-agarose, CoA-agarose as well as AMP-agarose all bound myocardial phospholipase to varying extents in the specified rank order (strongest-weakest, 60–10% binding). In contrast, D-ribos-5’-phosphate-agarose did not bind canine myocardial phospholipase A₂ activity. Although Blue Sepharose (CL-6B) quantitatively adsorbed enzymic activity (no activity was present in the void volume), recovery of phospholipase activity after elution with buffer containing ATP, ATP and 1 M NaCl or ATP, and 1 M K[PO₄] was poor (<5%). With the exception of Blue Sepharose (which nonspecifically adsorbed approximately 50% of the loaded proteins), greater than 99% of loaded proteins did not bind to these affinity matrices under the conditions employed. Furthermore, although classic calcium-dependent, low molecular weight phospholipases A₂ are known to bind to the nucleotide analog dye Cibacron Blue FBGA (35), none of the phospholipases A₂ examined (i.e. Naja naja, pancreatic, bcc venom, platelet cytosolic) adsorbed to the ATP resins used.

Kinetic Analyses of Purified Myocardial Phospholipase A₂—The homogenate polypeptide exhibited maximal enzymic activity in the presence of EGTA and possessed a pH optimum of 6.4 for each phospholipid substrate examined. Incubation of the purified enzyme with sn-2-radiolabeled phospholipid (e.g. plasmenylcholine, phosphatidylcholine, or phosphatidylethanolamine molecular species) resulted in the release of radiolabeled fatty acid with no observable radioactivity in lysophospholipid, diradylglycerol, or phosphatidic acid. The possibility that the release of sn-2 fatty acid from diradyl glycerophospholipids occurred by sequential phospholipases A₁ and lysophospholipase activities was eliminated by multiple independent techniques. First, myocardial phospholipase A₂ was incubated with 1–30 μM [³H-Me]choline-labeled DPPC, and the reaction products were isolated and quantified as described under "Experimental Procedures." For each concentration of substrate examined, the loss of PC and the accumulation of LPC was stoichiometric (Fig. 6) with no detectable radio-label in GPC. Second, when sn-2-[³H]labeled DPPC was utilized as substrate under identical assay conditions, the resultant increase in [³H-fatty acid equaled (±3%, n = 2) the increase in [³H-Me]LPC at each concentration examined (Fig. 6). Third, incubation of 1-[¹⁴C]palmitoyl-2-palmitoyl-GPE with purified enzyme resulted in the production of 1-[¹⁴C] palmitoyl-LPE without measurable amounts of radiolabeled palmitic acid, and the mass of phosphatidylethanolamine hydrolyzed was quantitatively accounted for by the mass of 1-acyl LPE produced (Fig. 6). Furthermore, no [¹⁴C]palmitate was released from 1-[¹⁴C]palmitoyl-2-palmitoyl-GPE in the presence of several detergents (i.e. Triton X-100, n-octyl glucoside, Lubrol-PX, or Tween-20). Finally, 100-fold molar excesses of LPC, diacylglycerol, and PA did not substantially diminish release of [³H-fatty acid from 1-O-(Z)-hexadec-1’-enyl-2-[9,10-³H]octadec-9’-enoyl-GPC. Thus, myocardial cytosolic phospholipase A₂ is specific for hydrolysis of the sn-2 ester linkage in choline and ethanolamine diradyl glycerophospholipids and is devoid of measurable phospholipase A₃, C, or D activities. Attempts to demonstrate significant reversibility of the reaction by incubation of purified enzyme with lysophospholipid and radiolabeled fatty acid (in the absence or presence of CoA) were unsuccessful.

Characterization of the phospholipid substrate specificity of purified myocardial cytosolic phospholipase A₂ was performed by kinetic analyses of the ATP eluent (52,000-fold purified, specific activity = 76 μmol/mg min) since the marked lability of Mono Q or hydroxyapatite eluates precluded their use. Examination of the choline glycerophospholipid subclass specificity of the 52,000-fold purified enzyme revealed that hydrolysis of plasmenylcholine substrate was more rapid than hydrolysis of alkyl-ether choline glycerophospholipid or phosphatidylethanolamine (Fig. 7, Table III). Comparisons of phospholipase A₂ activity utilizing phosphatidylethanolamine molecular species containing palmitate at the sn-1 position and either palmitic, oleic, or arachidonic acid at the sn-2 position as substrates demonstrated a rank order preference for cleavage of arachidonate > oleate > palmitate (Fig. 7, Table III).

Furthermore, substantial enzymic activity required the presence of a long chain acyl group at the sn-2 position since PAF...
was hydrolyzed three orders of magnitude more slowly than 1-O-hexadecyl-2-arachidonyl-GPC.

Since previous work has demonstrated that plasmenylcholine and phosphatidylcholine bilayers possess distinct molecular dynamics (36), additional experiments were performed to examine the substrate specificity of myocardial phospholipase A₂ in systems which minimize differences in the physical properties of aggregated substrate. In initial experiments, we prepared mixed micelles of phospholipids with selected detergents (e.g. Triton X-100, Tween-20, n-octyl glucoside, Nonidet P-40, CHAPS, Lubrol-PX, Brij-35, deoxycholate, and taurocholate) to compare hydrolytic rates for each choline phospholipid subclass in identical microenvironments. Unfortunately, myocardial phospholipase A₂ activity was completely abolished by each of these detergents. To circumvent this difficulty, additional experiments employing binary mixtures of plasmenylcholine and phosphatidylcholine in homogeneous systems, 2) equimolar mixtures of plasmenylcholine and phosphatidylcholine, and 3) vesicles whose physical properties and interfacial characteristics of aggregated substrate, to compare hydrolysis of each phospholipid subclass in a microenvironment possessing physical properties and interfacial characteristics of its phospholipid subclass counterpart, binary mixtures comprised of 10 mol% [³H]plasmenylcholine in phosphatidylcholine bilayers or 10 mol% [³H]phosphatidylcholine in plasmenylcholine bilayers were prepared. Purified myocardial phospholipase A₂ efficiently catalyzed the hydrolysis of plasmenylcholine when the physical characteristics of the vesicles were largely those of phosphatidylcholine. In contrast, phosphatidylcholine was not substantially hydrolyzed even when present in vesicles possessing the physical properties of the preferred substrate in homogeneous systems (i.e. plasmenylcholine) (Fig. 7). Since the purified enzyme selectively hydrolyzed plasmenylcholine in 1) homogeneous systems, 2) equimolar mixtures of plasmenylcholine/phosphatidylcholine, and 3) vesicles whose physical properties were largely those of the preferred substrate in homogeneous systems, we prepared mixed micelles of phospholipids with selected detergents (e.g. Triton X-100, Tween-20, n-octyl glucoside, Nonidet P-40, CHAPS, Lubrol-PX, Brij-35, deoxycholate, and taurocholate) to compare hydrolytic rates for each choline phospholipid subclass in identical microenvironments. Unfortunately, myocardial phospholipase A₂ activity was completely abolished by each of these detergents. To circumvent this difficulty, additional experiments employing binary mixtures of plasmenylcholine and phosphatidylcholine in homogeneous systems, 2) equimolar mixtures of plasmenylcholine and phosphatidylcholine, and 3) vesicles whose physical properties and interfacial characteristics of aggregated substrate, to compare hydrolysis of each phospholipid subclass in a microenvironment possessing physical properties and interfacial characteristics of its phospholipid subclass counterpart, binary mixtures comprised of 10 mol% [³H]plasmenylcholine in phosphatidylcholine bilayers or 10 mol% [³H]phosphatidylcholine in plasmenylcholine bilayers were prepared. Purified myocardial phospholipase A₂ efficiently catalyzed the hydrolysis of plasmenylcholine when the physical characteristics of the vesicles were largely those of phosphatidylcholine. In contrast, phosphatidylcholine was not substantially hydrolyzed even when present in vesicles possessing the physical properties of the preferred substrate in homogeneous systems (i.e. plasmenylcholine) (Fig. 7). Since the purified enzyme selectively hydrolyzed plasmenylcholine in 1) homogeneous systems, 2) equimolar mixtures of plasmenylcholine/phosphatidylcholine, and 3) vesicles whose physical
properties resemble those of phosphatidylcholine, these results demonstrate that myocardial phospholipase A₁ selectively hydrolyzes arachidonoylated plasmenylcholine in physiologically relevant matrices.

To further investigate the diversity of the substrate specificity of purified cytosolic myocardial phospholipase A₂, a battery of lipids was examined. When palmitoylarninic acid, sphingomyelin, acetylcholine, acetyl-CoA, triolein, l-palmityl-CoA, l-0-(Z)-hexadec-1”-enyl-2-octadec-9”-enyl-GPC) were incubated with the purified myocardial phospholipase A₁, no hydrolysis of these moieties was observed. Similarly, the purified enzyme did not catalyze the disproportion of LPC to PC and GPC. Remarkably, the purified enzyme hydrolyzed 1-[1-14C]palmitoyl lysophosphatidylcholine and 1-[1-14C]palmitoyl-CoA (Fig. 8, Table III) albeit at rates two to three orders of magnitude less than that manifest for choline or ethanolamine glycerophospholipids. Kinetic analyses demonstrated that monomorphic lysophosphatidylcholine and palmitoyl-CoA are both poor substrates and that the observed discontinuities in their substrate activity profiles (Fig. 8) closely correspond to the critical micellar concentration of each lipid (37, 38) underscoring the importance of the lipid-aqueous interface as a determinant of enzymic activity.

To verify that phospholipase A₂, lysophospholipase, and palmitoyl-CoA hydrolase activities were mediated by a single polypeptide with multiple catalytic activities, additional experiments were performed. First, parallel assays of phospholipase A₂, lysophospholipase, and palmitoyl-CoA hydrolase activities from each column fraction during Mono Q and hydroxyapatite chromatographies demonstrated that each activity precisely cochromatographed (Figs. 3 and 4) (see “Experimental Procedures” for details). Second, maximal catalytic activity for all three substrates was manifest in the fraction of EGTA and was reduced similarly in the presence of EGTA and was reduced similarly in the presence of EGTA.

To examine the potential physiologic relevance of lysophosphatidylcholine hydrolysis catalyzed by myocardial cytosolic phospholipase A₂, additional studies were performed. When bilayers containing 9 mol% lysophosphatidylcholine (6 μM 1-[1-14C]palmitoyl-LPC in 50 mM unlabeled 1-O-(Z)-hexadec-1’-enyl-2-octadec-9’-enyl-GPC) were incubated with purified myocardial phospholipase A₂, no radiolabeled fatty acid was released from LPC even though over 10% of plasmenylcholine was hydrolyzed. Similarly, since the loss of DPPE and DPPC and the accumulation of LPE and LPC were stoichiometric (Fig. 6), measurable amounts of lysophospholipid hydrolysis did not occur. Thus, under physiologically relevant conditions, myocardial cytosolic phospholipase A₂ hydrolyzes endogenous phospholipids to 1-acyl lysophospholipids and does not act effectively as a lysophospholipase.

**DISCUSSION**

The results contained herein constitute the first purification of a calcium-independent phospholipase activity which has absolute regiospecificity for cleavage of the sn-2 acyl linkage in diradyl glycerophospholipids. Although other calcium-independent phospholipases have previously been described (e.g., Refs. 39–42), detailed kinetic analyses have demonstrated that these phospholipases either specifically catalyze hydrolysis at the sn-1 position or indiscriminately hydrolyze acyl groups at both the sn-1 and sn-2 positions. Since phospholipase A₁ activity was not present utilizing multiple diradyl glycerophospholipid substrates in different physical states, these results demonstrate the absolute regiospecificity of myocardial cytosolic phospholipase A₂.

Myocardial cytosolic calcium-independent phospholipase A₁ is the major measurable phospholipase activity in myocardium and is a low abundance, high specific activity polypeptide which required a 154,000-fold purification to reach homogeneity. This degree of purification was facilitated by the unique, highly selective, and reversible adsorption of myocardial cytosolic phospholipase A₁ to ATP-agarose resin. The purity of the preparation was demonstrated by the presence of a single 40-kDa protein band visualized by the highly sensitive method of 125I autoradiography. Although attempts at obtaining phospholipase activity after polyacrylamide gel electrophoresis have failed (the enzyme is irreversibly inactivated by acrylamide), the high sensitivity and dynamic range of the visualization method employed, the high specific activity of the purified polypeptide (230 μmol/mg-min), as well as the concordant appearance and disappearance of 40-kDa mass with phospholipase activity, collectively demonstrate that the 40-kDa polypeptide catalyzes phospholipase A₁ activity.

Kinetic analyses demonstrated several novel features of the purified protein. Myocardial phospholipase A₂ is the first purified calcium-independent phospholipase A₂ which selectively hydrolyzes plasmenylglycerophospholipids and arachidonoylated glycerophospholipids. Remarkably, the purified polypeptide also contained intrinsic lysophospholipase and palmitoyl-CoA hydrolase activities, albeit at rates two to three orders of magnitude less than its phospholipase A₂ activity. The conclusion that phospholipase A₂, lysophospholipase, and palmitoyl-CoA hydrolase activities are catalyzed by a single polypeptide is substantiated by the coelution of each activity during multiple chromatographic steps to a single polypeptide, similar sensitivities of each activity to divalent cations and thiol oxidizing agents, and identical thermal denaturation profiles of each activity at different temperatures. The possibility that phospholipase A₂, lysophospholipase, and palmitoyl-CoA hydrolase activities are catalyzed by highly homologous yet distinct polypeptides of nearly identical molecular.
mass which copurify over 154,000-fold cannot be definitively excluded but seems unlikely.

Parenthetically, we note that venom phospholipase A₂ (the paradigm of sn-2 regiospecificity) possesses minute levels of lyso phospholipase activity (12). The highly regiospecific phospholipolysis catalyzed by the venom phospholipase A₂ and myocardial cytosolic phospholipase A₂ are in stark contrast to the lack of regiospecificity of the previously isolated 58-kDa calcium-independent phospholipase in guinea pig intestinal mucosa which possessed nearly identical phospholipase A₁, A₂, and lyso phospholipase activities (40). Although the 40-kDa polypeptide is the major measurable phospholipase A₂ in myocardium, its lyso phospholipase and palmitoyl-CoA hydrolase activities comprise only a small fraction of the total lyso phospholipase and palmitoyl-CoA hydrolysis activities in myocardium (4, 19, 21, 43, 44). Accordingly, based upon in vitro kinetic measurements with the purified protein as well as measurements of activities present in myocardial homogenates, it appears likely that this protein functions as a phospholipase A₂ and does not make substantial contributions to lyso phospholipid or palmitoyl-CoA hydrolysis in intact tissue.

The phospholipase A₂ purified in the present study is easily distinguished from other previously described myocardial cytosolic phospholipase activities. A calcium-dependent phospholipase A₁ activity is present in rat myocardial cytosol but specifically cleaves the sn-1 acyl linkage (41). A phospholipase B activity was reported in Syrian hamster myocardial cytosol (42) but differs from the enzyme purified in the present study by the following features: 1) it does not hydrolyze plasmenylethanolamine substrate; 2) its specific activity is three to four orders of magnitude less than the polypeptide purified herein; 3) it has a molecular weight of only 14 kDa; and 4) the regiospecificity of the hamster phospholipase B is predominantly directed toward the sn-1 position while the polypeptide purified in this report has absolute specificity for hydrolysis of the acyl group at the sn-2 position. It is important to note that these cytosolic phospholipase A₁ and B activities comprise less than 10% of the phospholipase A₂ activity present in myocardial cytosol (Table I) utilizing optimal homogenization methods and substrates for each activity (41, 42). Thus, cytosolic calcium-independent phospholipase A₂ is the major measurable phospholipase in myocardium and possesses separate and distinct physical characteristics and kinetic properties from other myocardial cytosolic phospholipase activities previously described.

Early experiments demonstrated that calcium-independent phospholipase A₂ was not present in serum or whole blood and that comparable levels of calcium-independent phospholipase A₂ activity were present in perfused and nonperfused hearts. However, comparisons of other calcium-independent lipases (which are predominantly localized in plasma) to the myocardial enzyme merit brief consideration. First, PAF acetylhydrolase possesses different chromatographic characteristics (binds to DEAE-Sephacel resin at pH 6.8), thermal stability (stable overnight at room temperature), detergent sensitivity (measurable activity in Triton X-100 or Tween-20), and a substantially different pH optimum (pH 7.8) (45) than the myocardial enzyme. Most importantly, PAF acetylhydrolase is highly specific for hydrolysis of alkyl-ether cholesterol glycerophospholipids containing acetyl groups at the sn-2 position (45). In contrast, myocardial phospholipase A₂ hydrolyzes alkyl ether choline glycerophospholipids with long chain sn-2 aliphatic constituents three orders of magnitude more rapidly than PAF. Second, phospholipase activity mediated by lecithincholesterol acyltransferase is distinguished from myocardial phospholipase A₂ since cholesterol acyltransferase is catalyzed by a 68-kDa polypeptide, requires a serum protein cofactor for expression of phospholipase activity (in its pure form), and exhibits no strict regiospecificity for phospholipid hydrolysis (46, 47). Third, endothelial cell-derived lipoprotein lipase is easily distinguished from myocardial phospholipase A₂ since myocardial lipoprotein lipase is a 34-kDa polypeptide, avidly binds to Heparin-Sepharose resin (unlike myocardial cytosolic phospholipase A₂), and tolerates aceton precipitation as well as homogenization in detergents (48), both of which completely ablate myocardial phospholipase A₂ activity. Fourth, plasma carboxylesterase possesses a different substrate selectivity, thermal stability profile, and molecular weight than myocardial phospholipase A₂ (49). Finally, cholesterol esterase has a different substrate specificity, a larger molecular mass (68 kDa), and has an absolute requirement for cofactors for lipolysis (29). Taken together, these results demonstrate that the cytosolic calcium-independent myocardial phospholipase A₂ purified in this report has physical and kinetic characteristics which discriminate it from other calcium-independent lipase activities previously studied.

We have recently demonstrated that myocardial sarcoclemma (the electrophysiologically active membrane in myocytes) is the primary target of accelerated phospholipid hydrolysis in myocytes subjected to simulated ischemia (14) and that myocardial sarcoclemma is predominantly comprised of plasmalogenes and plasmalogenethanolamine molecular species which are highly enriched in arachidonic acid (12). Since the myocardial phospholipase A₂ purified herein has direct physical access to the sarcoclemmal membrane and selectively hydrolyzes both plasmalogen substrate and arachidonoylated glycerophospholipids, this phospholipase has the catalytic potential to selectively hydrolyze the predominant phospholipid constituents present in myocardial sarcoclemma (i.e. arachidonoylated plasmalogens). Accordingly, activation of this polypeptide is anticipated to result in the selective release of arachidonic acid and the catabolism of sarcoclemmal membrane phospholipids similar to that seen during myocardial ischemia (4, 14). Although regulation of intracellular phospholipases activated by physiologic increments in calcium ion is now accepted (e.g. Refs. 50 and 51), the biochemical mechanisms responsible for regulation of calcium-dependent phospholipases A₂ are unknown. Accordingly, future efforts directed toward identification of the molecular mechanisms responsible for the activation of this calcium-independent phospholipase A₂ should provide direct insight into the biochemical mechanisms precipitating electrophysiologic dysfunction during myocardial ischemia.

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