Selective plasmalogen substrate utilization by thrombin-stimulated Ca\(^{2+}\)-independent PLA\(_2\) in cardiomyocytes

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McHowat, J ane, and Michael H. Creer. Selective plasmalogen substrate utilization by thrombin-stimulated Ca\(^{2+}\)-independent PLA\(_2\) in cardiomyocytes. Am J Physiol Heart Circ Physiol 278: H1933–H1940, 2000.—Thrombin stimulation of rabbit ventricular myocytes activates a membrane-associated, Ca\(^{2+}\)-independent phospholipase A\(_2\) (PLA\(_2\)) capable of hydrolyzing plasmalogen-containing phospholipids. Thrombin stimulation resulted in a selective decrease in arachidonoyl plasmalogen, with no change in arachidonoyl phosphatidylcholine. The decrease in arachidonoyl phosphatidylcholine was accompanied by an increase in phosphatidylethanolamine species containing linoleic and linolenic acids at the sn-2 position. A decrease in arachidonoyl plasmalogen was also observed after thrombin stimulation, with no concomitant change in arachidonoyl phosphatidylethanolamine. Thrombin stimulation resulted in the selective production of lysophosphatidylethanolamine. Arachidonic acid released after thrombin stimulation was rapidly oxidized to prostacyclin. Thus thrombin-stimulated Ca\(^{2+}\)-independent PLA\(_2\) selectively hydrolyzes arachidonoyl plasmalogen substrates, resulting in production of lysoplasmalogens and prostacyclin as the principal bioactive products.

phospholipase \textsubscript{A2}; prostacyclin; lysophospholipids

THROMBOTIC CORONARY ARTERY occlusion has been demonstrated to contribute directly to arrhythmogenesis during myocardial ischemia, suggesting that products released from or associated with an intracoronary thrombus may directly or indirectly influence the electrophysiological properties of ischemic cardiac myocytes (12). In isolated ventricular myocytes, thrombin has been shown to stimulate phospholipase A\(_2\) (PLA\(_2\)) activity (25) and generate lysophospholipids (24), which are potent arrhythmogenic amphiphilic lipid metabolites (29). Thus one of the potential factors contributing to arrhythmogenesis in the setting of myocardial ischemia may be the direct action of thrombin on ischemic myocytes, resulting in PLA\(_2\) activation and amphiphilic lipid metabolite generation.

Numerous types of mammalian PLA\(_2\)s have been identified and classified into several groups, each of which demonstrates unique characteristics (11). Secretory PLA\(_2\)-s (sPLA\(_2\)\(_s\)) require millimolar concentrations of Ca\(^{2+}\) for activity and consequently are only active extracellularly. At least two types of PLA\(_2\) are active intracellularly, a Ca\(^{2+}\)-dependent cytosolic PLA\(_2\) (cPLA\(_2\)) that requires micromolar concentrations of Ca\(^{2+}\) for translocation to intracellular membranes and a Ca\(^{2+}\)-independent PLA\(_2\) (iPLA\(_2\)). The three types of PLA\(_2\) have been shown to coexist in mammalian cells and may interact with each other (2). To date, published evidence suggests that iPLA\(_2\) may be involved in cell signaling and/or phospholipid remodeling, which appears to depend upon both the stimulus and the cell type involved (1, 2, 16, 24–26, 32).

The phospholipid composition of isolated cardiac myocytes is unique in being composed predominantly of plasmalogen molecular species (7, 28). Furthermore, plasmalogens are enriched in the electrically active membranes, the sarcolemma and sarcoplasmic reticulum (13, 14), and are important in modulating the kinetic properties of ion channels and ion transporters (10, 37). Previous studies suggest also that plasmalogen phospholipids may be the preferred substrates for ischemia-activated phospholipases (17). A plasmalogen-selective PLA\(_2\) has been described in several tissues, including myocardium (17, 25, 26), brain (18), and kidney (31), and may be involved in accelerated plasmalogen hydrolysis in response to agonist stimulation or ischemia. Accelerated plasmalogen hydrolysis in the ischemic myocardium may lead to the accumulation of lysoplasmalogen, a metabolite that has been shown to have profound effects on the electrophysiological properties of isolated cardiac myocytes (17, 28), which could lead to arrhythmogenesis in the ischemic heart.

The present study was undertaken to determine which individual phospholipid molecular species serve as endogenous substrates for PLA\(_2\) and to determine whether increased phospholipid hydrolysis leads to bioactive metabolite accumulation in thrombin-stimulated rabbit ventricular myocytes that may directly or indirectly contribute to the production of malignant ventricular arrhythmias in the ischemic heart.

MATERIALS AND METHODS

Materials. Methyl arachidonyl fluorophosphonate (MAFP) and arachidonyltrifluoromethyl ketone (AACOCF\(_3\)) were purchased from Calbiochem (La Jolla, CA). Bromoelol lactone (BEL) was a gift from Hoffmann-La Roche (Nutley, NJ). Collagenase (type II) was purchased from Worthington Bio-

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chemical (Lakewood, NJ). [3H]acetic acid, [3H]acetic anhy-
dride, and [14C]acetic anhydride were purchased from NEN
(Boston, MA); [3H]lysophosphatidylcholine was purchased
from Sigma Chemical (St. Louis, MO). Lysoplasmenylcholine was prepared by
alkaline hydrolysis of bovine heart choline glycerophospholip-
ids, as described previously (25). The majority of miscella-
neous reagents were purchased from Sigma.

Isolation of ventricular myocytes. Adult rabbits of either
sex weighing 2–3 kg were anesthetized with intravenous
pentobarbitone sodium (50 mg/kg), and the heart was rapidly
removed. The heart was mounted on a Langendorff perfusion
apparatus and perfused for 5.5 min with a Tyrode solution
containing (in mmol/l) NaCl 118, KCl 4.8, CaCl2 1.2, MgCl2
1.2, NaHCO3 24, KH2PO4 1.2, and glucose 11; the Tyrode
solution was saturated with 95% O2-5% CO2 to yield a pH of
7.4. This was followed by a 3.5-min perfusion with a Ca2+
free Tyrode solution containing EGTA (100 µM) and a final
perfusion for 20 min with the Tyrode solution containing 100
µM Ca2+ and 0.033% collagenase. The left and right vent-
tricles were cut into small pieces and placed in two Erlen-
meyer flasks containing fresh enzyme solution, which were
then shaken in a Dubnoff metabolic shaker at 37°C for 15
min, with 95% O2-5% CO2 blowing into each flask. The first
harvest of myocytes was discarded. Cells from the next three
harvests were combined and washed with a HEPES buffer
containing (in mmol/l) NaCl 133.5, KCl 4.8, MgCl2 1.2, KH2PO4
1.2, HEPES 10, and glucose 10 plus 300 µm CaCl2, with pH
adjusted to 7.4 with 10 N NaOH. Extracellular Ca2+ concen-
tration was increased to 1.2 mM in three stages at intervals of
20 min. Elongated myocytes were separated from rounded
nonviable cells by repeated differential sedimentation.

Stimulation of myocytes with thrombin. Aliquots of myo-
cytes (2 × 10^6 in 2 ml of 1.2 mM Ca2+ HEPES buffer) were
incubated at 37°C. AACOCF3, BEL, or MAPF was dissolved
in DMSO and diluted at least 1 in 1,000 in HEPES buffer
before addition to the cells. Thrombin was dissolved directly
in HEPES buffer and added to the myocytes with or without
prior PMA incubation. At the end of the stimulation period,
chloroform and methanol were added directly to the myocyte
suspension for measurement of phospholipids, lysophospholip-
ids, and platelet-activation factor (PAF). For measurement
of PLA2 activity, the surrounding buffer was removed and immedi-
ately replaced with ice-cold buffer containing (in mmol/l)
sucrose 250, KCl 10, imidazole 10, EDTA 5, and dithiothreitol
(DTT) 2 with 10% glycerol, pH = 7.8 (PLA2 assay buffer). For measurement
of 6-keto-PGF1α, the myocyte suspension was centrifuged and the supernatant was removed for assay of
6-keto-PGF1α content.

Extraction, separation, and analysis of phospholipid classes. Cellular phospholipids were extracted from isolated adult
rabbit ventricular myocytes by the method of Bligh and Dyer
(4) at 0–4°C. The chloroform layer was dried under N2, and
the lipid residue was resuspended in 1 ml chloroform-
methanol (1:1 vol/vol). Three 5-µl aliquots were removed for
measurement of total lipid phosphorus, and 200-µl aliquots were
injected onto an Ultrasphere-Si (5 µm silica), 4.6 ×
250-mm HPLC column (Beckmann Instruments). Phospholip-
ids were separated into different classes on the basis of
differences in polar headgroup composition with the use of
gradient elution with a mobile phase composed of hexane-
isopropanol-water as described previously (7). Phospholipid
classes were quantified in the isolated fractions by measure-
ment of lipid phosphorus by microphosphate assay (6). The
fatty acid composition of the isolated glycerophospholipid
classes was determined by gas chromatographic (GC) analy-

sis of the fatty acid methyl ester (FAME) and dimethylacet-
(95%) derivatives produced after acid-catalyzed methanol-
ysis. Identification of individual FAME species was established
by comparison of their GC retention times with commercial
standards (Alltech, Deerfield, IL). Individual DMA species
were identified by comparison of their GC retention times
with the DMA derivatives produced after acid-catalyzed methanolysis of lysoplasmenylcholine derived from bovine
heart choline glycerophospholipids (7). The alkylacyl glycer-
ophospholipid content of phosphatidylcholine and phosphati-
dylethanolamine was determined by quantification of lipid
phosphorus in the lysophospholipid fraction remaining after
sequential, exhaustive base- and acid-catalyzed hydrolysis of
the dirdial phospholipids (13).

Separation and quantification of individual choline and
ethanolamine glycerophospholipid molecular species. Indi-
individual choline and ethanolamine glycerophospholipid molecu-
lar species were isolated by reverse-phase HPLC with the use of an
Ultrasphere ODS (5 µm, C-18) column, 4.6 × 250 mm
(Beckmann Instruments). Individual molecular species were
separated with the use of a gradient elution system with a
mobile phase composed of acetonitrile-methanol-water with
20 mM choline chloride (27). The molecular identity of indi-
vidual molecular species was established by GC characteriza-
tion of the FAME and DMA derivatives produced after
acid-catalyzed methanolysis of the phospholipid species recov-
ered in column effluents and by comparison of absolute reten-
tion time, relative retention time, and order of elution of
different individual species with previously injected synthetic phospho-
lipids of known composition (27). Quantification of individual
phospholipid molecular species was achieved by determina-
tion of lipid phosphorus in reverse-phase HPLC column
effluents by the method of Iitaya and Ui (19).

PLA2 activity. Myocytes suspended in ice-cold PLA2 assay
buffer were sonicated on ice six times for 10 s, and the sonicate was centrifuged at 14,000 g for 10 min. The resultant
supernatant fraction was centrifuged at 100,000 g for 60 min
in the high-speed centrifuge to separate the membrane fraction (pellet) from the cytosolic fraction (supernatant). The membranes were washed twice and redissolved in PLA2 assay buffer. PLA2 activity in
subcellular fractions was assessed by incubating enzyme (8
µg membrane protein or 200 µg cytosolic protein) with 100
µM (16:0, [3H]18:1) plasmenylcholine, phosphatidylcholine, or
alkylacyl glycerophosphorylcholine (24) in assay buffer
containing 100 mM Tris, 4 mM EGTA, and 10% glycerol, pH = 7.0,
at 37°C for 5 min in a total volume of 200 µl. Reactions
were terminated by the addition of 100 µl butanol, and then
tubes were vortexed and centrifuged at 2,000 g for 5 min.
Released radiolabeled fatty acid was isolated by application of
25 µl of the butanol phase to a cellophane gel G plate,
development in petroleum ether-diethyl ether-acetic acid
(70:30:1 vol/vol/vol), and subsequent quantification by liquid
scintillation spectrometry. The reaction conditions selected
resulted in linear reaction velocities with respect to both time
and total protein concentration for each substrate examined.
Protein content of each sample was determined by the Lowry
method with the use of freeze-dried bovine serum albumin as
the protein standard, as described previously (23).

Measurement of choline lysophospholipids. Lysophosphati-
dylcholine and lysoplasmenylcholine measurements were made using a modification of a radiometric assay method
described previously (28). Lipids were extracted from the
myocyte suspension by the method of Bligh and Dyer (4), and
lysophospholipids were separated from other phospholipids
by HPLC. The purified lysophosphatidylcholine and lysoplas-
menylcholine fractions as well as known amounts of lysophosphatidylcholine and lysoplasmlyenylcholine standards were then acetylated with $^{[3]H}$acetic anhydride with the use of 0.33 M dimethylaminopropiridine as a catalyst. The acetylated lysophospholipid was then separated by TLC, and radioactivity was quantified by liquid scintillation spectrometry. Standard curves were constructed, and lysophosphatidylcholine and lysoplasmlyenylcholine content were derived for all samples and normalized according to the protein content of the myocytes as described previously (23). $^{[14]C}$lysophosphatidylcholine was added as an internal standard to all samples to correct for loss of sample that occurred during extraction, purification, and acetylation.

PGI$_2$ formation. After thrombin stimulation, the myocyte suspension was rapidly centrifuged, and the supernatant was removed. PGI$_2$ released from ventricular myocytes was measured in surrounding medium as its stable metabolite 6-keto-PGF$_1a$. The 6-keto-PGF$_1a$, in the sample was measured using a commercially available immunosassay kit (R&D Systems, Minneapolis, MN). The myocyte cell pellet was resuspended in distilled water, and the amount of protein was determined as described previously (23).

Production of PAF. Myocytes (10$^6$ in 1 ml) were incubated with 50 $\mu$Ci $^{[3]H}$acetic acid for 20 min. After thrombin stimulation for the selected time interval, lipids were extracted from the cell suspension by the method of Bligh and Dyer (4). The chloroform layer was concentrated by evaporation under $N_2$, applied to a silica gel 60 TLC plate, and developed in chloroform-methanol-acetic acid-water (50:25:8:4 vol/vol/vol/vol). The region corresponding to PAF was scraped and radioactivity was quantified with the use of liquid scintillation spectrometry. Loss of PAF during extraction and chromatography was corrected by adding a known amount of $^{[14]C}$PAF as an internal standard. $^{[14]C}$PAF was synthesized by acetylating the sn-2 position of lyso-PAF with $^{[3]H}$acetic anhydride (Amersham, Arlington Heights, IL) with the use of 0.33 M dimethylaminopropiridine as a catalyst. The synthesized $^{[14]C}$PAF was purified by HPLC. The specific activity of the final $^{[14]C}$PAF product was 300 $\mu$Ci/$\mu$mol. The amount of protein in each sample was measured as described previously (23).

Statistics. Statistical comparison of values was performed by Student's $t$-test or analysis of variance with the Fisher multiple-comparison test as appropriate. All results are expressed as means $\pm$ SE. Statistical significance was considered to be $P < 0.05$.

RESULTS

We have demonstrated previously that the majority of PLA$_2$ activity in isolated rabbit cardiac myocytes is Ca$^{2+}$-independent and membrane associated and exhibits a preference for arachidonylated phospholipid substrates (25, 26). Thrombin stimulation resulted in an increase in membrane-associated PLA$_2$ activity measured in the absence of Ca$^{2+}$ with the use of either plasmlyenylcholine, phosphatidylcholine, or alkylacyl glycerophosphorylcholine substrates (Fig. 1). Thrombin-stimulated PLA$_2$ activity was inhibited by pretreatment with BEL (a specific inhibitor of myocardial iPLA$_2$) but was unaffected by pretreatment with either AACOCF$_3$ or MAFP (inhibitors of both Ca$^{2+}$-dependent and -independent cytosolic PLA$_2$s) (Fig. 1).

To determine which phospholipids serve as endogenous substrates for the membrane-associated iPLA$_2$, we subsequently characterized the phospholipid molecular species composition of control and thrombin-stimulated ventricular myocytes. The effect of inhibition of iPLA$_2$ with BEL on phospholipid hydrolysis was also investigated.

The total phospholipid phosphorus in isolated rabbit ventricular myocytes was 141 $\pm$ 8 nmol/mg protein. The major phospholipid classes were choline and ethanolamine glycerophospholipids (Table 1). These were also the only classes found to contain plasmagas or alkylacyl glycerophospholipids (Table 1). Choline glycerophospholipids were composed of 45% plasmlyenylcholine and 55% phosphatidylcholine, whereas ethanolamine glycerophospholipids were composed of 50% plasmlyenylethanolamine (Table 1 and Fig. 2). Thrombin stimulation with or without pretreatment with BEL had no effect on the total mass of choline or ethanolamine phospholipids.

In choline glycerophospholipids, arachidonylated species were predominantly found in plasmagas; 55% of

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[Fig. 1. Effect of pretreatment of rabbit ventricular myocytes with specific phospholipase A$_2$ (PLA$_2$) inhibitors (10-min incubation) on membrane-associated PLA$_2$ under basal conditions or after thrombin stimulation (0.05 U/ml, 1 min) with use of (16:0,$^{[3]H}$18:1) plasmlyenylcholine, (16:0,$^{[3]H}$18:1) phosphatidylcholine, or (16:0,$^{[3]H}$18:1) alkylacyl glycerophosphorylcholine in absence of Ca$^{2+}$ (4 mM EGTA). Values are means $\pm$ SE of independent results derived from 6 separate animals. MAFP, methyl arachidonoyl fluorophosphonate; AACOCF$_3$, arachidonoyl trifluoromethyl ketone; BEL, bromoenol lactone. **$P < 0.01$ compared with corresponding PLA$_2$ activity in absence of inhibitor.]
plasmenylcholine contained arachidonic acid at the sn-2 position, but only 24% of phosphatidylinositol arachidonylated species were arachidonylated (Table 2). Thrombin stimulation of ventricular myocytes resulted in a significant decrease in the mass of arachidonylated plasmenylcholine (Fig. 3A) that reflected decreases in both (16:0, 20:4) and (18:1, 20:4) plasmenylcholine (Table 2). Thrombin-stimulated ventricular myocytes demonstrated no corresponding change in arachidonylated phosphatidylinositol (Table 2). The decrease in arachidonylated plasmenylcholine was accompanied by an increase in plasmenylcholine species containing linoleic (18:2) and linolenic (18:3) acids at the sn-2 position (Table 2 and Fig. 2), suggesting that the majority of lysoplasmeylcholine produced by PLA2 in response to thrombin stimulation in ventricular myocytes may be rapidly reacylated by CoA-dependent acyltransferase and/or CoA-independent transacylase enzymes. Pretreatment with BEL before thrombin stimulation abolished completely any thrombin-induced alterations in phospholipid molecular species (Fig. 2), demonstrating that these alterations are the direct result of PLA2 activation and not the result of metabolite flux through alternate, competing pathways. The majority of ethanolamine glycerophospholipid was found to be arachidonylated, with 62% of plasmenylethanolamine containing arachidonic acid at the sn-2 position (Fig. 3A). Thrombin stimulation of ventricular myocytes resulted in a decrease in total arachidonylated plasmenylethanolamine (Fig. 3A) that represented a decrease in the mass of each individual arachidonylated molecular

Table 1. Phospholipid composition of isolated adult rabbit ventricular myocytes

<table>
<thead>
<tr>
<th>Phospholipid Class</th>
<th>Phospholipid/mg Protein</th>
<th>% Plasmalogen Content</th>
<th>% Alklyacyl Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGP</td>
<td>64.9 ± 4.4</td>
<td>45.7 ± 3.9</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>EGP</td>
<td>51.9 ± 2.5</td>
<td>51.0 ± 3.4</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Plas Eth</td>
<td>5.1 ± 1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>5.9 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPG</td>
<td>8.5 ± 1.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPH</td>
<td>5.3 ± 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values represent means ± SE of separate measurements obtained using myocytes isolated from 6 different animals. CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipid; PI, phosphatidylinositol; PS, phosphatidylserine; DPG, diphosphatidylglycerol (cardiolipin); SPH, sphingomyelin.

Table 2. Composition of choline and ethanolamine glycerophospholipid molecular species in isolated rabbit ventricular myocytes

<table>
<thead>
<tr>
<th>Molecular Species</th>
<th>Control</th>
<th>Thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>(18:2, 18:3) Plas Cho</td>
<td>0.2 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>(18:2, 20:4) Plas Cho</td>
<td>0.2 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>(18:2, 18:3) Plas Cho</td>
<td>1.7 ± 0.2</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>
| (16:0, 18:3) Plas Cho | 0.1 ± 0.1 | 1.5 ± 0.2 |†
| (16:0, 20:4) Plas Cho | 5.7 ± 0.1 | 5.4 ± 0.1 |
| (16:0, 18:2) Plas Cho | 12.1 ± 10 | 13.0 ± 1.5 |
| (18:1, 18:2) Plas Cho | 3.1 ± 0.4 | 3.9 ± 0.5 |
| (16:0, 20:4) Plas Cho | 10.3 ± 6.6 | 5.9 ± 0.3 |
| (16:0, 18:2) Plas Cho | 6.7 ± 1.5 | 12.6 ± 1.4 |†
| (18:1, 20:4) Plas Cho | 6.9 ± 1.8 | 1.7 ± 0.1 |
| (16:0, 18:1) Plas Cho | 2.1 ± 0.1 | 3.5 ± 0.7 |
| (18:2, 20:4) Plas Cho | 3.7 ± 0.6 | 3.0 ± 0.3 |
| (18:2, 18:2) Plas Cho | 5.9 ± 0.5 | 6.7 ± 0.7 |
| (16:0, 20:4) Plas Cho | 1.9 ± 0.3 | 2.6 ± 0.3 |
| (18:1, 20:4) Plas Cho | 1.6 ± 0.3 | 1.4 ± 0.2 |
| (18:2, 18:2) Plas Cho | 0.8 ± 0.2 | 1.8 ± 0.3 |*|
| (18:0, 18:1) Plas Cho | 0.3 ± 0.1 | 0.4 ± 0.1 |

Values (in nmol PO4/mg protein) represent means ± SE of separate measurements obtained using rabbit ventricular myocytes isolated from 6 different animals. Composition of individual phospholipid molecular species is described by shorthand notation (a:b, c:d) where a and c represent chain length and b and d represent the number of C=C bonds for the aliphatic groups at the sn-1 and sn-2 positions, respectively. Plas Cho, plasmenylcholine; Ptd Cho, phosphatidylcholine; Plas Eth, plasmenylethanolamine; Ptd Eth, phosphatidylethanolamine; ND, none detected. *P < 0.05; †P < 0.01.

Fig. 2. Changes in mass of plasmenylcholine molecular species after thrombin stimulation (0.05 U/ml, 2 min) of isolated rabbit ventricular myocytes. Pretreatment with bromoenol lactone (BEL; 10 μM, 10 min) completely blocked catabolism of arachidonylated plasmenylcholine. Values are means ± SE of duplicate measurements for independent results obtained from 6 separate animals. *P < 0.05 and **P < 0.01 compared with untreated myocytes.
species (Table 2). No change in phosphatidylethanolamine species was observed in thrombin-stimulated myocytes (Fig. 3B). The decrease in arachidonoylated plasmenylethanolamine in thrombin-stimulated myocytes was significantly less than that seen in the choline phospholipid class and was accompanied by an increase in (18:1, 18:2) and (16:0, 18:3) plasmenylethanolamine (Table 2, Fig. 3A). Pretreatment with BEL blocked any thrombin-stimulated changes in ethanolamine glycerophospholipids (Fig. 3A), again confirming that these alterations are mediated by PLA₂ activation. Thus thrombin stimulation of isolated rabbit ventricular myocytes results in the selective hydrolysis of both choline and ethanolamine plasmenogen phospholipids by iPLA₂ with a preference for those plasmenogen species possessing a phosphocholine headgroup.

The selective hydrolysis of arachidonic acid from the sn-2 position of plasmenogen phospholipids in thrombin-stimulated myocytes suggests the selective concomitant production of lysoplasmalogens, which may or may not accumulate depending on the presence and activity of other enzymes catalyzing lysoplasmalogen catabolism or reacylation. Measurement of choline lysophospholipid content in thrombin-stimulated ventricular myocytes demonstrated a significant increase in lysoplasmalogen that was measured after 1 min of thrombin stimulation with no corresponding increase in lysophosphatidylcholine (Fig. 4). The increase in lysoplasmalogen was inhibited completely by BEL pretreatment (Fig. 4). Incubation of ventricular myocytes with thrombin for increasing time intervals resulted in a significant increase in lysoplasmalogen content for up to 5 min, after which time, lysoplasmalogen returned to basal values. The increase in lysoplasmalogen content was ~0.4 nmol/mg protein (Fig. 4), and there was an increase of 7–8 nmol/mg protein in choline glycerophospholipids containing either linoleic or linolenic acid at the sn-2 position (Fig. 2). Because PLA₂-catalyzed hydrolysis of plasmalogen resulted in a decrease of 8–10 nmol/mg protein of arachidonylated molecular species (Fig. 2), the majority of arachidonylated plasmenogen phospholipid hydrolyzed by thrombin-activated PLA₂ was reacylated, thereby limiting the accumulation of lysoplasmalogen within the cell.

The products formed after PLA₂ hydrolysis of membrane phospholipids, namely arachidonic acid and lysophosphatidylcholine, were measured in control and thrombin-stimulated ventricular myocytes. Pretreatment with BEL (10 µM, 10 min) significantly inhibited basal lysoplasmalogen and blocked completely increase in lysoplasmalogen produced by thrombin stimulation. Values are means ± SE for independent results from 6 separate animals. **P < 0.01 compared with untreated myocytes.
phospholipids, may affect membrane function directly (28, 29) or serve as precursors for biologically active metabolites. Lysophospholipids can serve as PAF precursors, whereas arachidonic acid is the precursor for eicosanoid production. To determine whether there is further metabolism of lysoplasmenylcholine and arachidonic acid to other bioactive products after thrombin stimulation of ventricular myocytes, PAF and prostacyclin production were measured under control and thrombin-stimulated conditions. Thrombin stimulation led to some increase in PAF production, but this was not statistically significant (Fig. 5), suggesting that little lysophospholipid is acetylated after production. It should be noted that with the use of this assay system, the amount of radiolabeled PAF produced is a relative measure of the acetylation of lysophospholipid rather than a direct measurement of the mass of biologically active PAF produced. Further metabolism of free arachidonic acid in ventricular myocytes was evidenced by the significant increase in prostacyclin production after thrombin stimulation (Fig. 6). This increase was blocked by inhibiting iPLA2 with BEL (Fig. 6).

**DISCUSSION**

Previous studies (24–26) have demonstrated that thrombin stimulation of rabbit ventricular myocytes results in a selective increase in membrane-associated PLA2 activity. On the basis of in vitro assay measurements, thrombin-stimulated PLA2 is able to hydrolyze plasmenylcholine, phosphatidylcholine, and alkylacyl glycerophosphorylcholine substrates in the absence of Ca2++; thus thrombin-stimulated PLA2 does not exhibit a catalytic requirement for Ca2+. However, it should be noted that myocytes suspended in a Ca2+-free buffer and stimulated with thrombin do not demonstrate increased PLA2 activity (data not shown), suggesting that although the catalytic activity of thrombin-stimulated PLA2 is Ca2+ independent, activation of PLA2 in intact myocytes requires the presence of intracellular Ca2+, possibly for the function of intracellular regulators of iPLA2. Thrombin-stimulated iPLA2 represents an integral membrane protein, because membrane-associated PLA2 activity cannot be removed by repeated sonication, washing in the presence of EGTA, high- or low-salt concentrations, or submicellar concentrations of detergent (26). The increase in membrane-associated PLA2 activity appears to be a result of activation of a latent membrane-associated PLA2, because increases in activity are observed after 30 s of thrombin stimulation, and no change in the amount of membrane-associated iPLA2 protein is observed by immunoblot analysis in thrombin-stimulated cells (data not shown).

Accompanying the increase in PLA2 is an increase in free arachidonic acid release from the myocytes and an accumulation of total choline lysophospholipids. Previous studies have not identified the endogenous diradyl phospholipid substrates targeted for hydrolysis by thrombin-stimulated PLA2. Similarly, previous measurements of thrombin-induced lysophospholipid production did not distinguish between lysophospholipids derived from diacyl phospholipids and lysoplasmalogens derived by PLA2-catalyzed plasmalogen hydrolysis. In addition, production of other "downstream" bioactive metabolites (e.g., PAF and eicosanoids) in response to thrombin activation of iPLA2 has not been investigated.

In this study, we have demonstrated that plasmalogen phospholipids serve as the endogenous substrates for thrombin-stimulated PLA2 activity. Thus, although in vitro measurements of PLA2 activity demonstrate...
increased hydrolysis of both plasmalogen and diacyl phospholipids, in vivo hydrolysis of ventricular myocyte phospholipids in response to thrombin stimulation occurs almost exclusively in plasmalogens. This suggests that plasmalogens serve as a highly metabolically active pool of membrane phospholipids that are readily hydrolyzed in response to agonist stimulation and may play a role in second messenger generation during signal transduction. Plasmalogens are the predominant phospholipids found in the sarclemma and sarcoplasmic reticulum of myocardial cells. The physiological consequences of plasmalogens in these subcellular pools remain to be fully established. However, plasmalogens play an important role in regulating transmembrane concentration gradients and myocardial electrical excitability. Additionally, plasmalogens have been demonstrated to regulate sodium-calcium exchange in cardiac sarcolemmal preparations, suggesting a role for plasmalogens in ion-transport regulation.

Myocardial ischemia is associated with the accelerated selective hydrolysis of plasmalysylinoline by phospholipase C and PL A2, suggesting the activation of multiple pathways for plasmalogen hydrolysis. The accumulation of lysoplasmenylcholine is the direct, specific result of accelerated PL A2-catalyzed hydrolysis of plasmalogen phospholipids and may be accompanied by other effects on the myocardium. For example, lysoplasmenylcholine has been shown to activate myocardial cAMP-dependent protein kinase, suggesting a role for lysoplasmenylcholine in signal transduction analogous to that of diacylglycerol activation of protein kinase C. Additionally, lysoplasmenylcholine has been shown to have direct effects on the function of integral proteins within the membrane. Schonefeld et al. have demonstrated a greater degree of inhibition of kidney cortex Na+/K+-ATPase with lysoplasmenylcholine than that observed with the structurally similar amphiphilic compounds lysophosphatidylcholine and palmityl carnitine. We have shown that lysoplasmenylcholine produces action potential derangements in cardiac myocytes at much lower concentrations than that described previously for either lysophosphatidylcholine or palmityl carnitine, suggesting a direct interaction of lysoplasmenylcholine with ionic channels in the membrane. The action potential derangements caused by lysoplasmenylcholine occur as a result of the action of this amphiphilic compound on multiple membrane currents. The increase in lysoplasmenylcholine observed in thrombin-stimulated myocytes (0.4 nmol/mg protein) is identical to the increase in lysophosphatidylcholine measured in the ischemic myocardium in vivo (0.5 nmol/mg protein) and the increase in vitro (0.4 nmol/mg protein) required to produce electrophysiological alterations. Thus, lysoplasmenylcholine is an important bioactive metabolite independent of its metabolism to other products. PAF has also been shown to exert multiple effects on the myocardium, either through a direct action modifying chronotropic and ionotropic activity or indirectly via the release of eicosanoids or cytokines.
PLASMALOGEN CATABOLISM BY MYOCARDIAL PLA₂

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