Assessing Phospholipase A_2 Activity toward Cardiolipin by Mass Spectrometry

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Abstract

Cardiolipin, a major component of mitochondria, is critical for mitochondrial functioning including the regulation of cytochrome c release during apoptosis and proper electron transport. Mitochondrial cardiolipin with its unique bulky amphipathic structure is a potential substrate for phospholipase A_2 (PLA_2) in vivo. We have developed mass spectrometric methodology for analyzing PLA_2 activity toward various cardiolipin forms and demonstrate that cardiolipin is a substrate for sPLA_2, cPLA_2 and iPLA_2, but not for Lp-PLA_2. Our results also show that none of these PLA_2s have significant PLA_1 activities toward dicyan-cardiolipin. To understand the mechanism of cardiolipin hydrolysis by PLA_2, we also quantified the release of monolysocardiolipin and dicyan-cardiolipin in the PLA_2 assays. The sPLA_2s caused an accumulation of dicyan-cardiolipin, in contrast to iPLA_2, which caused an accumulation of monolysocardiolipin. Moreover, cardiolipin inhibits iPLA_2 and cPLA_2, and activates sPLA_2 at low mol fractions in mixed micelles of Triton X-100 with the substrate 1-palmitoyl-2-arachidonyl-sn-phosphatidylcholine. Thus, cardiolipin functions as both a substrate and a regulator of PLA_2 activity and the ability to assay the various forms of PLA_2 is important in understanding its function.

Introduction

Over the past 40 years, scientists have devoted themselves to understanding the enzymes that can hydrolyze the sn-2 ester bond of phospholipids to release free fatty acids [1], leading to the discovery of the phospholipase A_2 (PLA_2) superfamily. The free fatty acids released by PLA_2 can be converted to eicosanoids, including the prostaglandins and leukotrienes, which are involved in inflammatory responses [2]. So far, six types including sixteen subtypes of PLA_2 have been identified (Table 1). The three main types of PLA_2, secreted PLA_2 (sPLA_2), cytosolic PLA_2 (cPLA_2) and Ca^{2+}-independent PLA_2 (iPLA_2), were found early and are well-studied; hence several enzymes have been categorized for each type. Platelet activating factor acetylhydrolase (PAF-AH), lysosomal PLA_2 and adenosine phosphate-activated PLA_2 (Ad-PLA_2) were later discovered and are now part of the phospholipase A_2 superfamily. These PLA_2s can be differentiated by their cellular localization, Ca^{2+} dependence of the activity and their physical properties [3]. The activities and membrane association of sPLA_2 and cPLA_2 are Ca^{2+} dependent, whereas the activity of iPLA_2, Lp-PLA_2, lysosomal PLA_2 and Ad-PLA_2 are independent of Ca^{2+} [1]. The activation of these PLA_2s has been vigorously investigated and shown to be involved in inflammation, atherosclerosis, cancer, diabetes and neurodegenerative diseases.

The complex anionic phospholipid, cardiolipin, is a major component of the inner membrane of mitochondria and bacterial membranes [4,5,6]. Cardiolipin has been shown to be a substrate of both sPLA_2 and cPLA_2 utilizing a fluorescent labeled cardiolipin [7,8] and implied to be a substrate for iPLA_2 [9]. Most cardiolipin resides in mitochondria and accounts for 10–20% of the total lipid in mammalian cells. Cardiolipin is an important membrane component maintaining the integrity of mitochondria and is critical for the production of ATP via the electron transport chain [10,11,12] by stabilizing the electron transport chain complex in the inner mitochondrial membrane [13,14]. Cardiolipin associates with the membrane-anchored cytochrome c under homeostatic conditions [15]. Oxidative stress causes peroxidation of cardiolipin and thus, hampers the electron transport chain and alters mitochondrial bioenergetics [16,17,18]. When programmed cell death is initiated, the interactions between cytochrome c and cardiolipin is disturbed [15,19,20]. Besides its functions in mitochondria, cardiolipin has been shown to be associated with several diseases. Cardiolipin causes the immune response to the anti-cardiolipin antibodies, which has been shown to be associated with increased risks of venous or arterial thrombosis and ischemic coronary and cerebral disease [21,22,23,24]. Cardiolipin is also a significant and normal physiologic component present in human plasma lipoproteins including LDL and HDL [25]. Recently, cardiolipin release from damaged mitochondria has been shown to exacerbate breathing problems of pneumonia patients [26]. Uniquely and more importantly, cardiolipin is a substrate for the PLA_2 superfamily of enzymes; however, their activities in vitro have not yet been characterized.

An X-linked tafazzin gene mutation causes the alterations of mitochondrial cardiolipin, including modified acyl chains and accumulation of monolysocardiolipin, and with a deficiency in
cardiolipin remodeling, many patients further develop Barth syndrome. [9,27,28]. The 85-kDa GVIA iPLA2 (also known as iPLA2β) has been shown to localize to mitochondria in many types of cells [29,30,31,32], which indicates iPLA2 is localized in the proximity of cardiolipin. iPLA2 was characterized and it showed potent phospholipase, lysophospholipase and transacylase activities toward phosphatidylcholine (PC) which could be blocked by various inhibitors [33,34,35,36]. iPLA2 has been suggested to be responsible for cardiolipin deacylation and monolyso-cardiolipin accumulation in Barth syndrome and hypertensive heart failure [9,37]. Inhibition of iPLA2 can suppress the phenotype of tafazzin knockouts in drosophila [9]. The discovery that cardiolipin is involved in Barth syndrome has suggested that the catabolism of cardiolipin by PLA2 plays a pivotal role in mitochondria maintenance. iPLA2 inhibition could be a potential treatment for Barth syndrome patients.

Because radiolabeled cardiolipin is challenging to synthesize, our goal in this study was to develop a mass spectrometry methodology to quantitate PLA2 activity toward natural and synthetic non-radiolabeled cardiolipin and further understand the activity of PLA2 toward cardiolipin. We utilized mass spectrometry (GC/MS and LC/MS) to monitor hydrolysis of cardiolipin by the four major types of PLA2, including GI and GV sPLA2, cPLA2, iPLA2 and Lp-PLA2 and also determined whether the hydrolysis occurs at the sn-1 and/or sn-2 positions. Additionally, we obtained further information regarding the interfacial catalytic activities of these PLA2 enzymes.

Table 1. The phospholipase A2 superfamily.

| Type | Group | Subgroups | Size (kDa) | Ca²⁺ | Cellular Location | Catalytic Residues |
|------|-------|-----------|-----------|------|-------------------|-------------------|
| GI   | A, B  | 13–15     |           |      |                   |                   |
| GII  | A, B, C, D, E, F | 13–17 |           |      |                   |                   |
| GIII | 15–18 |           |           |      |                   |                   |
| GV   | 14    |           |           |      |                   |                   |
| iPLA2| GIX   | 14        | Yes       | Secreted | His/Asp         |                   |
| GX   | 14    |           |           |      |                   |                   |
| GXI  | 12–13 |           |           |      |                   |                   |
| GXII | 19    |           |           |      |                   |                   |
| GXIII| <10   |           |           |      |                   |                   |
| GXIV | 13–19 |           |           |      |                   |                   |
| cPLA2| GIV   | A(α), B(β), C(γ) | 60–114 | Yes | Cytosol | Ser/Asp |
| iPLA2| GVI   | A, B, C, D, E, F | 84–90 | No   | Cytosol Mito. | Ser/Asp |
| PAF-AH| GVII | A(Lp-PLA2), B(PAF-AH II) | 40–45 | No | LDL, HDL | Ser/His/Asp |
|     | GVIII | π₁, π₂, β | 26–40 | No | Lysosome | Ser/His/Asp |
| Lysoosomal PLA2 | GXV | 45 | No | Lysosome | Ser/His/Asp |
| Adipose-specific PLA2 (AdiPLA2) | GXVI | 18 | No | Adipocyte | His/Cys |

Adapted from Dennis et al [1].

doi:10.1371/journal.pone.0059267.t001

Cardiolipin

1',3'-Bis-[1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho]-sn-glycerol

Figure 1. Structure of Cardiolipin. The structure of cardiolipin, 1',3'-Bis-[1, 2-di-(9Z-octadecenoyl)-sn-glycero-3-phospho]-sn-glycerol, is drawn and adapted from LIPID MAPS (www.lipidmaps.org).

doi:10.1371/journal.pone.0059267.g001
Materials and Methods

Materials
Natural and synthetic cardiolipin and 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (PAPC) were from Avanti Polar Lipids. 1-palmitoyl-2-arachidonoyl-3-[arachidonyl-1-14C]–phospho-sn-glycerol, and its PLA2 hydrolysis products, monolysocardiolipin and dilyso-cardiolipin were purified by Bligh and Dyer lipid extraction methods [41]. The extracted cardiolipin, monolysocardiolipin and dilyso-cardiolipin were dried with nitrogen gas at room temperature. Samples were resuspended in a buffer composed of 100 mM HEPES at pH 7.5. The mixed micelles were composed of 0.1 mM PAPC (containing 14C labeled PAPC with 80,000 cpm) and 0.4 mM Triton X-100 in a final volume of 500 μl. Different compositions of lipid mixed-micelles containing PAPC, cardiolipin and PI(4,5)P2 were mixed and dried, and then prepared utilizing the same method. ATP, Ca2+ and DTT were added depending on the requirement of the particular PLA2. GIA sPLA2 and GV sPLA2 assays were carried out in 5 mM Ca2+; GIVA cPLA2 assays were carried out in 3% PI(4,5)P2, 2 mM DTT and 0.1 mM Ca2+, and GIVA iPLA2 assays were carried out in 1 mM EDTA, 2 mM ATP and 4 mM DTT. The reaction was initiated by adding PLA2 to mixed micelles and incubated at 40°C for 30 min. After incubation, the reaction was quenched, and the fatty acids were extracted using a modified Dole assay protocol as previously described [38,39].

PLA2 Activity Assay

For the basal specific activity of PLA2, assays were performed in a buffer composed of 100 mM HEPES at pH 7.5. The mixed micelles were composed of 0.1 mM PAPC (containing 14C labeled PAPC with 80,000 cpm) and 0.4 mM Triton X-100 in a final volume of 500 μl. Different compositions of lipid mixed-micelles containing PAPC, cardiolipin and PI(4,5)P2 were mixed and dried, and then prepared utilizing the same method. ATP, Ca2+ and DTT were added depending on the requirement of the particular PLA2. GIA sPLA2 and GV sPLA2 assays were carried out in 5 mM Ca2+; GIVA cPLA2 assays were carried out in 3% PI(4,5)P2, 2 mM DTT and 0.1 mM Ca2+, and GIVA iPLA2 assays were carried out in 1 mM EDTA, 2 mM ATP and 4 mM DTT. The reaction was initiated by adding PLA2 to mixed micelles and incubated at 40°C for 30 min. After incubation, the reaction was quenched, and the fatty acids were extracted using a modified Dole assay protocol as previously described [38,39].

Results and Discussion

PLA2 Activity Toward Cardiolipin
Cardiolipin has been shown to be a substrate for both cPLA2 and sPLA2 using fluorescence labeling substrate [7]. This indicates that the phospholipid sn-2 position can be recognized by phospholipase A2, despite its unique structure containing four fatty acyl chains with a bulky and negatively charged head group (Fig. 1). Whether cardiolipin is also a substrate for iPLA2 and Lp-PLA2 was not known. The direct measurement and comparisons of the PLA2 activity by following fatty acid hydrolysis has not been reported. Here we showed that the hydrolysis of tetraoleoyl(18:1)-cardiolipin can be monitored by measuring the oleic acid released by both GC/MS and LC/ESI-MS/MS (Fig. 2) without fluorescent or radio-labeling. Three common contaminants from single-use glassware during fatty acid derivatization, 16:0, 18:2 and 18:0 do not affect the quantification of 18:1 on GC/MS. Because oleic acid (18:1) is the most abundant fatty acyl chain in the biological samples, measurement of the oleic acid release is the best way to quantify cardiolipin hydrolysis. The advantage of the oleic acid measurement on LC-MS/MS is to avoid those fatty acid contaminants and the oleic acid peak can be isolated in the precursor mode.
We further examined the cardiolipin activities of five different PLA2 enzymes representing four different PLA2 types by GC mass spectrometry (Fig. 3). The activities of these five highly purified enzymes, GVIA iPLA2, GIA sPLA2, GV sPLA2, GIVA cPLA2 and Lp-PLA2 have been well characterized previously [38,39]. The results show that cardiolipin is a substrate for GVIA iPLA2, GIA sPLA2, GV sPLA2 and GIVA cPLA2, but not Lp-PLA2. The specific activities are 2.0 μmol/min/mg for GVIA iPLA2, 36.9 μmol/min/mg for GIA sPLA2, 5.0 μmol/min/mg for GV sPLA2 and 2.3 μmol/min/mg for GIVA cPLA2. The negatively charged PI(4,5)P2 binds to cPLA2 in a 1:1 stoichiometry to increase its enzymatic activity [42]. A lysine pocket in the catalytic domain (Lys488, Lys541, Lys543, and Lys544) was shown to be essential for PIP2-dependent activity increases [43]. PIP2 therefore was recognized as an activator of cPLA2 and was included in the standard assay. Without PIP2 in the cardiolipin assay, GIVA cPLA2 showed an activity of 1.4 μmol/min/mg, consistent with the activation of cPLA2 by PIP2, though less so than with PAPC as substrate, suggesting perhaps that PIP2 may have less impact on the negatively charged surface of cardiolipin. Interestingly, GIA sPLA2 purified from snake venom shows the highest activity among all PLA2s. The sPLA2 in the snake venom can cause excessive inflammation when added to tissues through the hydrolysis of phospholipid membranes [44,45].

**Lyso-cardiolipin Analysis by LC/MS**

The unique structure and the negative charge of cardiolipin stabilize the electron transport chain complex and maintain the structure of mitochondria membranes. Hydrolysis of cardiolipin will inevitably affect the structure and function of mitochondria. We have demonstrated that cardiolipin is a substrate for sPLA2, cPLA2 and iPLA2 (Fig. 2). The hydrolysis of cardiolipin should generate lyso-cardiolipin products and we employed tandem mass spectrometry to monitor the production of these species (Fig. 4).

The dominant fatty acyl on cardiolipin (18:1) peak in mass spectrometry is seen as a doubly charged ion in the negative mode (Fig. 4A). These two negative charges of cardiolipin are from the two negative charges on the phosphate groups. The cardiolipin was hydrolyzed by iPLA2 in a mixed-micelle assay and the cardiolipin was extracted by the Bligh/Dyer method and then analyzed by LC/ESI-MS/MS (Fig. 4B). We identified the monolyso-cardiolipin (m/z = 596.4) and the dilyso-cardiolipin (m/z = 463.9) peaks by monitoring oleic acid (m/z = 281.4) in a precursor ion scan experiment. Both lyso-cardiolipins are also doubly charged as is cardiolipin. We have not detected any trilysocardiolipins, which would probably be very hydrophilic and not extracted by the current extraction method. Alternatively, iPLA2 may never cleave the sn-1 fatty acyl chain. Interestingly, sPLA2, which is known to have only sn-2 specific activity, only cleaves two fatty acyl chains off the cardiolipin resulting in the accumulation of dilyso-cardiolipin (Fig. 4C). This indicates the two sn-2 positions also have significantly higher rates of hydrolysis by PLA2 than the two sn-1 positions for sPLA2. We further purified the dilyso-cardiolipin from the GIA sPLA2 assay of cardiolipin. The hydrolyzed products have 90% dilyso-cardiolipin, which is the sn-1(1'-diacyl-lysocardiolipin, and contain less than 10% of monolysocardiolipin; the latter may arise from migration of the sn-1 fatty acid to the sn-2 position as occurs with PC. Assaying the five phospholipase A2s for PLA1 activity against dilyso-cardiolipin gave no activity.

**Differential PLA2 Activities**

The presence of four sterically distinct fatty acyl chains in cardiolipin presents challenges for kinetic studies of PLA2 hydrolysis. These phospholipase A2s [8] have specific activity against the two sn-2 fatty acyl chains, but no activity toward the sn-1 chains. Previously, iPLA2 has been reported to catalyze a transacylase reaction [35]. Cardiolipin, monolysocardiolipin and dilyso-cardiolipin can be measured in one mass spectrometry run (Fig. 3). Here, we aimed at understanding how PLA2 hydrolyzes cardiolipin to produce lyso-cardiolipins over a 2-hour time scale.
On the other hand, iPLA2 showed a completely different pattern. After initial 20 min and then reached a plateau. Dilyso-cardiolipin production continued through the whole time course. After 60 min, the dilyso-cardiolipin became the most abundant species. The two sPLA2s showed a very similar pattern with the hydrolysis of this particular cardiolipin species to the total ion counts (Fig. 5). The maximum increase was 4 fold for both GIA sPLA2 and GV sPLA2. In the presence of 50% cardiolipin, the activity for both enzymes toward PAPC was at the same level as if cardiolipin was not added. Note that Lp-PLA2 did not show significant activity toward PAPC or cardiolipin confirming that it has specificity for short chain containing and/or oxidized phospholipids [46].

Because both cardiolipin and PAPC are substrates for PLA2, the activation or inhibition curves could be altered by a surface charge effect, a competition effect and/or a surface dilution effect, we further examined the activity differences between these two substrates (Fig. 7). For iPLA2 and cPLA2, the activity decreased along with the increase of cardiolipin. The activity of iPLA2 toward cardiolipin was 2 μmol/min/mg, which was 3.8 times higher than toward PAPC in this assay. However, cPLA2 showed the opposite relative activity toward cardiolipin and PAPC. Under activating conditions, GIA sPLA2 showed a 3 fold greater activity toward PAPC than toward cardiolipin. GV sPLA2 showed a higher activity toward cardiolipin (18:1) than toward the PAPC substrate. The competition and surface dilution effects did not appear significant when the overall cardiolipin was below 20%. When cardiolipin was increased above 20%, cardiolipin became a major substrate, and then the competition between cardiolipin and PAPC became apparent. sPLA2 is particularly interesting in that it has high activity against cardiolipin and cardiolipin presence can increase its activity toward phosphatidylcholine. Negative charge may play a key role of the increase of activity, suggesting the hydrolysis of the cardiolipin in mitochondria may speed up the breakdown of other phospholipids by any secreted PLA2 generated.

Cardiolipin Effects on PLA2 Activities
Cardiolipin is not only a substrate of various PLA2s, but it may also play a key role in regulating PLA2 activities at the membrane surface. The catalytic activities of PLA2 and phospholipid metabolism can be affected by the presence of the bulky and negatively charged cardiolipin. Hence, we have utilized an in vitro mixed micelle assay to determine the effect of cardiolipin on the enzymatic activities of five different PLA2s acting on PAPC (Fig. 6). 1-palmitoyl(16:0)-2-arachidonoyl(20:4)-sn-phosphatidylcholine (PAPC) was chosen because it does not interfere with the measurement of the oleic acid (18:1) release from cardiolipin. A total of 100 μM phospholipid and 400 μM Triton X-100 was used as substrate. The results showed two major types of cardiolipin effects, activation or inhibition, on PLA2 activities. The inhibition by cardiolipin was observed for the activity of iPLA2 and cPLA2 toward PAPC (Fig. 6A, D). At 50% cardiolipin, iPLA2 activity was inhibited 80% and cPLA2 activity was inhibited 90%. The decrease caused by the addition of cardiolipin was not linear. The decreased level was higher than surface dilution of PAPC would predict assuming equal surface areas for Triton X-100, PC and cardiolipin molecules. Of course, the bulky volume of cardiolipin makes the calculation of surface dilution more complex than with simpler lipids in the PAPC/Triton X-100 mixed micelle system. In contrast, activation effects were observed with GIA and GV sPLA2 acting on PAPC which occurred when the phospholipid composition contained 0–20% cardiolipin (Fig. 6B, C). The PLA2 activity decreased when the cardiolipin content was above 20%. The decreased rate of activity was similar to that expected for surface dilution of the PAPC, but represents a complex mixture of effects. The maximum increase was 4 fold for both GIA sPLA2 and GV sPLA2. In the presence of 50% cardiolipin, the activity for both enzymes toward PAPC was at the same level as if cardiolipin was not added. Note that Lp-PLA2 did not show significant activity toward PAPC or cardiolipin confirming that it has specificity for short chain containing and/or oxidized phospholipids [46].

Figure 4. Cardiolipin and Lyso-cardiolipin Analysis by LC/MS. A. The dominant cardiolipin (18:1) peak in mass spectrometry was observed as a doubly charged ion in the negative ion mode. B. Cardiolipin was hydrolyzed by iPLA2 in a mixed-micelle assay and the cardiolipin was extracted by the Bligh/Dyer method and then analyzed by LC/MS. C. GIA sPLA2 hydrolysis of cardiolipin results in the accumulation of mainly dilyso-cardiolipin. doi:10.1371/journal.pone.0059267.g004

The mixed micelle assays contained 100 μM cardiolipin and 400 μM of Triton. The cardiolipin, monolyso-, and dilyso-cardiolipin were measured in the same samples by LC-MS/MS (Fig. 4B). The total ion counts for all cardiolipin species were calculated in each sample. The percentages of cardiolipin and lyso-cardiolipin were determined by the ratio of the ion counts for this particular cardiolipin species to the total ion counts (Fig. 5). The two sPLA2s showed a very similar pattern with the hydrolysis of cardiolipin occurring relatively early in the assay (Fig. 5B, C). The production of monolyso-cardiolipin increased during the initial 20 min and then reached a plateau. Dilyso-cardiolipin production continued through the whole time course. After 60 min, the dilyso-cardiolipin became the most abundant species. On the other hand, iPLA2 showed a completely different pattern. The cardiolipin percentage remained above 50% and lyso-cardiolipin levels stayed below 30%. The accumulation of dilyso-cardiolipin observed in the sPLA2 experiments did not occur for iPLA2 hydrolysis. This indicates that iPLA2 is regulated by a different mechanism than sPLA2 to maintain cardiolipin and lyso-cardiolipin at constant levels.
Conclusions

Each of the five enzymes studied are thought to have a different physiological function and the PLA2 activities toward cardiolipin as a substrate are significantly different for each (Fig. 7). The unique structure of cardiolipin also differentially affects the PLA2 activities toward other phospholipids (Fig. 6). Two major features of cardiolipin are the large head group formed by two glycerol backbones and its two negative charges from the two phosphates. One would assume that the large head group would affect the uniform lipid surface and affect activity as seen with iPLA2 and cPLA2, but not sPLA2 and Lp-PLA2. Interestingly, cPLA2 and iPLA2 are the two enzymes with high molecular weights and both contain a regulatory domain and a catalytic domain. Even 5% cardiolipin can decrease their activity toward PC. The other complication is the surface dilution effect. The addition of cardiolipin dilutes the concentration of PAPC on the surface of the Triton X-100 mixed micelles. The inhibition toward cPLA2 is more significant than iPLA2. Note that the cPLA2 mixed micelle assay contains the negatively charged PIP2 and the surface dilution effects by cardiolipin may be enhanced by PIP2 dilution. On the other hand, sPLA2 is only affected above 20% cardiolipin. Interestingly, cardiolipin is predominantly found in mitochondria, which contains 10–20% cardiolipin. In the mixed micelle assays, the cardiolipin forms a negatively charged surface and sPLA2 has been shown to favor anionic phospholipid substrates. The increased affinity toward the anionic surface may play an important role. The surface dilution effect starts to take place and decrease the PLA2 activities when the cardiolipin content is

Figure 5. Differential Cardiolipin Hydrolysis by iPLA2 and sPLA2. The hydrolysis of cardiolipin by A. GVIA iPLA2, B. GIA sPLA2 and C. GV sPLA2 were examined in mixed micelle assays containing 100 μM cardiolipin and 400 μM Triton X-100 over a 100 min time course. The cardiolipin (green), monolysocardiolipin (red) and dilyso-cardiolipin (blue) were measured in the same samples by LC/MS. The percentages of cardiolipin and lyso-cardiolipin are based on ion intensity counts.

doi:10.1371/journal.pone.0059267.g005

Figure 6. Cardiolipin Effects on PLA2 Activity toward PAPC. The in vitro mixed micelle assay was utilized to determine if cardiolipin affects the enzymatic activities of A. GVIA iPLA2, B. GIA sPLA2, C. GV sPLA2, and D. GIVA cPLA2 toward PAPC. Mixed micelles composed of 100 μM phospholipid and 400 μM Triton X-100 was employed as substrate containing the mole % of cardiolipin to PAPC indicated.

doi:10.1371/journal.pone.0059267.g006
The fold differences between cardiolipin and PAPC were calculated and are shown. (red) are shown. The assays were conducted in mixed micelle assays containing either 100 μM cardiolipin or 100 μM PAPC and 400 μM Triton X-100. The fold differences between cardiolipin and PAPC were calculated and are shown.

above 20% in the GIA and GV sPLA2 assays. Overall, cardiolipin alters membrane dynamics, which affects these different enzymes binding to micelles and catalyzing lipid hydrolysis. The fatty acid composition at the sn-2 positions may also be a factor. Among these enzymes, sPLA2 and iPLA2 do not have much specificity for the specific fatty acid while cPLA2 is selective for arachidonic acid and Lp-PLA2 is very specific for short fatty acyl chains and oxidized fatty acids. Because the availability of commercial reagents/materials/analysis tools: YH DD JC ED. Wrote the paper: YH

Figure 7. Comparison of PLA2 activities. The activities of GIVA iPLA2, GIA sPLA2, GV sPLA2, and GIVA cPLA2 toward cardiolipin (blue) and PAPC (red) are shown. The assays were conducted in mixed micelle assays containing either 100 μM cardiolipin or 100 μM PAPC and 400 μM Triton X-100. The fold differences between cardiolipin and PAPC were calculated and are shown.

doi:10.1371/journal.pone.0059267.g007

Author Contributions
Conceived and designed the experiments: YH DD JC ED. Performed the experiments: YH. Analyzed the data: YH DD JC ED. Contributed reagents/materials/analysis tools: YH DD JC ED. Wrote the paper: YH DD JC ED.

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