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Review

Distinguishing phospholipase A_2 types in biological samples by employing group-specific assays in the presence of inhibitors

Karin Killermann Lucas, Edward A. Dennis *

University of California, San Diego School of Medicine, Department of Chemistry, Biochemistry and Pharmacology, 9500 Gilman Drive, La Jolla, San Diego, CA 92093 0601, USA

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Abstract

This manuscript reviews and updates radiolabel-based enzyme assays designed to distinguish the activity of phospholipase A_2 (PLA_2) types in biological samples. This approach should be useful in lipidomics studies. The assays were originally designed to differentiate between Group IVA cytosolic PLA_2 (GIVA $cPLA_2$), Group VIA calcium-independent PLA_2 (GVIA $iPLA_2$), Group IIA secreted PLA_2 (GIIA $sPLA_2$) and Group V secreted PLA_2 (GV $sPLA_2$). The specificity of these assays has now been confirmed using purified, recombinant human PLA_2s and the utility of these assays is demonstrated with rat spinal cord homogenate as an example of a biological tissue sample of interest to the neuroscience community. Modifications to the original assays by the addition of group-specific inhibitors are presented to ensure the specificity of the assays and to further differentiate between recently identified PLA_2s. Specific tests are suggested to confirm the specificity of each assay. Additionally, it was discovered that one commonly used GIVA $cPLA_2$/GVIA $iPLA_2$ inhibitor, methyl arachidonyl fluorophosphonate (MAFP) from one commercial source, was found to inhibit GIIA $sPLA_2$ and GV $sPLA_2$, but not GIVA $cPLA_2$, presumably due to oxidation of the compound during shipment, resulting in a different molecule with altered specificity.

Keywords: Lipidomics; Phospholipase A_2; MAFP; BEL; Indoxam; Dole assay; PLA_2 activity assay; PIP_2

* Corresponding author. Tel.: +1 858 534 3055; fax: +1 858 534 7390.
E-mail address: edennis@ucsd.edu (E.A. Dennis).

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Contents

1. Introduction

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of fatty acids from the *sn*-2 position of phospholipids. The release of fatty acids including arachidonic acid (AA) and lysophospholipids from the cell membrane by PLA_2 activity leads to a cascade of lipid second messengers that regulates a wide variety of physiological responses. Interest in the PLA_2 field has increased in the past decade with the discovery of the important role of lipid messengers in diseases such as cancer [1–3] and atherosclerosis [4,5]. While lipidomics approaches should help elucidate the intricate relationships of the numerous lipid messengers, the ability to also block and measure the activity of the specific PLA_2 enzymes that initiate lipid second messenger release should aid in lipidomics studies.

In 1999, our laboratory published a manuscript on group-specific assays describing four specific PLA_2 assays [6] that were designed to distinguish between the four major mammalian PLA_2 enzymes that had been identified at that time. The assays took advantage of differences in preferred lipid substrate and activators, calcium dependence and susceptibility to disulfide bond reduction to distinguish between the Group IVA cytosolic PLA_2 (GIVA $cPLA_2$), the Group VIA calcium-independent PLA_2 (GVIA $iPLA_2$) and two secreted $sPLA_2$s (GIIA and GV $sPLA_2$). The power of these assays lies in their use in determining the activity of each of these enzymes in a biological sample.

In the decade following the research that contributed to the manuscript's preparation, major advances have occurred in the PLA_2 field. Two new paralogs of the GIVA $cPLA_2$ were

discovered and characterized [7,8] as well as new forms of iPLA$_2$ [9], and a number of novel sPLA$_2$ enzymes, bringing the current count to 10 different mammalian sPLA$_2$s [9–12]. It should be noted that at this time, 14 groups and numerous subgroups of PLA$_2$s have been described [9,13]. These 14 groups include four main "types" of PLA$_2$s including sPLA$_2$s, cPLA$_2$s, iPLA$_2$s and PAF-acetyl hydrolases. This manuscript focuses on group-specific assays for a mammalian example of each of the first three types. The fourth type, the PAF-acetyl hydrolase enzymes (Groups VII and VIII), acts on PAF and/or oxidized phospholipids and not on long chain "normal" phospholipids. Thus, the assays described herein should not detect this type of PLA$_2$. However, some of the types of enzymes described herein could act on PAF and oxidize phospholipids, so if group-specific assays for PAF-acetyl hydrolases are desired, analogous strategies to those described herein could be devised for PAF-acetyl hydrolases.

Herein, a review and update of the PLA$_2$ activity assays is provided in light of these recent findings on unique PLA$_2$s. Additionally, new information regarding specific inhibitors of the various PLA$_2$s has been developed so that we now include inhibitors in the assays that improve the specificity of the assays. Practical tips and technical details are offered for each assay that are hoped to be of use to the PLA$_2$ novice and expert alike. In addition, we illustrate the usefulness of these assays on a typical biological sample of interest to the neuroscience community, namely rat spinal cord homogenates.

2. Materials and methods

2.1. Materials

Non-radioactive lipids were purchased from Avanti Polar Lipids: 1-palmitoyl-2-arachidonoyl-diacyl-*sn*-glycero-3-phosphatidylcholine (PAPC), 1-palmitoyl-2-palmitoyl-diacyl-*sn*-glycero-3-phosphatidylcholine (DPPC), 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylethanolamine (PLPE) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyl-serine (POPS). Phosphatidylinositol 4,5-bisphosphate (PIP$_2$) was purchased from Roche Applied Science. Radioactive PAPC was purchased from Perkin-Elmer Life Sciences: phosphatidylcholine, L-α-1-palmatoyl-2-arachidonyl and [arachidonyl-1-^{14}C]. Radioactive DPPC and PLPE were purchased from Amersham Biosciences: phosphatidylcholine, 1-palmitoyl-2-[^{14}C] palmitoyl and 1-palmitoyl-2-[^{14}C] linoleoyl-phosphatidyl ethanolamine. Triton X-100 was purchased from CalBiochem. Bromoenol lactone (BEL) was purchased from Cayman Chemical. Methyl arachidonyl fluorophosphonate (MAFP) was purchased from Cayman Chemical (shipped in methyl acetate solution) as well as from Biomol (shipped as a dry powder). LY311727 was a kind gift from Lilly Research Laboratories. Indoxam was a gift from Shionogi Research Laboratories.

2.2. Phospholipase A_2 enzymes

Synthetic human Group IIA sPLA$_2$ was a generous gift of Drs. T. Hackeng and J. Griffin at the Research Institute of Scripps Clinic and was chemically synthesized and folded as described [14]. Recombinant human Group V sPLA$_2$ was expressed, purified and folded in

our laboratory (Six et al., in preparation). The human Group IVA $cPLA_2$ was a generous gift from Drs. R. Kramer and J. Sharp at Lilly Research Laboratories [15]. The human Group VIA-2 $iPLA_2$ was expressed and purified in our laboratory (Stevens et al., in preparation).

2.3. Preparation of rat spinal cord homogenate and rat cerebral spinal fluid

Prior to sacrifice, the rats were deeply anesthetized and after decapitation, the spinal cords were ejected from the spinal column by a saline-filled syringe. The lumbar part of the spinal cord was frozen on dry ice and stored at −70 °C. Frozen spinal cords were pulverized using a BioPulverizor (Biospec Products) pre-chilled on dry ice. Pulverized tissue was then transferred to a microcentrifuge tube and mixed with 750 μL lysis buffer: 10 mM Hepes, pH 7.5, 1 mM EDTA and 0.34 M sucrose. Twenty microlitres of mammalian protease inhibitor cocktail (Sigma) was added immediately. Samples were vortexed and sonicated until homogenous and then centrifuged at 16,000 × *g*, 4 °C, 40 min. Supernatants were transferred to a fresh eppendorf tube and the pellet was discarded.

2.4. Phospholipase A_2 assays

2.4.1. Lipid preparation

Lipid was alliquotted, dried under N_2 and lyophilized for at least 1 h to remove all traces of chloroform. For mixed micelles, the lipid was then resuspended in 100 mM Hepes and Triton X-100 and micelles were created by repeated vortexing and heating in hot water until the solution clarified. For small unilamellar vesicles (SUVs), the lipid was resuspended in 100 mM Hepes, vortexed for 10 min and then sonicated on ice until the solution cleared. The preparation was then tested by centrifuging an aliquot at 15,000 × *g*, RT for 5 min. The radioactivity of a 5 μL sample of the centrifuged samples as well as a 5 μL sample of the original preparation was determined by scintillation counting and compared. If SUVs do not form properly, they will precipitate upon centrifugation and the respective radioactive counts will be significantly different. Substrate was used only if the counts were within 200 cpm of each other.

2.4.2. Assay conditions

Group IV $cPLA_2$ assay—100 μM lipid PAPC/PIP_2 (97/3) doping with 100,000 cpm ^{14}C labeled PAPC in 400 μM Triton X-100 mixed micelles, 100 mM Hepes, pH 7.5, 0.08 mM $CaCl_2$, 0.1 mg/mL BSA and 2 mM DTT. Group VI $iPLA_2$ assay—100 μM DPPC doping with 100,000 cpm ^{14}C labeled DPPC in 400 μM Triton X-100 mixed micelles, 100 mM Hepes, pH 7.5, 5 mM EDTA and 1 mM ATP. Group V $sPLA_2$ assay—100 μM DPPC/POPS (3/1) doping with 100,000 cpm ^{14}C labeled DPPC SUV's, 100 mM Hepes, pH 7.5, 5 mM Ca^{2+} and 1 mg/mL BSA. Group IIA $sPLA_2$ assay—100 μM PLPE/POPS (1/1) doping with 100,000 cpm ^{14}C labeled PLPE SUVs, 100 mM Hepes, pH 7.5, 1 mM Ca^{2+} and 1 mg/mL BSA.

The total volume for each assay is 500 μL—200 μL lipid, 250 μL assay buffer and 50 μL sample. For the biological samples, the amount of calcium or EDTA added was adjusted to account for the addition of EDTA in the lysis buffer to give the final concentrations listed above. Pure enzymes were incubated with substrate for 30 min while biological samples

were incubated with substrate for 1 h at 40 °C in a shaking bath. The assay was then terminated by addition of 2.5 mL Dole Reagent (isopropyl alcohol:heptane:0.5 M sulfuric acid, 400:100:20, v/v/v). Silica gel (0.1–0.2 mg) was added to each tube followed by 1.5 mL heptane and 1.5 mL deionized water. Each tube was vortexed for 15 s. One millilitre of the organic phase was removed and passed through a Pasteur pipet filled with silica gel (0.1–0.2 mg). This column was then washed with 1 mL diethyl ether. Five millilitres of scintillation cocktail (Biosafe II, RPI) was then added to the eluent and the radioactivity was determined by scintillation counting.

2.4.3. Data analysis

Unless otherwise indicated, experiments were performed in at least two independent sets, each in duplicate. A representative experiment is shown. Results are presented as the mean ± standard deviation. Differences between groups were compared with one-way ANOVA using Prism statistical software.

3. Results and discussion

3.1. Group-specific assays on pure enzymes

The original manuscript in which the group-specific PLA_2 assays were described [6] utilized four PLA_2 enzymes: synthetic human GIIA $sPLA_2$, recombinant human GIVA $cPLA_2$, recombinant human GV $sPLA_2$ and GVIA $iPLA_2$ purified from rat brain. The current manuscript improves on these sources in two ways. First, we have recently developed a method to express and purify recombinant human GVIA-2 $iPLA_2$ so that we can now test the assays using all purified, human PLA_2 enzymes. Additionally, the GV $sPLA_2$ that was originally used contained extra, non-native amino acids at the N-terminus. We have now developed a method to express and purify GV $sPLA_2$ without these extraneous amino acids that shows higher intrinsic activity. Fig. 1 shows a coomassie-stained SDS-PAGE gel of the four enzymes used to retest the assays. The activity of each of these enzymes in the four assays is shown in Fig. 2. Using these purified, human enzymes, the assay results are in good agreement with the original findings.

3.2. Proof of principle: testing a biological sample

While these assays work well to differentiate the activity of pure PLA_2 enzymes, the purpose of their development was to differentiate between mixtures of PLA_2s found in a biological sample. The specificity of each assay can be tested by observing the effect of the addition of PLA_2 inhibitors on activity. The inhibitors and compounds used to decrease PLA_2 activity in this study are summarized in Table 1.

Rat spinal cord homogenate was chosen as a test material for assay specificity because it is known to express at least one PLA_2 enzyme from each of the three main types of PLA_2 ($sPLA_2$, $cPLA_2$ and $iPLA_2$) [16,17]. For each assay, 50 μL of rat spinal cord homogenate was incubated with substrate for 1 h in the presence or absence of 0.8 mol% PLA_2 inhibitor, 10 mM DTT or 5 mM EDTA. Only 0.8 mol% inhibitor is added to the substrate because

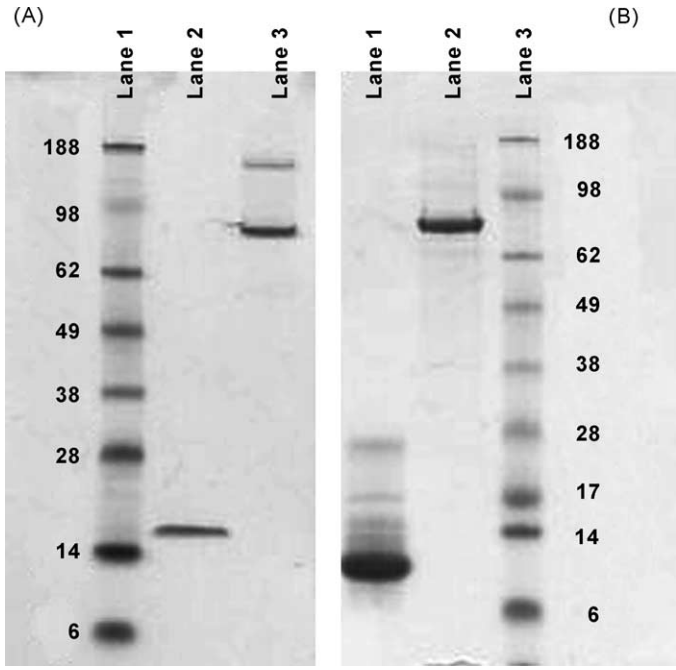

Fig. 1. Coomassie-stained SDS-PAGE of purified enzymes. (A) Lane 1: molecular weight ladder; lane 2: GIIA $sPLA_2$; lane 3: GIVA $cPLA_2$. (B) Lane 1: GV $sPLA_2$; lane 2: GVIA-2 $iPLA_2$; lane 3: molecular weight ladder.

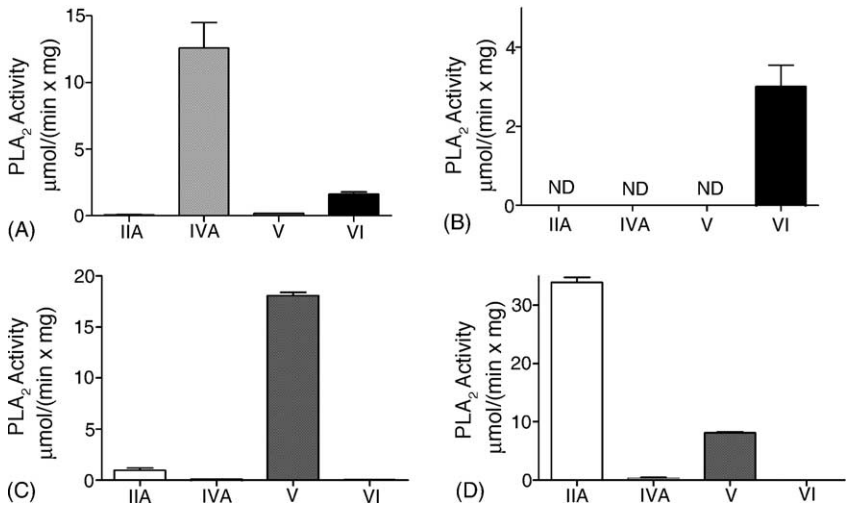

Fig. 2. Group-specific assays tested on purified enzymes. (A) GIVA $cPLA_2$ specific assay: 100 μM PAPC/PIP_2 (97/3)/400 μM Triton X-100 mixed micelles; (B) GVI $iPLA_2$ specific assay: 100 μM DPPC/400 μM Triton X-100 mixed micelles, ND indicates activity was not detected; (C) GV $sPLA_2$ specific assay: 100 μM DPPC/POPS (3/1) SUVs and (D) GIIA $sPLA_2$ specific assay: 100 μM PLPE/POPS (1/1) SUVs.

Table 1
PLA_2 inhibitors

Inhibitor/compound	PLA_2s inhibited	Method of inhibition
MAFP	GIVA, GIVB, GIVC $cPLA_2$ GVIA $iPLA_2$	Enzyme inhibitor
BEL	GVIA, GVIB $iPLA_2$	Enzyme inhibitor
LY311727	All $sPLA_2$s	Enzyme inhibitor
Indoxam	All $sPLA_2$s	Enzyme inhibitor
EDTA	All $sPLA_2$s, GIVA, GIVB $cPLA_2$	Chelates calcium required for catalysis or binding of the C2 domain
DTT	All $sPLA_2$s	Reduces disulfide bonds to denature protein structure

higher concentrations could interfere with the presentation of the substrate, resulting in a non-specific decrease of the activity. As a result of this low inhibitor concentration, complete inhibition of activity is not expected. The results of these assays are presented in Figs. 3 and 4 and are discussed below.

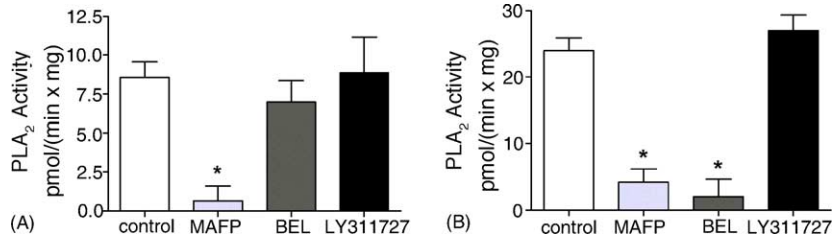

Fig. 3. PLA_2 activity of rat spinal cord homogenate on the GIVA $cPLA_2$ and GVI $iPLA_2$ specific assays, $n = 3$. Samples are tested on substrate alone (control) or in the presence of 0.8 mol% PLA_2 inhibitor (MAFP, BEL or LY311727). (A) GIVA $cPLA_2$ specific assay: 100 μM PAPC/PIP_2 (97/3)/400 μM Triton X-100 mixed micelles, (B) GVI $iPLA_2$ specific assay: 100 μM DPPC/400 μM Triton X-100 mixed micelles. $^{*}P < 0.001$ compared with control. Portions of this figure are reprinted with permission from the *British Journal of Pharmacology* [27].

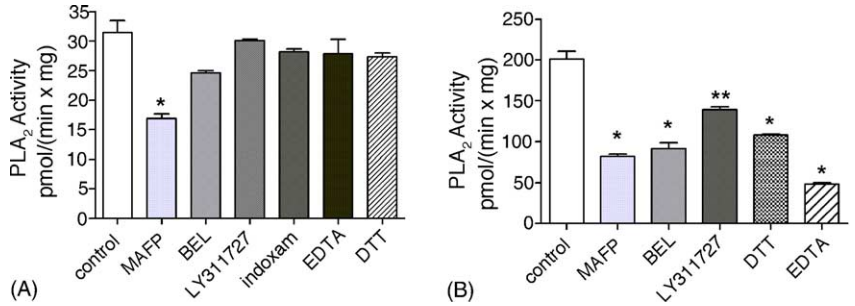

Fig. 4. PLA_2 activity of rat spinal cord homogenate tested on the $sPLA_2$ specific assays, $n = 3$. Samples are tested on substrate alone (control) or in the presence of 0.8 mol% PLA_2 inhibitor (MAFP, BEL, LY311727 or Indoxam), or in the presence of reducing agent (10 mM DTT) or a cation chelator (5 mM EDTA). (A) GV $sPLA_2$ specific assay: 100 μM DPPC/POPS (3/1) SUVs, (B) GIIA $sPLA_2$ specific assay: 100 μM PLPE/POPS (1/1) SUVs. $^{*}P < 0.001$, $^{**}P < 0.05$, compared with control. Portions of this figure are reprinted with permission from Neuroscience [17].

3.3. Specificity improved assays

The results obtained from this study demonstrate the advantage of adding inhibitors to the assays to improve their specificity for use in testing biological samples. This can be accomplished by the addition of specific PLA_2 inhibitors to the assay buffers. However, even with these improvements, it is strongly recommended that researchers verify the specificity of these assays for each biological sample type to be tested. Table 2 summarizes the substrate and buffer conditions of each assay as well as compounds that can be used to inhibit the activity of that PLA_2 subgroup. For each assay, it is recommended that the specificity be verified both by inhibiting the activity of interest (for example, decreasing GIVA $cPLA_2$ activity with MAFP) as well as by excluding the possibility of crossover from other PLA_2s (for example, also testing for $iPLA_2$ activity with BEL and $sPLA_2$ activity with DTT). The principles, improvements and specificity of each of these assays are discussed below.

3.3.1. GIVA $cPLA_2$

The GIVA $cPLA_2$ assay takes advantage of the enzymes known preference for arachidonic acid at the *sn*-2 position of phospholipid [18] as well as its demonstrated ~100-fold increase in activity in the presence of PIP_2 in a Triton X-100 mixed micelle substrate [19]. The buffer includes 80 μM Ca^{2+}, which is enough for GIVA $cPLA_2$ to be active but not the $sPLA_2$s, as well as 2 mM DTT, which acts to reduce the disulfide bonds of the $sPLA_2$s to denature these enzymes. The two $sPLA_2$s tested had negligible activity in this assay, but as was found previously, GVIA-2 $iPLA_2$ does have measurable, although low activity (Fig. 2A). These findings are further supported by the results of the biological sample testing (Fig. 3A). The activity of rat spinal cord homogenate in the GIVA $cPLA_2$ assay was almost entirely inhibited by MAFP, a widely used $iPLA_2$ and $cPLA_2$ inhibitor [20], while the $sPLA_2$ inhibitor, LY311727, had no effect. The Group VI $iPLA_2$ inhibitor, BEL, reduced the activity slightly, demonstrating the GVI $iPLA_2$ crossover in the assay, although this decrease was not statistically significant.

The original manuscript [6] describes a mathematical method to subtract the GVI $iPLA_2$ activity from the total activity to determine the contribution specific to GIVA $cPLA_2$. This method has two major disadvantages. First, it requires that biological samples be assayed

Table 2
Inhibitor-based PLA_2 assays and tests for specificity

PLA_2 class	$cPLA_2$	$iPLA_2$	$sPLA_2$
Substrate	100 μM PAPC/PIP_2 (97/3), 400 μM Triton X-100 mixed micelles	100 μM DPPC, 400 μM Triton X-100 mixed micelles	100 μM PLPE/POPS (1/1) SUVs (or 100 μM DPPC/POPS (3/1) SUVs)
Buffer conditions	100 mM Hepes, 0.08 mM $CaCl_2$, 0.1 mg/mL BSA, 2 mM DTT, 0.8 μM BEL	100 mM Hepes, 5 mM EDTA, 1 mM ATP, 2 mM DTT	100 mM Hepes, 1 (or 5)mM $CaCl_2$, 1 mg/mL BSA, 0.8 μM MAFP
Test compounds	MAFP, EDTA	MAFP, BEL	EDTA, DTT, indoxam, LY311727

both for $cPLA_2$ as well as $iPLA_2$ activity even if only the $cPLA_2$ activity is of interest. Secondly, it assumes that the specific activity of the GVI $iPLA_2$ is constant. Because there may be significant differences in the specific activities of the GVI $iPLA_2$ splice variants as well as species differences between orthologs, this is a risky assumption. We instead suggest including 0.8 mol% bromoenol lactone in the $cPLA_2$ assay to eliminate any $iPLA_2$ crossover. BEL specifically inhibits the GVI $iPLA_2$, but does not inhibit any of the GIV $cPLA_2$ paralogs [21]. It should be noted that while BEL is specific for $iPLA_2$ within the PLA_2 family, it is also known to potently inhibit phosphatidic acid phosphohydrolase (PAP-1) [22].

As was mentioned in Section 1, two novel $cPLA_2$ paralogs have recently been described. Neither GIVB nor GIVC $cPLA_2$ are activated by PIP_2 [21]; therefore, these enzymes should have negligible activity in this assay. Furthermore, GIVB $cPLA_2$ has very low intrinsic activity so that a vast quantity would have to be present in a sample to produce any appreciable activity in this assay. However, it should be noted that it is not possible to rule out the contribution of GIVB activity to this assay with absolute certainty.

To verify that GIVC $cPLA_2$ is not contributing to the activity, a biological sample can be tested in the presence of BEL and EDTA. Because GIVC $cPLA_2$ has no calcium dependence and is not inhibited by BEL, it is the only known PLA_2 that will have catalytic activity under these conditions. If activity is found, it can be subtracted from the activity of the Group IVA $cPLA_2$ assay to determine the GIVA contribution. Recently, a pyrrolidine-based molecule has been reported to inhibit GIVA $cPLA_2$ activity while having significantly less inhibitory action against GIVC $cPLA_2$ that might be used to distinguish the two activities [23]. Unfortunately, this inhibitor is not commercially available.

3.3.2. GVI $iPLA_2$

The $iPLA_2$ assay is the most specific of the four assays. None of the other purified enzymes had any activity in this assay (Fig. 2B) and the rat spinal cord homogenate activity in this assay was inhibited by BEL and MAFP but not LY311727 (Fig. 3B). The assays specificity can be attributed to the 5 mM EDTA in the assay buffer that acts as a calcium chelator. All $sPLA_2$s tested thus far have an absolute calcium requirement for catalysis. The GIVA and GIVB $cPLA_2$ both have a C2 domain that sequesters the enzymes to the membrane upon calcium binding. However, there is one other PLA_2 enzyme in addition to the $iPLA_2$ subfamily that is calcium independent, Group IVC $cPLA_2$. In initial experiments utilizing cell lysates overexpressing GIVC $cPLA_2$, this enzyme has been found to have significant activity in the $iPLA_2$ assay (unpublished observations). Therefore, it is possible that GIVC $cPLA_2$ can contribute to the activity in this assay if it is present in a biological sample. To rule out any contribution by this PLA_2 subtype, the sample can be assayed with and without the addition of up to 2 mol% BEL, which will specifically inhibit GVI $iPLA_2$ activity but not GIVC $cPLA_2$. If significant activity remains in the presence of BEL, it is likely due to GIVC $cPLA_2$. Unfortunately, a compound has not yet been found which inhibits GIVC $cPLA_2$ but not GVI $iPLA_2$. However, by performing the assay both with and without BEL, the contribution to the observed activity from GIVC $cPLA_2$ can be determined and then subtracted from the total activity.

3.3.3. $sPLA_2$

At the time the original group-specific assay manuscript [6] was written, the two major $sPLA_2$ enzymes found in biological samples were Groups IIA and V. The authors developed an assay for each of these enzymes that would distinguish one from the other as well as from the GVI $iPLA_2$ and GIVA $cPLA_2$. These assays maintained their specificity when tested with purified, human PLA_2s (Fig. 2C and D). The original manuscript [6] describes a mathematical method to subtract the GV $sPLA_2$ activity from the GIIA assay and vice versa in order to determine the true GIIA and GV $sPLA_2$ activity in a biological sample. Today, 10 different mammalian $sPLA_2$s have been identified and as their activity under these conditions is unknown, this simple mathematical subtraction is no longer valid nor is the specification as "Group IIA" or "Group V" assay. Rather, both of these assays should be regarded as general $sPLA_2$ assays. For ease of discussion, these historical distinctions will be adhered to in this manuscript.

Both of these $sPLA_2$ assays proved to be less specific when used to assay the spinal cord homogenate. The "GV" $sPLA_2$ assay, which uses DPPC/POPS (3/1) as its substrate, did not show a decrease in activity with the addition of BEL, LY311727 or indoxam, but was decreased somewhat by MAFP (Fig. 4A). Addition of EDTA or pre-incubation of the spinal cord homogenate for 30 min with 10 mM DTT also failed to decrease the observed activity (Fig. 4A), indicating that the observed activity cannot be attributed to any $sPLA_2$.

The rat spinal cord homgenate activity in the "GIIA" $sPLA_2$ assay, which uses PLPE/POPS (1/1) as its substrate, was decreased by all three PLA_2 inhibitors (Fig. 4B). In addition, DTT and EDTA only partially decreased the activity. This evidence indicates that in the presence of a biological sample, the GIIA assay is no longer $sPLA_2$ specific and in fact presents substrate that can be hydrolyzed by all three PLA_2 classes. This change is likely due to the presence of phospholipid from the spinal cord homogenate, which alters the presentation of the phospholipid substrate. Even after centrifugation, some phospholipid will remain in the supernatant of most biological samples. It is important to recognize the ability of phospholipid from the biological sample to interact with the phospholipid substrate of the assay and to limit the addition of physiological phospholipid as much as possible. In addition to altering the specificity of the assay, phospholipid addition can also result in an apparent reduction in activity because the ratio of radioactive lipid to non-labeled lipid will decrease resulting in the release of less radiolabel due to surface dilution.

The GIIA $sPLA_2$ assay can be modified by the addition of 0.8 mol% MAFP to restore its $sPLA_2$ selectivity. The addition of MAFP will act to inhibit all of the GIV $cPLA_2$ paralogs as well as any GVI $iPLA_2$ activity. The remaining activity can thus be confidently attributed to $sPLA_2$. The suitability of this assay was demonstrated by testing rat spinal cord homogenate in the presence of substrate alone, in the presence of either MAFP or LY311727, or in the presence of both MAFP and LY311727 (at 0.8 mol% each) (Fig. 5). When combined, the inhibitors had an additive effect, thereby demonstrating that the PLA_2 activity is indeed attributable to multiple enzymes and verifying that the addition of MAFP results in the measurement of only $sPLA_2$ activity.

A number of recent publications have utilized indoxam, a novel, potent inhibitor of $sPLA_2$ to successfully decrease $sPLA_2$ activity [24–26]. Although indoxam is not sold commercially at this time, it may be used to distinguish $sPLA_2$ activity if it is available.

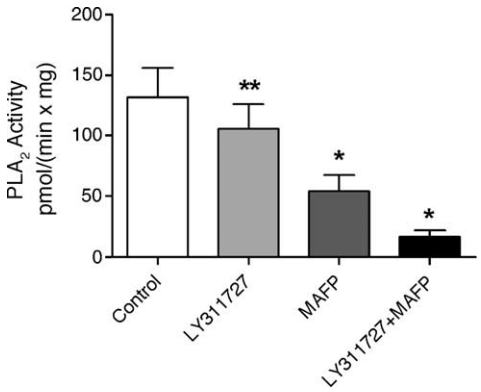

Fig. 5. PLA_2 activity of rat spinal cord homogenate on the GIIA $sPLA_2$ specific assay, $n = 9$. Samples are tested on substrate alone (control) or in the presence of 0.8 mol% PLA_2 inhibitor (MAFP, LY311727, LY311727 + MAFP at 0.8 mol% each). $^{*}P < 0.001$, $^{**}P < 0.05$ compared with control. Portions of this figure are reprinted with permission from *Neuroscience* [17].

3.4. Important precautions

When testing the $sPLA_2$ activity of a biological sample, special precaution should be taken to avoid any blood contamination as blood contains a high level of GIIA $sPLA_2$. We found a very substantial amount of $sPLA_2$ activity in rat cerebral spinal fluid which was highly sensitive to LY311727 only to determine later that it was due entirely to blood contamination. When the samples were cleanly removed from the animal, we found no $sPLA_2$ activity at all (Fig. 6).

Much of the specificity of these assays is established by the concentration of calcium. It is very important to achieve the final calcium concentrations listed in Table 2. Many lysis buffers and most protease inhibitor cocktails include significant concentrations of EDTA or EGTA, which will chelate the calcium in the assay. It is of vital importance to compensate for this chelation with the addition of equimolar calcium to the assay. Endogenous calcium

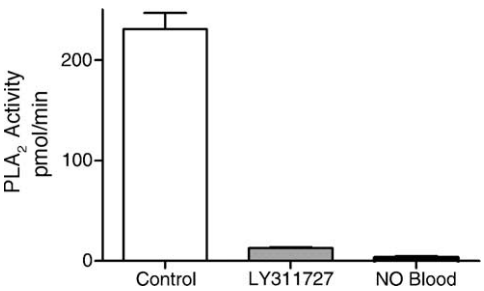

Fig. 6. PLA_2 activity of rat cerebral spinal fluid on the GIIA $sPLA_2$ specific assay. Samples contaminated with blood demonstrated significant activity (control, $n = 2$) that was inhibited by LY311727 ($n = 2$). Samples that were not contaminated with blood did not have significant activity ($n = 25$).

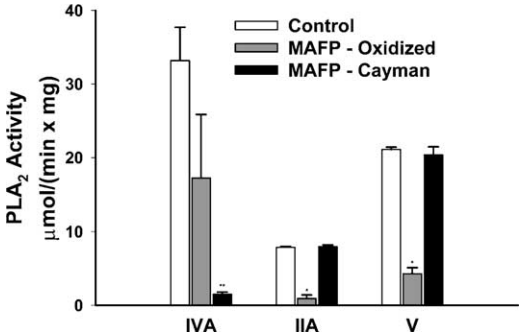

Fig. 7. MAFP inhibition profile. Purified enzymes were tested on substrate alone (control) or in the presence of 0.8 mol% inhibitor (oxidized MAFP or Cayman MAFP). $^{*}P < 0.001$, $^{**}P < 0.05$ compared with control.

from the biological sample has not interfered with any of these assays in studies performed thus far.

Reproducibility is also an important issue of which to be aware. Even the most experienced phospholipid chemist is hard pressed to prepare substrate on two different days, which will produce the same specific activity for a given sample. We have found that the activity of biological samples can vary up to 5-fold from substrate preparation to preparation. The best way to compare the PLA_2 activity in a number of biological samples is to test all the samples on the same day using the same substrate. If this is not possible, an internal standard of pure PLA_2 enzyme may be used to adjust for the variation in phospholipid preparations.

Finally, a special precaution must be mentioned regarding the source of the PLA_2 inhibitor MAFP. During our studies, we tested two sources of MAFP, one was shipped as a dry powder while the other was shipped in a solution of methyl acetate. Both inhibitors were tested on the pure enzymes and it was discovered that the MAFP that had been shipped dry had the exact opposite inhibition profile than was expected. These samples of MAFP inhibited the activity of the GIIA and GV $sPLA_2$s but not GIVA $cPLA_2$. NMR analysis of these samples indicated that the MAFP that had been shipped dry was highly oxidized (data not shown). Cayman chemical ships MAFP in a methyl acetate solution and this preparation exhibits the expected inhibition profile (Fig. 7). Because few researchers test the specificity of commercial inhibitors, this finding is especially alarming and could bring into question the conclusions of many published reports that utilized this source of MAFP.

4. Conclusion

As interest in the inflammatory component of disease increases, so will the desire to elucidate the role different PLA_2 types play in the initiation of the inflammatory response. This update establishes the utility of the PLA_2 group-specific assays in light of new developments in the PLA_2 field and is hoped to be of use to the research community.

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