Localization and functional interrelationships among cytosolic Group IV, secreted Group V, and Ca\(^{2+}\)-independent Group VI phospholipase A\(_2\)s in P388D\(_1\) macrophages using GFP/RFP constructs

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Abstract

P388D\(_1\) cells exposed to bacterial lipopolysaccharide (LPS) mobilize arachidonic acid (AA) for prostaglandin synthesis in two temporally distinct pathways. The “immediate pathway” is triggered within minutes by receptor agonists such as platelet-activating factor (PAF) but only if the cells have previously been primed with LPS for 1 h. The “delayed pathway” occurs in response to LPS alone over the course of several hours. We have now investigated the subcellular localization of both the Group IV cytosolic phospholipase A\(_2\) (cPLA\(_2\)) and the Group V secreted PLA\(_2\) (sPLA\(_2\)) during these two temporally distinct routes of AA release. We have prepared cells overexpressing fusion proteins of sPLA\(_2\)-GFP and cPLA\(_2\)-RFP. In the resting cells, cPLA\(_2\)-RFP was uniformly located throughout the cytoplasm, and short-term treatment with LPS did not induce translocation to perinuclear and/or Golgi membranes. However, such a translocation occurred almost immediately after the addition of PAF to the cells. Long-term exposure of the cells to LPS led to the translocation of cPLA\(_2\)-RFP to intracellular membranes after 3 h, and correlates with a significant release of AA in a cPLA\(_2\)-dependent manner. At the same time period that the delayed association of cPLA\(_2\) with perinuclear membranes is detected, an intense fluorescence arising from the sPLA\(_2\)-GFP was found around the nucleus in the sPLA\(_2\)-GFP stably transfected cells. In parallel with these changes, significant AA release was detected from the sPLA\(_2\)-GFP transfectants in a cPLA\(_2\)-dependent manner, which may reflect cross-talk between sPLA\(_2\) and cPLA\(_2\). The subcellular localization of the Group VIA Ca\(^{2+}\)-independent PLA\(_2\) (iPLA\(_2\)) was also investigated. Cells overexpressing iPLA\(_2\)-GFP showed no fluorescence changes under any activation condition. However, the iPLA\(_2\)-GFP-expressing cells showed relatively high basal AA release, confirming a role for iPLA\(_2\) in basal deacylation reactions. These new data illustrate the subcellular localization changes that accompany the distinct roles that each of the three kinds of PLA\(_2\) present in P388D\(_1\) macrophages play in AA mobilization.

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1. Introduction

The mobilization of free arachidonic acid (AA) for eicosanoid synthesis is mainly regulated by phospholipase A\(_2\) (PLA\(_2\)), a superfamily of enzymes that includes 14 groups, most of them containing several subgroups [1,2]. From a functional point of view, the PLA\(_2\) enzymes can be divided into three major classes, namely the cytosolic Ca\(^{2+}\)-dependent PLA\(_2\)\(_S\) (cPLA\(_2\)), the secreted PLA\(_2\)\(_S\) (sPLA\(_2\)) and the cytosolic Ca\(^{2+}\)-independent PLA\(_2\)\(_S\) (iPLA\(_2\)) [2,3].
In major eicosanoid-producing inflammatory cells such as mast cells and macrophages, AA mobilization and attendant prostaglandin production usually occurs in two phases [4,5]. The “immediate pathway” takes place within minutes and is strikingly characterized by its dependence on \( \text{Ca}^{2+} \) mobilization from intracellular stores. The second “delayed pathway” takes several hours and involves synthesis of new protein effectors [4,5].

Our laboratory has been delineating the molecular events involved in PLA\(_2\) activation and AA mobilization in murine P388D\(_1\) macrophage-like cells under different stimulation conditions [6–18]. In both the immediate and delayed pathways for AA release, two PLA\(_2\)s have been implicated, i.e., the Group IV cPLA\(_2\) and the Group V sPLA\(_2\). The former enzyme plays a critical role as an initiator and main regulator of the signaling leading to AA mobilization. The importance of cPLA\(_2\) in AA mobilization and prostaglandin production by immunoinflammatory cells such as mast cells and macrophages has been highlighted by recent studies utilizing cells from cPLA\(_2\) knock-out mice [19–22].

Beyond this essential role of cPLA\(_2\) in the initiation of stimulus-coupled AA mobilization, the sPLA\(_2\) also has the potential to participate in the process and may augment the cPLA\(_2\)-dependent AA release [2–5], as evidenced in the Group V sPLA\(_2\) knockout mice [23], but it may play other roles instead (U. Kessen et al., manuscript in preparation). The molecular nature of the sPLA\(_2\) involved appears to vary depending on species and cell type. In murine macrophage and mast cells, the dominant form involved in AA release appears to be the Group V enzyme [9,22,24–26].

The third PLA\(_2\) form present in macrophages, i.e., the iPLA\(_2\), appears not to participate in the AA release process directly, as shown by studies in P388D\(_1\) macrophages [7,16,17], mesangial cells [27], peritoneal macrophages [28], neutrophils [29,30] and U937 cells [31,32], where selective inhibition of the iPLA\(_2\) did not affect the stimulus-induced AA release. Rather, the iPLA\(_2\) appears to play an important role in regulating basal phospholipid remodeling via fatty acid deacylation reactions [33,34]. Recent evidence has suggested the participation of iPLA\(_2\) in the enhanced destruction of membrane phospholipid secondary to oxidative stress [35] or to the cells entering apoptosis [36,37] and may have other roles in other cells and tissues [2].

Despite all the progress in understanding the biochemical events regulating AA release in macrophages, little is still known about the location and movement of the three kinds of PLA\(_2\)s during this process. In this work, we have constructed fusion proteins of Group IVA cPLA\(_2\), Group V sPLA\(_2\) and Group VIA iPLA\(_2\) with green or red fluorescent protein and investigated their subcellular localization during the immediate and delayed pathways of AA mobilization in stimulated P388D\(_1\) macrophages.

### 2. Experimental procedures

#### 2.1. Materials

LPS (E. coli 0111: B4) and PAF were purchased from Sigma (St. Louis, MO). Iscove’s modified Dulbecco’s medium (IMDM) and fetal bovine serum (FBS) were from Whittaker laboratories (Walkersville, MD) and Hyclone Scientific (Santa Ana, CA), respectively. Geneticin (G418) was from Life Technologies (Rockville, MD). [5,6,8,9,11,12,14,15-\(^{3}H\)]arachidonic acid (98.6 mCi/mmol) and 1-palmitoyl-2-\(^{14}C\)arachidonyl-sn-glycero-3-phosphocholine (48 mCi/mmol) were from NEN Life Science Products (Boston, MA). 1-Palmitoyl-2-\(^{14}C\)palmitoyl-sn-glycero-3-phosphocholine (54 mCi/mmol) was from American Pharmacia Biotech (Buckinghamshire, England). Texas red- or fluorescein-conjugated wheat-germ agglutinin (WGA) was from Molecular Probes (Eugene, OR). Methyl arachidonoyl fluorophosphonate (MAFP) was from BIO-MOL Research Laboratories (Plymouth meeting, PA). Anti-GFP monoclonal and RFP polyclonal antibodies were from Clontech (Palo Alto, CA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were from Life Technologies (Rockville, MD). The plasmids encoding human Group V sPLA\(_2\) and mouse Group VIA iPLA\(_2\) were provided by Dr. Michelle Winstead (University of California at San Diego, La Jolla, CA), and the one encoding human Group IVA cPLA\(_2\) was from Dr. David Six (University of California at San Diego, La Jolla, CA). The pEGFPNI plasmid containing a CMV promoter was obtained from Clontech (Palo Alto, CA). Plasmid BS704, which encodes RFP (DsRed) and has a SV40 promoter, was kindly provided by Dr. Saito (Kobe University, Japan).

#### 2.2. Culture conditions

The MAB clone of P388D\(_1\) macrophage-like cells [12] was maintained at 37 °C in a humidified atmosphere at 10% CO\(_2\) in IMDM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. P388D\(_1\) cells transiently expressing the PLA\(_2\) fusion protein with either GFP or RFP were maintained under the same conditions. In the case of the stable transfectants, the medium was supplemented with 1 mg/ml of genetin.

#### 2.3. Construction of plasmids encoding Group V PLA\(_2\)-GFP, Group VI PLA\(_2\)-GFP and Group IV PLA\(_2\)-RFP

The cDNA encoding human Group V sPLA\(_2\) with the signal peptide at the N-terminus were produced by PCR with hPLA\(_2\)-10 [38] as the template. The sense primer was 5’-TTGAGATCTGAGATGAAGGCCTTCTCCA-3’ and the antisense primer was 5’-AAGAGATCTGGAGAAGGAGATTGGG-3’. The PCR product of Group V sPLA\(_2\) was digested with BglII followed by subcloning into the BglII site in pEGFPN1. For the production of the cDNA
encoding Group VI iPLA₂, the sense primer was 5′-TTAGATCTAGATCGTTCTGGGACGC-3′, the antisense primer was 5′-AAAGATCTAGAGGAGACGAGCCAAGCTG-3′, and the template was Group VI PLA₂ in pCDNA3.1. The PCR product was digested with BglII and then subcloned into the BglII site in pEGFP-N1, which contains the CMV promoter. Similarly, Group IV cPLA₂ was amplified using the oligonucleotides 5′-GAGAAGTAATGCATTATGCAGACCT-3′ and 5′-TTTAGAGCATGAGTTCTTCGGACGC-3′ as the sense and antisense primers, respectively, with human Group IV cPLA₂ in pALTER as the template. Finally, BglII-digested PCR product was subcloned into the BglII site in BS704. All PCR products were verified by sequencing.

2.4. Transient expression of Group IV cPLA₂-RFP

Plasmids of Group IV cPLA₂-RFP (approximately 5.5 μg) were mixed with 15 μl of TransIT™-LT1 (Mirus Co., Madison, WI) in 1000 μl of Opti-MEM for 10 min at room temperature and then added to 0.8 × 10⁶ P388D₁ cells in 60-mm dishes. After incubation at 37 °C for 4 h, the medium was changed to normal medium and cultured for an additional 18–26 h.

2.5. Production of transfectants stably expressing Group V-GFP, Group VI-A-GFP and GFP

Plasmids of Group V sPLA₂-GFP, Group VI cPLA₂-GFP or pEGFP (approximately 5.5 μg) were transfected with TransIT™-LT1 into P388D₁ cells as described above. To obtain the transfectants stably expressing Group V sPLA₂-GFP, Group VI iPLA₂-GFP or GFP, the transfected cells were cloned by limiting dilution and were cultured in a medium supplemented with 1 mg/ml of geneticin using 96-well plates. After 2 weeks, wells containing a single colony were chosen for further expansion and the fluorescence of the cells was checked under an epifluorescence microscope. Among the clones having the GFP fluorescence, stable-transfectants of Group V sPLA₂-GFP, Group VI iPLA₂-GFP or GFP were selected by immunoblotting and measurement of PLA₂ activity.

2.6. Measurement of AA release from the stable transfectants

For radiolabeling, the cells (10⁶) were seeded in 6-well plates and incubated with 0.5 μCi/ml [³H]AA in culture medium containing 1 mg/ml of geneticin for 18 h. After washing with PBS containing 1 mg/ml bovine serum albumin (BSA), the cells were preincubated for 1 h in serum-free IMDM containing 0.1 mg/ml BSA and then incubated with LPS (100 ng/ml). The medium was collected at appropriate time points and the radioactivity was measured with liquid scintillation counting. For the LPS/PAF stimulations, the radiolabeled cells were preincubated with LPS (200 ng/ml) for 1 h prior to the addition of PAF (100 nM) for the time indicated.

2.7. Immunoblotting

The stable transfectants were harvested with PBS and centrifuged. The cell pellet was resuspended in 150 μl PBS containing 1% Triton X-100 and 1 mM PMSF. After vortexing and centrifugation, the supernatants were subjected to a 12% SDS-PAGE, followed by blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific binding sites were blocked by incubation with 5% skim milk in PBS containing 0.3% Triton X-100 (PBS-T) for 1 h at room temperature. The membrane was then incubated with anti-GFP antibodies (diluted 1:1000) for 1 h. After washing with PBS-T, the membrane was incubated with peroxidase-labeled anti-rabbit IgG or anti-mouse IgG for 30 min. After 3 rinses with PBS-T, the immunoreactive bands were visualized using a chemiluminescence detection kit (ECL, Amersham, Buckinghamshire, England).

2.8. PLA₂ assays

Plasmids (5.5 μg) encoding each subtype of GIV cPLA₂-RFP, GV sPLA₂-GFP or GV iPLA₂-GFP were transfected into 0.5 × 10⁶ HEK 293 cells using Trans IT as described above. After being cultured overnight, the cells were harvested with PBS and centrifuged. The cell pellet was resuspended in 150 μl PBS containing 1% Triton X-100 and 1 mM PMSF. To measure PLA₂ activity in vitro, aliquots of the cell lysate were assayed using group-specific assays, exactly as described elsewhere [39].

2.9. Visualization of the PLA₂ fusion proteins with GFP or RFP by confocal microscopy

An appropriate number of P388D₁ cells transiently or stably expressing the PLA₂ fusion proteins were placed on a glass-bottomed dish (MetTek, Ashland, MA) and cultured overnight. After 1 h preincubation in serum-free medium, LPS (100 ng/ml) was directly added into the medium. In the case of LPS/PAF stimulation, the cells were incubated with LPS (200 ng/ml) for 1 h at 37 °C and then treated with PAF (100 nM) under confocal microscopy (Biorad, MRC-1024 or Carl Zeiss, LSM 510). The fluorescence of GFP was monitored at 488-nm argon excitation using a 522–535 nm or a 505–530 nm band pass barrier filter, while that of RFP was monitored at 568-nm argon excitation using a 522–535 nm or a 505–530 nm band pass barrier filter, or observed at 543-nm argon excitation using a 590-nm long pass barrier filter.

2.10. Co-detection of the WGA binding sites and Group V sPLA₂-GFP

Texas red- or fluorescein-conjugated WGA was used to monitor the Golgi network. After incubation with LPS, the
cells were fixed with 4% paraformaldehyde in PBS for 30 min. After washing 3 times with PBS, the fixed cells were treated with PBS-T and 10% normal goat serum for 20 min. The cells were then incubated with 0.5 μg/ml Texas red-conjugated WGA in PBS-T for 30 min. Finally, the fluorescence of GFP and Texas Red were observed under a confocal laser scanning fluorescent microscope at 488-nm argon excitation using a 522–535 nm band pass barrier filter and at 568-nm argon excitation using a 585-nm long pass barrier filter, respectively.

2.11. Immunofluorescence

After each treatment, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. Cells were then permeabilized with 0.3% Triton X-100/PBS for 5 min at room temperature, and blocked for 1 h in 10% normal goat serum in PBS. Incubations with anti-GVI cPLA2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI) were performed at a 1:100 dilution overnight. To enhance the signal, biotin-labeled anti-mouse IgG (Vector laboratories Inc., Burlingame, CA) was used for secondary antibodies and the final fluorescent signal was detected by Alexa 488-conjugated avidin (Molecular Probe, Eugene, OR). For double staining, anti-GM130 antibody and Cy3 labeled anti-rabbit IgG were used for primary and secondary antibodies, respectively. Extensive washes in between incubations with antibodies were performed in PBS. Specimens were mounted in anti-fade medium and viewed with the Carl Zeiss, LSM 510 laser-scanning confocal system. The fluorescence of Alexa488 was monitored at 488-nm argon excitation using a 505–550 nm band pass barrier filter, while that of Cy3 was monitored at 543-nm argon excitation using a 590-nm long pass barrier filter.

3. Results and discussion

3.1. GFP/RFP PLA2 transfectants and activity

We have demonstrated in P388D1 macrophages exposed to bacterial lipopolysaccharide (LPS) the existence of two distinct routes for AA mobilization and prostaglandin production. The first one, which we have called the “primed immediate pathway” [6–8,11], takes place in minutes and is elicited by Ca\(^{2+}\)-mobilizing agonists such as platelet-activating factor (PAF), but requires the cells to be exposed first to LPS. The second route, the “delayed pathway” [12,13], is elicited by LPS alone for periods of time requiring several hours. To examine the regulation and possible relationship between both cPLA2 and sPLA2 during LPS activation of P388D1 macrophages, we have now prepared cells overexpressing the different PLA2s that these cells contain. To help focus on the subcellular location of these enzymes during the activation process, PLA2 fusion proteins with GFP or RFP have been prepared.

Both fusion proteins of Group V sPLA2-GFP and Group IV cPLA2-RFP possess PLA2 activity. HEK cells transiently expressing GV sPLA2-GFP or GIV cPLA2-RFP showed an approximately 6.0-fold or 11-fold increase in cellular PLA2 activity, respectively, as measured utilizing vesicle-based in vitro assays (Fig. 1). The molecular mass of GV sPLA2-GFP and GIV cPLA2-RFP was 41 kDa and 120 kDa, respectively. Transfection efficiency in the transient transfectants of HEK cells was 60–70%, as measured by confocal microscopy. Finally, we have obtained two clones of P388D1 macrophages stably expressing GV sPLA2-GFP and three clones of GFP. The GV sPLA2-GFP in the stable transfectant also showed appropriate molecular mass to that found in HEK cells and similar PLA2 activity to that shown in Fig. 1. In spite of several attempts, we were unable to obtain a stable transfectant of the cPLA2-RFP fusion protein. Therefore, in this case, we used transient transfectants.

3.2. Primed immediate pathway

In resting cells, cPLA2-RFP was localized uniformly throughout the cytoplasm with no nuclear staining. After 1 h pretreatment with LPS—a condition that in itself does not induce AA release but primes the cells for a subsequent
stimulation by a Ca\(^{2+}\)-mobilizing stimulus—the localization of cPLA\(_2\)-RFP did not appreciably change. Under these conditions, we have found that short-term treatment with LPS induces the complete phosphorylation of the enzyme by MAP kinase family members [14]. This suggests that complete phosphorylation of the cPLA\(_2\) at Ser\(^{505}\) is not sufficient to induce translocation of the enzyme to intracellular membranes, although it may be necessary, as recently suggested by Cho and coworkers [40].

Exposure of the LPS-primed cells to the Ca\(^{2+}\)-mobilizing stimulus PAF resulted in the expected translocation of the cPLA\(_2\)-RFP to perinuclear membranes. Translocation was already detected at 30 s and was mostly reversed after 1 min (Fig. 2A). Under the LPS/PAF activation conditions, the cPLA\(_2\)-RFP was found to colocalize with WGA (Fig. 2B). Such kinetics of translocation of the enzyme to the membranes correlated very well with the time-course of accumulation of intracellular free AA [7], a phenomenon that was attributed to the exclusive participation of cPLA\(_2\), on the basis of its inhibition by inhibitors of cPLA\(_2\) but not of sPLA\(_2\) [8]. Studies utilizing the sPLA\(_2\)-GFP transfectants

![Figure 2](image-url)  
**Fig. 2.** Effect of LPS/PAF on the localization of Group IV cPLA\(_2\)-RFP and Group V sPLA\(_2\). After preincubation with 200 ng/ml LPS and 100 nM PAF, the localization of (A) Group IV cPLA\(_2\)-RFP or (C) Group V sPLA\(_2\)-GFP was recorded at different times after PAF addition. (B) The colocalization of cPLA\(_2\)-RFP with fluorescein-conjugated WGA was also measured at 0.5 min after PAF addition. Bars: 20 μm.

![Figure 3](image-url)  
**Fig. 3.** Time course of [\(^3\)H]AA release from the Group V sPLA\(_2\)-GFP-expressing cells. [\(^3\)H]AA-labeled stable transfectants of Group V sPLA\(_2\)-GFP were treated with (open circles) or without (closed circles) 100 nM PAF after 1 h preincubation with 200 ng/ml LPS. After appropriate incubation, the medium was collected and the radioactivity was measured. Each data point represents the mean and S.D. from 4 independent experiments.

![Figure 4](image-url)  
**Fig. 4.** Effect of LPS on the localization of Group IV cPLA\(_2\)-RFP. After incubation for the indicated time with LPS (100 ng/ml), the transfectants transiently expressing Group IV cPLA\(_2\)-RFP were fixed with 4% paraformaldehyde and the fluorescence was observed with confocal microscope. Left panels show the fluorescence of Group IV cPLA\(_2\)-RFP and the panels on the right show the Nomarski images. Images at 0, 1.5, 3 and 6 h were taken with same magnification. Bar: 20 μm.
did not reveal any evident changes in the distribution of this fusion protein after addition of PAF (Fig. 2C).

Immediately following this intracellular accumulation of free AA, a sustained release of AA into the incubation medium is detected upon PAF activation. This release was partially inhibited by sPLA2 inhibitors, which points to the Group V sPLA2 as a possible participant in this process [8]. To assess that the transfected cells behave just like the regular P388D1 cells in terms of AA signaling, AA release experiments were conducted. As shown in Fig. 3, the LPS/PAF-treated cells released AA with a time-course comparable to that of normal P388D1 cells [7, 8], i.e., a rapid release of extracellular radiolabel was observed immediately after the addition of PAF, reaching a plateau at 10–15 min. In agreement with the data for regular, wild type cells, extracellular AA release in the transfectants was suppressed by the cPLA2 inhibitor MAFP (data not shown).

3.3. The delayed pathway

Study of the localization of the cPLA2-RFP at longer times revealed a clear association of the enzyme with perinuclear membranes at 3 h (and perhaps as early as 1.5 h). Such an association ceased at 6 h (Fig. 4). To compare this with the localization of endogenous cPLA2 during the delayed pathway, we performed immunofluorescence staining. Fig. 5 clearly shows that endogenous cPLA2 accumulated near the nucleus and the nuclear membranes at 3 h after LPS treatment, and then returned to the initial state. Some of the accumulated cPLA2 colocalized with GM 140, a Golgi marker protein, indicating that at least some of the cPLA2 was translocated to the Golgi complex by LPS treatment. This pattern is consistent with the kinetics of long-term AA release, where extracellular release of AA and of PGE2 barely increased above background in the first 3 h of treatment with LPS, rising afterward [12]. Given that the long-term stimulation of the P388D1 macrophages with LPS is assumed to occur in the absence of changes in the intracellular Ca2+-concentration, it is not clear at this time what biochemical signal(s) drives the delayed association of cPLA2 to perinuclear membranes. We have recently described the importance of phosphatidylinositol 4,5-bisphosphate (PIP2) levels for the translocation/activation of cPLA2 to model membranes and membranes of ultraviolet light-treated macrophages at concentrations of Ca2+ equal to those present in unstimulated cells [15, 41, 42]. It is interesting to note that cPLA2-activating stimuli that induce...
AA release in the absence of intracellular Ca\(^{2+}\) increases (e.g. phorbol esters, okadaic acid) have been shown to increase the intracellular levels of PIP\(_2\) as well [43]. Whether this is the mechanism through which LPS induces the delayed association of cPLA\(_2\) to intracellular membranes is currently under investigation.

An intense fluorescence arising from the sPLA\(_2\)-GFP was detected around the perinuclear membranes in the sPLA\(_2\)-GFP stably transfected cells at 3 h (Fig. 6A), a time at which the delayed association of cPLA\(_2\) with perinuclear membranes also occurred. Colocalization of sPLA\(_2\)-GFP with WGA was detected (Fig. 7). Importantly, however, some of the sPLA\(_2\)-containing granules showed no-colocalization with WGA, and this was particularly evident after 18 h stimulation with LPS. Instead, the sPLA\(_2\) granules showed colocalization with caveolin-enriched granules [18]. These results indicate that there are two populations of sPLA\(_2\) granules: one, whose role is to convey Group V sPLA\(_2\) to the plasma membrane from the Golgi complex, and the other, whose role is to internalize and convey Group V sPLA\(_2\) to the perinuclear region.

As a control for these experiments, the fluorescence of GFP in the GFP-stable transfectants was determined and was found to be uniformly distributed along the cytoplasm and the nucleus, irrespective of the cells being treated or not with LPS (Fig. 6B). Interestingly, an increase in protein level was observed in the sPLA\(_2\)-GFP-expressing cells compared to the GFP-expressing cells (Fig. 6C), suggesting the induction of sPLA\(_2\)-GFP by LPS. It is not clear at this time why the sPLA\(_2\)-GFP is increased with LPS and the GFP alone is not since they are both under the control of identical promoters. It is possible that the sPLA\(_2\)-GFP in secretory vesicles is protected from degradation. This issue needs further investigation.

The addition of MAFP blocked the accumulation of sPLA\(_2\)-GFP in the Golgi area (Fig. 8A); 67 ± 2% (n = 60) of the cells treated with LPS for 3 h showed significant Golgi staining, whereas 27 ± 2% (n = 31) of the cells showed Golgi staining in the presence of MAFP. In parallel with these changes in the fluorescence of sPLA\(_2\)-GFP, the protein level of sPLA\(_2\)-GFP was reduced to about 50% by MAFP treatment (Fig. 8B), although it should be under the control of the CMV promoter. In addition, significant release of AA was detected from the sPLA\(_2\)-GFP transfectants in a MAFP-sensitive manner (Fig. 8C). It is noteworthy that AA release from the stable transfectants of Group V sPLA\(_2\)-

![Fig. 7. Colocalization of Group V sPLA\(_2\)-GFP with WGA. After incubation with 100 ng/ml LPS for (A) 3 h, (B) 6 h and (C) 18 h, the stable transfectants of Group V sPLA\(_2\)-GFP were fixed with 4% paraformaldehyde. The fixed cells were treated with Texas red-conjugated WGA as described under Experimental procedures. Group V sPLA\(_2\)-GFP is shown on the left, in green. WGA binding sites are shown in the middle column (red). The merged images of GFP and Texas red appear in yellow on the right column. Bars: 10 \(\mu\)m.](image-url)
Fig. 8. Effect of MAFP on the localization and activity of Group V sPLA₂-GFP. (A) After incubation for 3 h with LPS (100 ng/ml) in the presence and absence of 50 μM MAFP, the Group V sPLA₂-GFP stable transfectants were fixed with 4% paraformaldehyde and the fluorescence was observed under confocal microscope. Bars: 20 μm. (B) After incubation for 18 h with LPS (100 ng/ml) in the presence or absence of 50 μM MAFP, the protein levels of GV sPLA₂-GFP or GFP were detected by immunoblotting using anti-GFP antibody. (C) Each stable cell line pre-labeled with [3H]-AA was treated with (closed circles) or without (open circles) 100 ng/ml LPS, or in the presence of both 10 μM MAFP and 100 ng/ml LPS (closed triangle). The amount of AA released into the medium was shown as percentage of the AA released during the 1-h preincubation in serum-free medium. Each data point represents the mean and S.D. of 12 separate determinations from 4 independent experiments. The asterisk (*) represents probability, P < 0.001. (D) Group V sPLA₂-GFP stable transfectants pre-labeled with [3H]AA were incubated with 100 ng/ml LPS in the presence or absence of 10 μM MAFP for 3 h. Control was incubated in the absence of both LPS and MAFP. Each point represents the mean ± S.D. of three separate experiments.
GFP was detected at 3 h, whereas significant AA release at 3 h was not detected from the GFP alone-transfected cells. The early AA release from the stable transfectants of Group V sPLA2-GFP was also inhibited by MAFP (Fig. 8D). This could be explained if cPLA2, by an unknown mechanism, activates Group V PLA2-GFP, or alternatively, if secreted Group V PLA2-GFP activates Group IV PLA2, possibly by binding to an sPLA2 receptor. While it has been demonstrated and generally assumed that MAFP inhibits cPLA2 and/or iPLA2, it was recently found [44] that oxidized MAFP preparations may also inhibit sPLA2, so we cannot rule out that the MAFP inhibition observed here is due to direct sPLA2 inhibition.

3.4. The role of iPLA2

P388D1 macrophages contain a third PLA2 enzyme, the calcium-independent Group VIA PLA2 (iPLA2). Although data in several non-myeloid systems have been published to implicate this enzyme in stimulus–response coupling leading to AA release, in macrophages and other immunoinflammatory cells, most of the evidence suggests that iPLA2 plays no role in such a process [27–32]. We have re-evaluated again the involvement of iPLA2 in P388D1 macrophage AA release by utilizing stable transfectants of the iPLA2-GFP fusion protein.

We obtained a clone of iPLA2-GFP whose PLA2 activity was approximately 7-fold above that of untransfected P388D1 cells, as assessed by in vitro assay utilizing a mixed micelle assay in the absence of calcium (Fig. 1). The iPLA2-GFP stable transfectant expressed recombinant protein at the expected molecular mass. Under the microscope, the cells presented a morphology comparable to those of untransfected cells.

We did not detect any significant change in the intracellular localization of iPLA2-GFP under any stimulation condition tested (Fig. 9), suggesting a lack of responsivity of the enzyme to cellular activation. Interestingly, cells overexpressing iPLA2-GFP showed abnormally high basal levels of AA. This result is consistent with the purported role of this enzyme as a major player of basal phospholipid deacylation reactions in macrophages. Importantly, however, the spontaneous AA release observed under unstimulated conditions was so high that it masked any subsequent increase in AA release induced by LPS (Fig. 10). Positive controls stimulating the cells with Ca2+ ionophore plus phorbol myristate acetate, a condition that results in massive AA release, i.e., several fold higher than that observed with receptor agonists [45], did result in the expected increased AA release from the iPLA2-GFP-overexpressing cells (Fig. 10). Thus, that LPS fails to elicit an elevated AA response from the iPLA2-GFP-expressing cells is not because of a loss of responsivity of the cells subsequent to iPLA2 overexpression, but rather to the elevated background AA release that these cells apparently exhibit.

Fig. 9. Localization of iPLA2-GFP. (A) The cells were treated with 200 ng/ml LPS followed by 100 nM PAF at the indicated times. (B) The cells were treated with 200 ng/ml LPS for the indicated times. Images were recorded without fixation. Bars: 20 μM.

Fig. 10. AA mobilization from the iPLA2-GFP stable transfectants. (A) The cells labeled with [3H]AA were incubated without (open symbols) or with (closed symbols) exposed to 200 ng/ml LPS for the indicated times. (B) The iPLA2-GFP-expressing cells or the cells expressing only GFP (see abscissa) were treated without (open bars) or with (closed bars) 500 nM phorbol myristate acetate plus 1 μM ionophore A23187 for 15 min.
3.5. Concluding remarks

We have studied the localization of the signaling PLA2s, Group IV cPLA2 and Group V sPLA2 in activated P388D1 macrophages utilizing GFP and RFP constructs, and integrated the information obtained by this approach with the biochemical data that we have described in recent years. Specifically, the present study provides new information about temporal–spatial activation of Group IV cPLA2 and Group V sPLA2 and their interrelationships during both the primed immediate and the delayed AA pathway. Our results provide new data on the regulation of AA signaling in macrophages that is consistent with the participation of the three major PLA2 classes, each one playing a different role.

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