

Functional crosstalk between phospholipase D₂ and signaling phospholipase A₂/cyclooxygenase-2-mediated prostaglandin biosynthetic pathways

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Abstract We performed reconstitution analyses of functional interaction between phospholipase A₂ (PLA₂) and phospholipase D (PLD) enzymes. Cotransfection of HEK293 cells with cytosolic (cPLA₂) or type IIA secretory (sPLA₂-IIA) PLA₂ and PLD₂, but not PLD₁, led to marked augmentation of stimulus-induced arachidonate release. Interleukin-1-stimulated arachidonate release was accompanied by prostaglandin E₂ production via cyclooxygenase-2, the expression of which was augmented by PLD₂. Conversely, activation of PLD₂, not PLD₁, was facilitated by cPLA₂ or sPLA₂-IIA. Thus, our results revealed functional crosstalk between signaling PLA₂s and PLD₂ in the regulation of various cellular responses in which these enzymes have been implicated. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Phospholipase A₂; Phospholipase D; Arachidonic acid; Prostaglandin; Cyclooxygenase-2

1. Introduction

Phospholipase A₂ (PLA₂) catalyzes the hydrolysis of membrane phospholipids, liberating free arachidonic acid (AA) and lysophospholipids, which are precursors for bioactive lipid mediators. Of a number of PLA₂ enzymes identified to date, cytosolic PLA₂α (cPLA₂) and several secretory PLA₂ (sPLA₂) enzymes have been implicated in stimulus-induced AA release, which is coupled with cyclooxygenase (COX)-catalyzed prostaglandin (PG) formation [1–6]. Activation of cPLA₂ is tightly regulated by post-receptor signal transduction events, including Ca²⁺ signaling promoted by phosphoinositide-specific phospholipase C (PLC) enzymes and phosphorylation directed by the mitogen-activated protein kinase (MAPK) members [1,2]. In response to elevated cytoplasmic Ca²⁺ levels, cPLA₂ translocates from the cytosol to the peri-

nuclear membrane [7]. Type IIA sPLA₂ (sPLA₂-IIA), a signaling sPLA₂, accumulates in caveolae signalsomes through binding to glycosylphosphatidylinositol-anchored heparan sulfate proteoglycan glypican [8] and plays an augmentative role in PG biosynthesis in autocrine, paracrine and juxtacrine fashions [3–5,8,9]. The AA-releasing function and expression of sPLA₂-IIA and its relative sPLA₂-V depend on prior activation of cPLA₂ [3,6,10,11].

Phospholipase D (PLD) catalyzes the hydrolysis of phosphatidylcholine to produce phosphatidic acid (PA), which by itself acts as a second messenger or is metabolized to other signaling molecules, such as lysophosphatidic acid and diacylglycerol (DAG). PLD activation has been implicated in a wide range of cellular responses, including vesicular transport, mitogenesis and respiratory burst (reviewed in [12]). There are at least two PLD isoforms, PLD₁ and PLD₂, in mammals [13–17]. PLD₁, which exists as two splicing variants (PLD_{1a} and PLD_{1b}), has low basal activity and is activated by small G proteins (ARF, Rho and Rac), phosphatidylinositol 4,5-bisphosphate (PIP₂), and protein kinase Cα in vitro [13–16]. PLD₁ is distributed primarily on intracellular membranes, where it may regulate the formation of transport intermediates [17]. PLD₂ is constitutively active and is further stimulated by PIP₂ in vitro [18,19], and is localized in caveolae [20]. Each enzyme undergoes unique posttranslational modifications, including fatty acylation [21] and tyrosine phosphorylation [22,23]. However, segregated roles of the two PLD isoforms in cellular responses are still poorly understood.

Several lines of evidence have suggested a functional correlation between PLA₂ and PLD enzymes during cell activation [24–30]. We [24] and others [25–27] have reported that stimulus-induced AA release, which appears to be mediated by cPLA₂, is suppressed by pharmacological inhibition of PLD. Moreover, PLD₂, not PLD₁, activity has been reported to be modulated by cPLA₂ or its reaction products [28–30]. Antisense suppression of cPLA₂ expression leads to a concomitant reduction of PLD activity in several cell lines predominantly expressing PLD₂ [30]. Since PLA₂ and PLD are often simultaneously activated following various stimuli, it would be of great importance to understand their functional interrelationship.

To confirm functional interaction between the two phospholipases critically involved in signal transduction, we performed reconstitution analyses by cotransfecting HEK293 cells with signaling PLA₂s (cPLA₂ and sPLA₂-IIA) and either of the two PLD enzymes. Our results have provided evidence that PLD₂, but not PLD₁, has the ability to enhance signaling PLA₂-promoted AA release and attendant COX-2-dependent

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Abbreviations: PLD, phospholipase D; PLA₂, phospholipase A₂; cPLA₂, cytosolic PLA₂; sPLA₂, secretory PLA₂; PLC, phospholipase C; AA, arachidonic acid; OA, oleic acid; COX, cyclooxygenase; PG, prostaglandin; MAPK, mitogen-activated protein kinase; PA, phosphatidic acid; DAG, diacylglycerol; PIP₂, phosphatidylinositol 4,5-bisphosphate; PEt, phosphatidylethanol; PMA, phorbol myristate acetate; FCS, fetal calf serum; IL-1, interleukin-1

PGE₂ generation, and conversely, activation of PLD₂, but not PLD₁, is facilitated by signaling PLA₂s.

2. Materials and methods

2.1. Materials

cDNAs for mouse cPLA₂, mouse sPLA₂-IIA, and human COX-2 were described previously [3,4]. cDNAs for human PLD_{1b} and human PLD₂ subcloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen) were kindly provided by Dr. S.H. Ryu (Pohang University of Science and Technology). The mammalian expression vector pcDNA3.1/zeo(+), geneticin and zeocin were purchased from Invitrogen. A23187 was purchased from Calbiochem. Human interleukin-1 β (IL-1 β) was obtained from Genzyme. [³H]AA and [³H]oleic acid (OA) were purchased from Amersham Pharmacia Biotech. TRIzol reagent, Lipofectamine PLUS and Opti-MEM were from Life Technologies. Phorbol myristate acetate (PMA) was purchased from Sigma. Other chemicals were obtained from Wako. Culture of HEK293 cells was described previously [3].

2.2. Establishment of PLA₂/PLD transfectants

Human PLD_{1b} and PLD₂ cDNAs subcloned into pcDNA3.1(+) were each transfected into HEK293 cells using Lipofectamine PLUS according to the manufacturer's instruction. Briefly, 1 μ g of the plasmid was mixed with 4 μ l of Lipofectamine and 6 μ l of PLUS reagent in 200 μ l of Opti-MEM medium, left for 15 min, and then added to cells that had attained 70% confluency in 6-well plates (Iwaki Glass) in 1 ml of Opti-MEM. After incubation for 6 h, 2 ml of fresh culture medium (RPMI 1640 (Nissui Pharmaceutical) containing 10% fetal calf serum (FCS)) was added. After 18 h, the medium was replaced with 2 ml of fresh medium, and culture was continued for 3 days. Then the cells were cloned by limiting dilution in 96-well plates (Iwaki Glass) in culture medium supplemented with 800 μ g/ml geneticin. After culture for 3 weeks, wells containing a single colony were chosen, and the expression of each PLD was assessed by RNA blotting. Establishment of HEK293 cells stably overexpressing mouse cPLA₂ or mouse sPLA₂-IIA was described previously [3].

In order to establish double transfectants stably coexpressing PLA₂ and PLD enzymes, cells expressing each PLD were subjected to a second transfection with cPLA₂ or sPLA₂-IIA cDNA subcloned into pcDNA3.1/zeo(+). After selection in culture medium containing 50 μ g/ml zeocin in 96-well plates, single colonies were picked up and expanded. Expression of each enzyme was assessed by RNA blotting.

2.3. Activation of PLA₂

All procedures were described in our previous reports [3,4]. Briefly, HEK293 cells (5×10^4 /ml) were seeded into each well of 24-well culture plates (Iwaki Glass) in 1 ml of culture medium. After culture for 3 days, the cells were prelabeled with 10 μ Ci/ml [³H]AA and cultured for another day. Then the cells were washed once with culture medium and incubated with 250 μ l of 10 μ M A23187 in medium containing 1% FCS for 30 min or 1 ng/ml IL-1 β in medium containing 10% FCS for 4 h. The percentage release of [³H]AA was calculated using the formula $[S/(S+P)] \times 100$, where S and P are the radioactivities measured in equal portions of the supernatant and cell pellet, respectively.

2.4. Activation of PLD

PLD activity was assessed by its unique transphosphatidylolation reaction [12]. Cells (5×10^4 /ml) were seeded into 12-well plates (Iwaki Glass) in 1.5 ml of culture medium, cultured for 3 days, and then incubated with 1 μ Ci/ml [³H]OA in culture medium for another day. After washing twice with medium containing 1% FCS, the cells were cultured with or without 10 μ M A23187 and 2 μ M PMA in medium containing 1% FCS for 30 min in the presence of 1% ethanol. After removing the supernatants, the cells were lysed promptly by adding a mixture of 2 ml of methanol, 1 ml of chloroform and 300 μ l of 0.1% sodium dodecyl sulfate. Total lipids were then extracted by the method by Bligh and Dyer [31] and developed by thin-layer chromatography on silica 60 gel plates (Merck DuPont) with a solvent system of ethyl acetate/trimethyl pentane/acetic acid/water = 52/8/12/40. After drying, the plates were developed again to the same direction with the same solvent. The zones on the silica gel corresponding to phos-

phatidylethanol (PEt) were identified by comparing the mobility with that of authentic standard, which was visualized with iodine vapor, and were scraped into a vial. The associated radioactivity was counted using a liquid β -scintillation counter (Aloka).

2.5. RNA blotting

Approximately equal amounts (~ 5 μ g) of total RNA obtained from the transfected cells were applied to individual lanes of 1.2% (w/v) formaldehyde-agarose gels, electrophoresed, and transferred to Immobilon-N membranes (Millipore). The resulting blots were then probed with the relevant cDNA probes, which had been labeled with [³²P]dCTP (Amersham Pharmacia Biotech) by random priming (Takara Biomedicals). All hybridizations were carried out as described previously [32].

3. Results

3.1. AA release

The expression levels of each enzyme in HEK293 cells transfected with PLA₂s (cPLA₂ and sPLA₂-IIA) and PLDs (PLD₁ and PLD₂) alone or in combination, as assessed by RNA blotting, are shown in Fig. 1. When cells transfected with cPLA₂ alone were stimulated with A23187 for 30 min (immediate response) or with IL-1 for 4 h (delayed response), AA release was increased significantly relative to that by replicate control cells in both responses (Fig. 2), as described previously [3,4]. AA release by cells expressing PLD₁ or PLD₂ alone was comparable to that by control cells. Remarkably, AA release by cells expressing cPLA₂ in combination with PLD₂, but not with PLD₁, was markedly augmented, reaching a several-fold higher level than that by cells expressing cPLA₂ alone in both A23187- and IL-1-induced responses (Fig. 2). Since PLD₁ has been shown to be activated by PMA [13–16], we next stimulated cPLA₂/PLD₁ cotransfectants with A23187 in the presence of PMA. Despite increased PLD₁ activity (see below), however, AA release by these cells was similar to that by replicate cPLA₂-expressing cells (data not shown). Expression of sPLA₂-IIA also led to significant increase in A23187- and IL-1-induced AA release (Fig. 3), as described previously [3–5,8]. This sPLA₂-mediated AA release

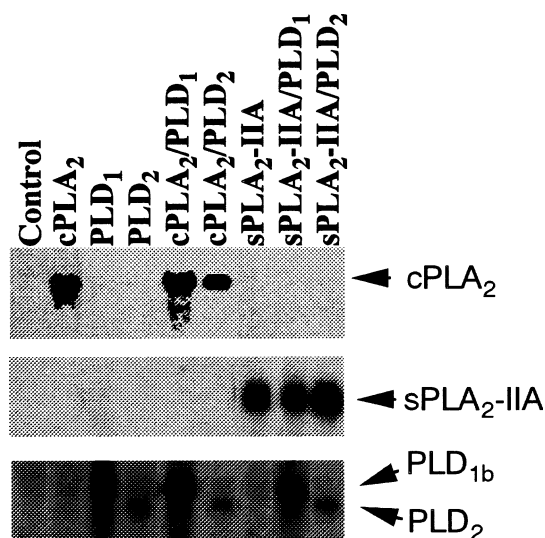


Fig. 1. Expression of PLA₂ and PLD enzymes in HEK293 transfectants. Expression of cPLA₂, sPLA₂-IIA, PLD₁ and PLD₂ in HEK293 transfectants was assessed by RNA blotting.

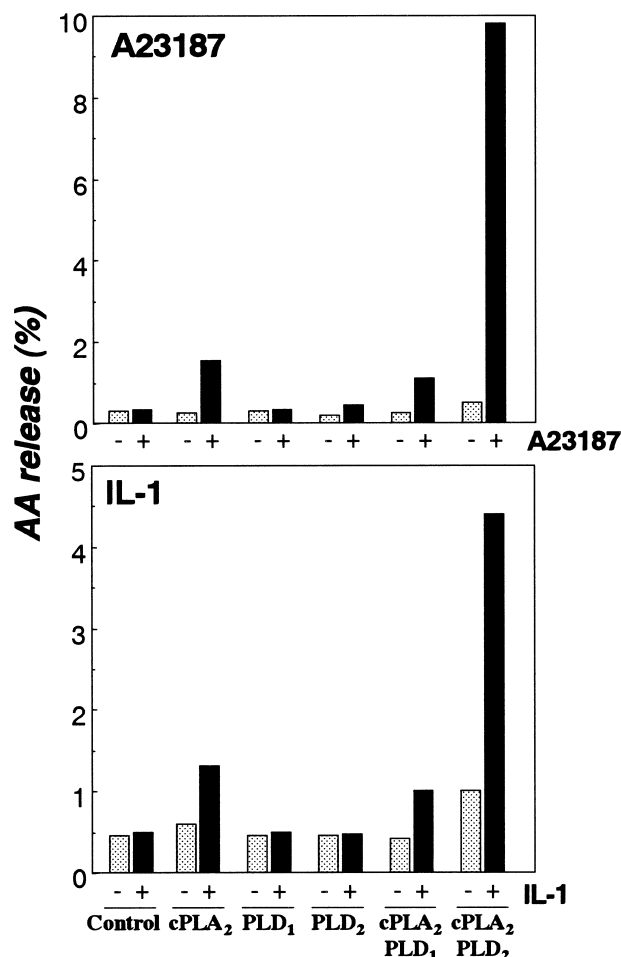


Fig. 2. AA release by cPLA₂/PLD transfectants. [³H]AA-prelabeled HEK293 transfectants expressing cPLA₂, PLD₁ and PLD₂, alone or in combination, were stimulated for 30 min with A23187 or for 4 h with IL-1. A representative result of four independent experiments is shown.

was also enhanced markedly by coexpression with PLD₂, but not with PLD₁ (Fig. 3).

3.2. COX-2-dependent PGE₂ generation

IL-1-stimulated increase in AA release by cPLA₂- or sPLA₂-IIA-transfected HEK293 cells was accompanied by accumulation of PGE₂ in the culture medium [3–5,8]. As shown in Fig. 4, PGE₂ generation by cells expressing cPLA₂ or sPLA₂-IIA alone increased modestly compared with that by control cells, and that by cells coexpressing cPLA₂ or sPLA₂-IIA and PLD₂ reached levels several-fold higher than that by cells expressing each PLA₂ alone. In contrast, no significant increase in PGE₂ generation was observed in cells expressing PLD₁ or PLD₂ alone, and PGE₂ generation by cells coexpressing cPLA₂ and PLD₁ was comparable to that by cells expressing cPLA₂ alone.

IL-1-dependent delayed PGE₂ generation depends entirely on inducible COX-2 [3–5,8]. Of interest, the expression level of COX-2 in cells expressing PLD₂ alone was higher than that in control cells or in cells expressing cPLA₂ and PLD₁, alone or in combination (Fig. 4, top panel). COX-2 expression in cells coexpressing cPLA₂ and PLD₂ was similar to that in cells expressing PLD₂ alone, indicating that the PLD₂-mediated,

but not cPLA₂-mediated, signals crucially influence the COX-2 induction machinery. Cells expressing PLD₂ alone did not produce PGE₂ appreciably irrespective of increased COX-2 expression (Fig. 4), most likely because AA supply was limited in these cells (Fig. 2). sPLA₂-IIA alone had the ability to enhance COX-2 expression (Fig. 4, top panel), as reported previously [5,9], and coexpression of sPLA₂-IIA and PLD₂ augmented it further (Fig. 4, top panel). COX-2 expression was undetectable in all clones without IL-1 stimulation (data not shown).

3.3. PLD activation

We next examined the effect of PLA₂s on PLD activation. To this end, cells were treated for 30 min with A23187 and PMA in the presence of ethanol, and the formation of PEt, a reaction product of PLD-mediated transphosphatidylolation [12], was assessed. As shown in Fig. 5, PLD activity in cells transfected with PLD₁ and PLD₂ increased 2- and 1.4-fold, respectively, compared with that in control cells or cells expressing each PLA₂ alone. Notably, more PEt was produced

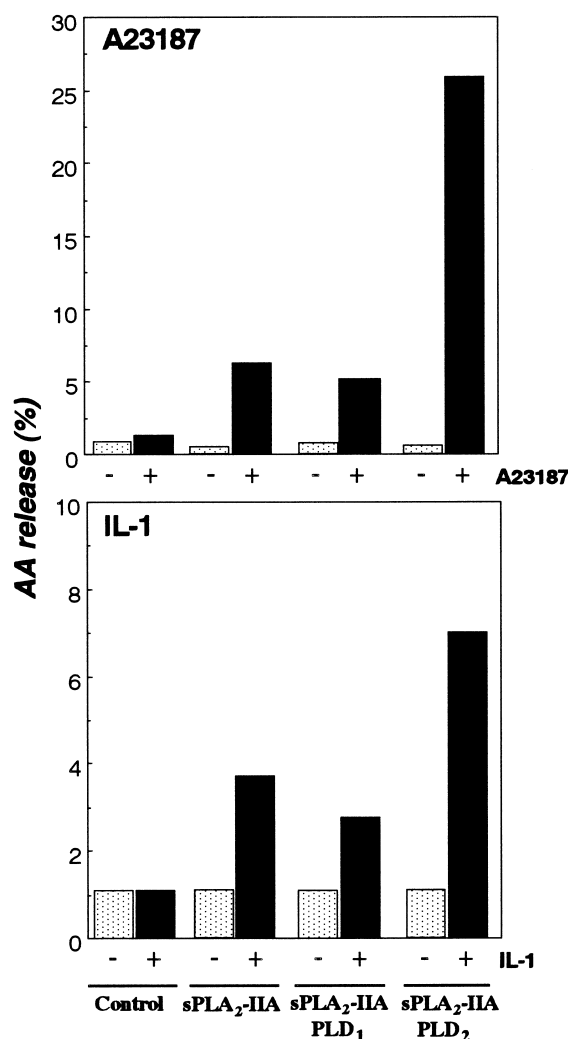


Fig. 3. AA release by sPLA₂-IIA/PLD transfectants. [³H]AA-prelabeled HEK293 transfectants expressing sPLA₂-IIA, PLD₁ and PLD₂, alone or in combination, were stimulated for 30 min with A23187 or for 4 h with IL-1. A representative result of three independent experiments is shown.

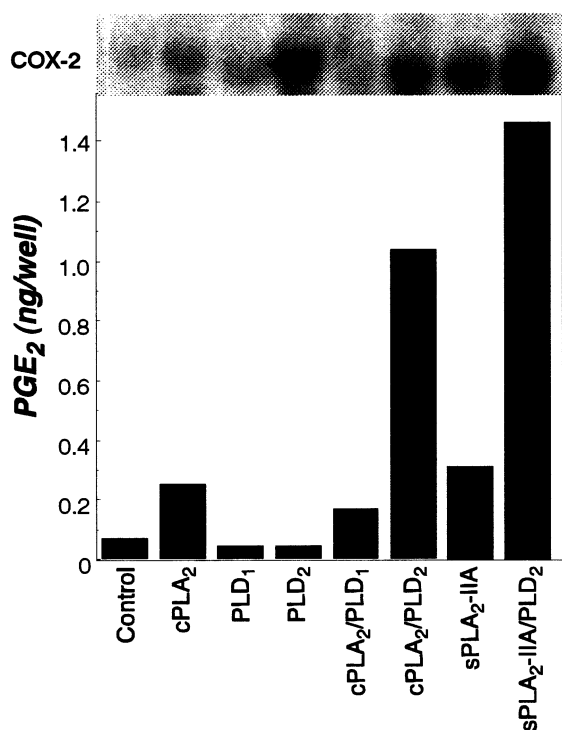


Fig. 4. IL-1-induced PGE₂ generation and COX-2 expression in PLA₂/PLD transfectants. HEK293 transfectants expressing cPLA₂, sPLA₂-IIA, PLD₁ and PLD₂, alone or in combination, were stimulated for 4 h with IL-1, and PGE₂ production and COX-2 mRNA expression (assessed by RNA blotting; top panel) were examined. A representative result of three independent experiments is shown.

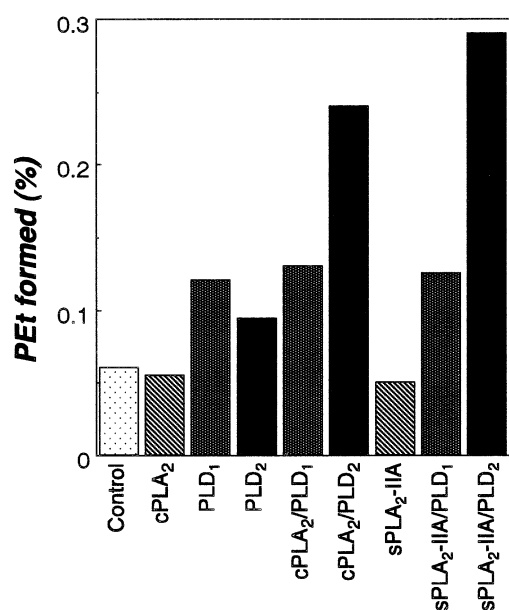


Fig. 5. PLD activation in PLA₂/PLD transfectants. [³H]OA-prelabeled HEK293 transfectants expressing cPLA₂, sPLA₂-IIA, PLD₁ and PLD₂, alone or in combination, were stimulated for 30 min with A23187 and PMA, and [³H]PEt formation was examined. Values indicate the percent radioactivity associated with PEt relative to that incorporated into total phospholipids. A representative result of three independent experiments is shown.

in cells coexpressing PLD₂ and either cPLA₂ or sPLA₂-IIA than in cells expressing PLD₂ alone, whereas the PLD₁-mediated increase in PEt formation was not altered significantly when combined with either PLA₂.

4. Discussion

Interrelationships between different classes of phospholipase have been shown to significantly affect signal transduction systems in activated cells. PLC-mediated production of inositol triphosphate triggers intracellular Ca²⁺ mobilization, which is a prerequisite for cPLA₂ activation [1]. Conversely, the tyrosine kinase-coupled PLC-γ isozymes are stimulated by AA, which is supplied by cPLA₂, in the presence of the microtubule-associated protein tau or tau-like protein AHNAK [33,34]. Prior activation of cPLA₂ modulates the action of signaling sPLA₂s probably through destabilizing sPLA₂-targeted membrane structures [3,10,11,35], while sPLA₂s can activate cPLA₂ through the sPLA₂ receptor-dependent [36,37] and -independent [38] processes.

Although the precise regulatory mechanisms for intracellular activation of PLD enzymes are not yet fully understood, several previous works have supported the idea that PLA₂ and PLD also display some functional crosstalk. For instance, PLD has been suggested to act as an upstream regulator of PLA₂ activity in neutrophils, where AA release was suppressed by alcohol and PA phosphatase inhibitors, which disturb the PLD pathway, and was restored by PA and DAG [24–26]. However, these pharmacological studies do not necessarily define the involvement of particular PLD isozymes in the cPLA₂-regulatory processes. More recently, a study using dominant-negative forms of PLD variants has revealed the involvement of PLD₂, but not PLD₁, in the insulin-induced production of PA, which promotes membrane translocation of raf-1 and subsequent activation of MAPK [27], thereby leading in turn to cPLA₂ activation [2]. A requirement for PLA₂ for PLD activation has been suggested by the demonstrations that PLD₂, but not PLD₁, was activated by unsaturated fatty acids, such as OA and AA, in concert with PIP₂ in vitro [28], and that inhibitors and antisense for cPLA₂ reduced A23187-induced PLD₂ activation in vivo [30]. Moreover, reduced PLD₂ activation by cPLA₂ inhibitors was restored by supplementing the cells with lysophosphatidylcholine, a primary product of cPLA₂ [30]. In marked contrast, activation of cPLA₂ and PLD₁ occurred independently in human monocytes [29].

Our present study, by employing a cotransfection strategy, has confirmed the functional interaction between signaling PLA₂s (cPLA₂ and sPLA₂-IIA) and PLD₂. Cotransfection of either of the signaling PLA₂s and PLD₂, but not PLD₁, markedly augmented AA release (indicative of PLA₂ action) and PEt formation (indicative of PLD activation), providing further support for the hypothesis that both pathways are functionally interrelated. Moreover, we have shown that PLD₂, but not PLD₁, facilitates the induced expression of COX-2, which lies downstream of cPLA₂ and sPLA₂-IIA in the delayed PG biosynthetic pathway [3–5,8]. Thus, the PLD₂ pathway significantly affects cellular capacity to produce PGs.

The molecular mechanisms whereby signaling PLA₂s and PLD₂ affect each other's functions are unknown at present. The findings that the activity of PLD₂, but not PLD₁, is modulated by the PLA₂ reaction products [28,30] may ex-

plain, at least in part, why only PLD₂ can be stimulated by signaling PLA₂s. Moreover, activation of the MAPK pathway via the PLD₂-dependent route [27] could be sequentially linked to cPLA₂ activation [2] as well as COX-2 induction [39]. Since the action of sPLA₂-IIA depends on prior activation of cPLA₂ [3,10,11], PLD₂-mediated activation of a low level of endogenous cPLA₂ may eventually cause subsequent activation of sPLA₂-IIA in HEK293 cells. Interestingly, both PLD₂ [20] and sPLA₂-IIA [8] have been shown to reside in caveolae. Colocalization of these enzymes in this particular membrane compartment may assist adequate presentation of the PLA₂ reaction products, which would facilitate the activation of PLD₂. Since PA, a PLD product, is a preferred substrate for sPLA₂-IIA [40], its production by PLD₂ in the caveolar membrane may further accelerate sPLA₂-IIA-directed membrane hydrolysis. This speculation is in line with the notion that sPLA₂-IIA requires stimulus-induced membrane modifications for its proper hydrolytic action [41]. Thus, the PLA₂/PLD₂ crosstalk may represent an unexplored positive-feedback amplification loop of the signal transduction cascade.

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