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Functional crosstalk between phospholipase D_2 and signaling phospholipase A_2/cyclooxygenase-2-mediated prostaglandin biosynthetic pathways

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Abstract We performed reconstitution analyses of functional interaction between phospholipase A_2 (PLA_2) and phospholipase D (PLD) enzymes. Cotransfection of HEK293 cells with cytosolic ($cPLA_2$) or type IIA secretory ($sPLA_2$-IIA) PLA_2 and PLD_2, but not PLD_1, led to marked augmentation of stimulus-induced arachidonate release. Interleukin-1-stimulated arachidonate release was accompanied by prostaglandin E_2 production via cyclooxygenase-2, the expression of which was augmented by PLD_2. Conversely, activation of PLD_2, not PLD_1, was facilitated by $cPLA_2$ or $sPLA_2$-IIA. Thus, our results revealed functional crosstalk between signaling PLA_2s and PLD_2 in the regulation of various cellular responses in which these enzymes have been implicated.

Key words: Phospholipase A_2; Phospholipase D; Arachidonic acid; Prostaglandin; Cyclooxygenase-2

1. Introduction

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of membrane phospholipids, liberating free arachidonic acid (AA) and lysophospholipids, which are precursors for bioactive lipid mediators. Of a number of PLA_2 enzymes identified to date, cytosolic $PLA_2\alpha$ ($cPLA_2$) and several secretory PLA_2 ($sPLA_2$) enzymes have been implicated in stimulus-induced AA release, which is coupled with cyclooxygenase (COX)-catalyzed prostaglandin (PG) formation [1–6]. Activation of $cPLA_2$ is tightly regulated by post-receptor signal transduction events, including Ca^{2+} 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 Ca^{2+} levels, $cPLA_2$ translocates from the cytosol to the perinuclear membrane [7]. Type IIA $sPLA_2$ ($sPLA_2$-IIA), a signaling $sPLA_2$, 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 $sPLA_2$-IIA and its relative $sPLA_2$-V depend on prior activation of $cPLA_2$ [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, PLD_1 and PLD_2, in mammals [13–17]. PLD_1, 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 Ral), phosphatidylinositol 4,5-bisphosphate (PIP_2), and protein kinase Cα in vitro [13–16]. PLD_1 is distributed primarily on intracellular membranes, where it may regulate the formation of transport intermediates [17]. PLD_2 is constitutively active and is further stimulated by PIP_2 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 PLA_2 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 $cPLA_2$, is suppressed by pharmacological inhibition of PLD. Moreover, PLD_2, not PLD_1, activity has been reported to be modulated by $cPLA_2$ or its reaction products [28–30]. Antisense suppression of $cPLA_2$ expression leads to a concomitant reduction of PLD activity in several cell lines predominantly expressing PLD_2 [30]. Since PLA_2 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 PLA_2s ($cPLA_2$ and $sPLA_2$-IIA) and either of the two PLD enzymes. Our results have provided evidence that PLD_2, but not PLD_1, has the ability to enhance signaling PLA_2-promoted AA release and attendant COX-2-dependent

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Abbreviations: PLD, phospholipase D; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $sPLA_2$, secretory PLA_2; PLC, phospholipase C; AA, arachidonic acid; OA, oleic acid; COX, cyclooxygenase; PG, prostaglandin; MAPK, mitogen-activated protein kinase; PA, phosphatidic acid; DAG, diacylglycerol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PEt, phosphatidylethanol; PMA, phorbol myristate acetate; FCS, fetal calf serum; IL-1, interleukin-1

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PGE_2 generation, and conversely, activation of PLD_2, but not PLD_1, is facilitated by signaling PLA_2s.

2. Materials and methods

2.1. Materials

cDNAs for mouse $cPLA_2$, mouse $sPLA_2$-IIA, and human COX-2 were described previously [3,4]. cDNAs for human PLD_{1b} and human PLD_2 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-1β (IL-1β) was obtained from Genzyme. [^{3}H]AA and [^{3}H]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 PLA_2/PLD transfectants

Human PLD_{1b} and PLD_2 cDNAs subcloned into pcDNA3.1(+) were each transfected into HEK293 cells using Lipofectamine PLUS according to the manufacturer's instruction. Briefly, 1 μg of the plasmid was mixed with 4 μl of Lipofectamine and 6 μl of PLUS reagent in 200 μl 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 μg/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 $cPLA_2$ or mouse $sPLA_2$-IIA was described previously [3].

In order to establish double transfectants stably coexpressing PLA_2 and PLD enzymes, cells expressing each PLD were subjected to a second transfection with $cPLA_2$ or $sPLA_2$-IIA cDNA subcloned into pcDNA3.1/zeo(+). After selection in culture medium containing 50 μg/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 PLA_2

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 μCi/ml [^{3}H]AA and cultured for another day. Then the cells were washed once with culture medium and incubated with 250 μl of 10 μM A23187 in medium containing 1% FCS for 30 min or 1 ng/ml IL-1β in medium containing 10% FCS for 4 h. The percentage release of [^{3}H]AA was calculated using the formula $[S/(S+P)]\times100$, 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 transphosphatidylation 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 μCi/ml [^{3}H]OA in culture medium for another day. After washing twice with medium containing 1% FCS, the cells were cultured with or without 10 μM A23187 and 2 μM 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 μl 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 phosphatidylethanol (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 μg) 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 [^{32}P]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 PLA_2s ($cPLA_2$ and $sPLA_2$-IIA) and PLDs (PLD_1 and PLD_2) alone or in combination, as assessed by RNA blotting, are shown in Fig. 1. When cells transfected with $cPLA_2$ 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 PLD_1 or PLD_2 alone was comparable to that by control cells. Remarkably, AA release by cells expressing $cPLA_2$ in combination with PLD_2, but not with PLD_1, was markedly augmented, reaching a several-fold higher level than that by cells expressing $cPLA_2$ alone in both A23187- and IL-1-induced responses (Fig. 2). Since PLD_1 has been shown to be activated by PMA [13–16], we next stimulated $cPLA_2$/PLD_1 cotransfectants with A23187 in the presence of PMA. Despite increased PLD_1 activity (see below), however, AA release by these cells was similar to that by replicate $cPLA_2$-expressing cells (data not shown). Expression of $sPLA_2$-IIA also led to significant increase in A23187- and IL-1-induced AA release (Fig. 3), as described previously [3–5,8]. This $sPLA_2$-mediated AA release

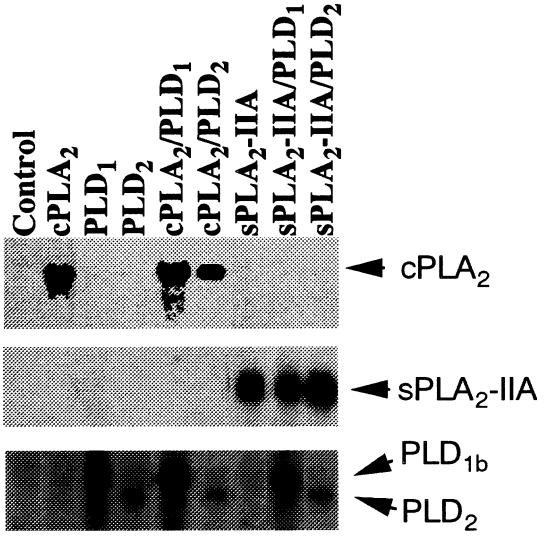

Fig. 1. Expression of PLA_2 and PLD enzymes in HEK293 transfectants. Expression of $cPLA_2$, $sPLA_2$-IIA, PLD_1 and PLD_2 in HEK293 transfectants was assessed by RNA blotting.

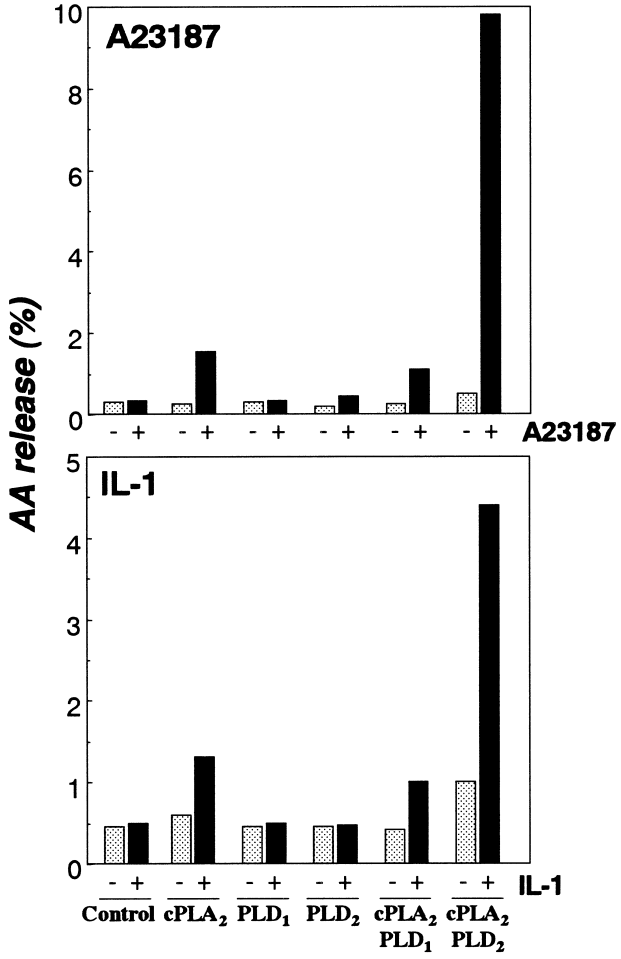

Fig. 2. AA release by $cPLA_2$/PLD transfectants. [^{3}H]AA-prelabeled HEK293 transfectants expressing $cPLA_2$, PLD_1 and PLD_2, alone or in combination, were stimulated for 30 min with A23187 or for 4 h with IL-1. A representative result of four independent experiments is shown.

was also enhanced markedly by coexpression with PLD_2, but not with PLD_1 (Fig. 3).

3.2. COX-2-dependent PGE_2 generation

IL-1-stimulated increase in AA release by $cPLA_2$- or $sPLA_2$-IIA-transfected HEK293 cells was accompanied by accumulation of PGE_2 in the culture medium [3–5,8]. As shown in Fig. 4, PGE_2 generation by cells expressing $cPLA_2$ or $sPLA_2$-IIA alone increased modestly compared with that by control cells, and that by cells coexpressing $cPLA_2$ or $sPLA_2$-IIA and PLD_2 reached levels several-fold higher than that by cells expressing each PLA_2 alone. In contrast, no significant increase in PGE_2 generation was observed in cells expressing PLD_1 or PLD_2 alone, and PGE_2 generation by cells coexpressing $cPLA_2$ and PLD_1 was comparable to that by cells expressing $cPLA_2$ alone.

IL-1-dependent delayed PGE_2 generation depends entirely on inducible COX-2 [3–5,8]. Of interest, the expression level of COX-2 in cells expressing PLD_2 alone was higher than that in control cells or in cells expressing $cPLA_2$ and PLD_1, alone or in combination (Fig. 4, top panel). COX-2 expression in cells coexpressing $cPLA_2$ and PLD_2 was similar to that in cells expressing PLD_2 alone, indicating that the PLD_2-mediated, but not $cPLA_2$-mediated, signals crucially influence the COX-2 induction machinery. Cells expressing PLD_2 alone did not produce PGE_2 appreciably irrespective of increased COX-2 expression (Fig. 4), most likely because AA supply was limited in these cells (Fig. 2). $sPLA_2$-IIA alone had the ability to enhance COX-2 expression (Fig. 4, top panel), as reported previously [5,9], and coexpression of $sPLA_2$-IIA and PLD_2 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 PLA_2s 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 transphosphatidylation [12], was assessed. As shown in Fig. 5, PLD activity in cells transfected with PLD_1 and PLD_2 increased 2- and 1.4-fold, respectively, compared with that in control cells or cells expressing each PLA_2 alone. Notably, more PEt was produced

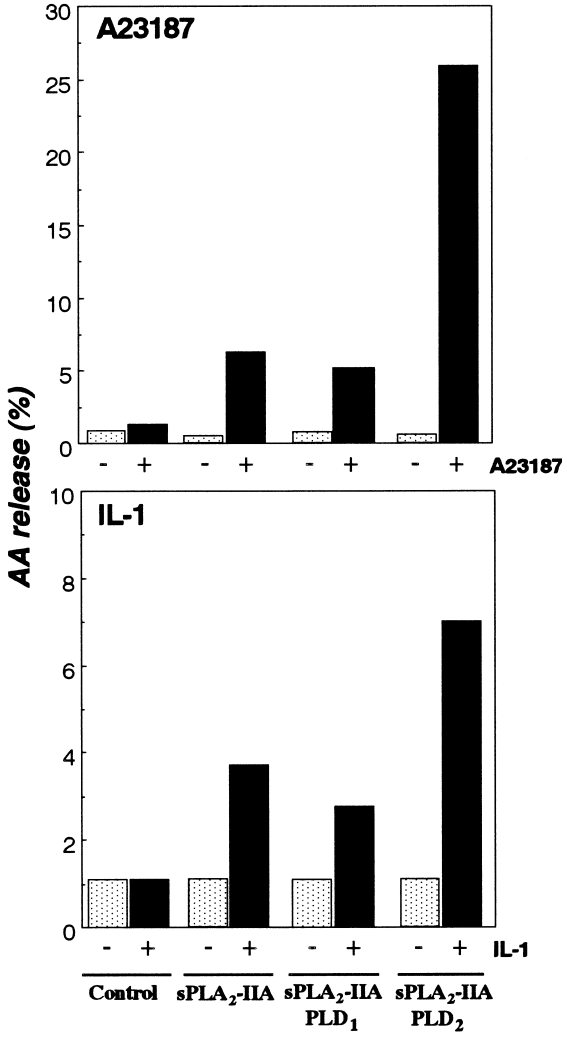

Fig. 3. AA release by $sPLA_2$-IIA/PLD transfectants. [^{3}H]AA-prelabeled HEK293 transfectants expressing $sPLA_2$-IIA, PLD_1 and PLD_2, alone or in combination, were stimulated for 30 min with A23187 or for 4 h with IL-1. A representative result of three independent experiments is shown.

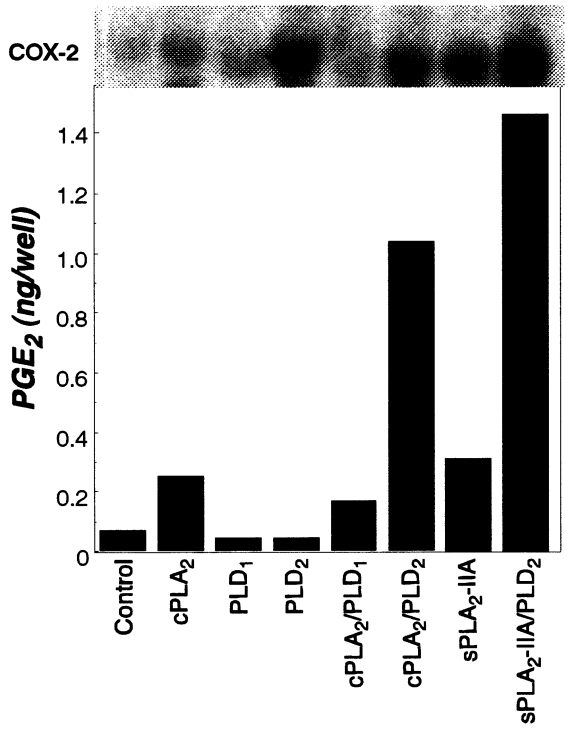

Fig. 4. IL-1-induced PGE_2 generation and COX-2 expression in PLA_2/PLD transfectants. HEK293 transfectants expressing $cPLA_2$, $sPLA_2$-IIA, PLD_1 and PLD_2, alone or in combination, were stimulated for 4 h with IL-1, and PGE_2 production and COX-2 mRNA expression (assessed by RNA blotting; top panel) were examined. A representative result of three independent experiments is shown.

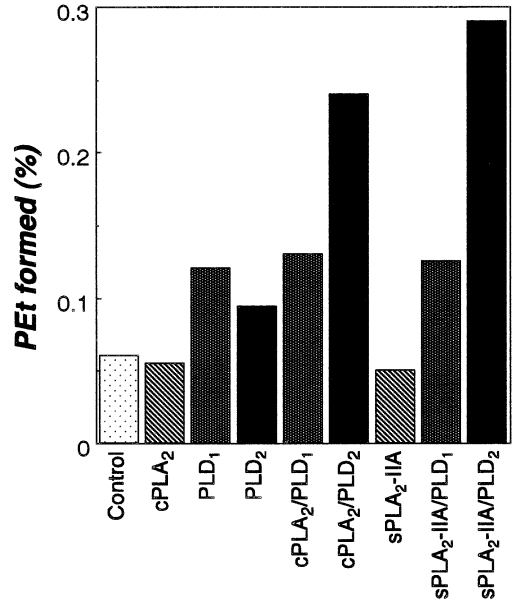

Fig. 5. PLD activation in PLA_2/PLD transfectants. [^{3}H]OA-prelabeled HEK293 transfectants expressing $cPLA_2$, $sPLA_2$-IIA, PLD_1 and PLD_2, alone or in combination, were stimulated for 30 min with A23187 and PMA, and [^{3}H]PEt formation was examined. Values indicate the percent radioactivity associated with PEt relative to that incorporated into total phospholipids. A representative result of three independent experiments is shown.

in cells coexpressing PLD_2 and either $cPLA_2$ or $sPLA_2$-IIA than in cells expressing PLD_2 alone, whereas the PLD_1-mediated increase in PEt formation was not altered significantly when combined with either PLA_2.

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 Ca^{2+} mobilization, which is a prerequisite for $cPLA_2$ activation [1]. Conversely, the tyrosine kinase-coupled PLC-γ isozymes are stimulated by AA, which is supplied by $cPLA_2$, in the presence of the microtubule-associated protein tau or tau-like protein AHNAK [33,34]. Prior activation of $cPLA_2$ modulates the action of signaling $sPLA_2$s probably through destabilizing $sPLA_2$-targeted membrane structures [3,10,11,35], while $sPLA_2$s can activate $cPLA_2$ through the $sPLA_2$ 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 PLA_2 and PLD also display some functional crosstalk. For instance, PLD has been suggested to act as an upstream regulator of PLA_2 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 $cPLA_2$-regulatory processes. More recently, a study using dominant-negative forms of PLD variants has revealed the involvement of PLD_2, but not PLD_1, 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 $cPLA_2$ activation [2]. A requirement for PLA_2 for PLD activation has been suggested by the demonstrations that PLD_2, but not PLD_1, was activated by unsaturated fatty acids, such as OA and AA, in concert with PIP_2 in vitro [28], and that inhibitors and antisense for $cPLA_2$ reduced A23187-induced PLD_2 activation in vivo [30]. Moreover, reduced PLD_2 activation by $cPLA_2$ inhibitors was restored by supplementing the cells with lysophosphatidylcholine, a primary product of $cPLA_2$ [30]. In marked contrast, activation of $cPLA_2$ and PLD_1 occurred independently in human monocytes [29].

Our present study, by employing a cotransfection strategy, has confirmed the functional interaction between signaling PLA_2s ($cPLA_2$ and $sPLA_2$-IIA) and PLD_2. Cotransfection of either of the signaling PLA_2s and PLD_2, but not PLD_1, markedly augmented AA release (indicative of PLA_2 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 PLD_2, but not PLD_1, facilitates the induced expression of COX-2, which lies downstream of $cPLA_2$ and $sPLA_2$-IIA in the delayed PG biosynthetic pathway [3–5,8]. Thus, the PLD_2 pathway significantly affects cellular capacity to produce PGs.

The molecular mechanisms whereby signaling PLA_2s and PLD_2 affect each other's functions are unknown at present. The findings that the activity of PLD_2, but not PLD_1, is modulated by the PLA_2 reaction products [28,30] may ex-

plain, at least in part, why only PLD_2 can be stimulated by signaling PLA_2s. Moreover, activation of the MAPK pathway via the PLD_2-dependent route [27] could be sequentially linked to $cPLA_2$ activation [2] as well as COX-2 induction [39]. Since the action of $sPLA_2$-IIA depends on prior activation of $cPLA_2$ [3,10,11], PLD_2-mediated activation of a low level of endogenous $cPLA_2$ may eventually cause subsequent activation of $sPLA_2$-IIA in HEK293 cells. Interestingly, both PLD_2 [20] and $sPLA_2$-IIA [8] have been shown to reside in caveolae. Colocalization of these enzymes in this particular membrane compartment may assist adequate presentation of the PLA_2 reaction products, which would facilitate the activation of PLD_2. Since PA, a PLD product, is a preferred substrate for $sPLA_2$-IIA [40], its production by PLD_2 in the caveolar membrane may further accelerate $sPLA_2$-IIA-directed membrane hydrolysis. This speculation is in line with the notion that $sPLA_2$-IIA requires stimulus-induced membrane modifications for its proper hydrolytic action [41]. Thus, the PLA_2/PLD_2 crosstalk may represent an unexplored positive-feedback amplification loop of the signal transduction cascade.

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