Perinuclear localization of cytosolic phospholipase $A_2\alpha$ is important but not obligatory for coupling with cyclooxygenases

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Abstract In response to Ca^{2+} signaling, cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) translocates from the cytosol to the perinuclear membrane, where downstream eicosanoid-synthetic enzymes, such as cyclooxygenase (COX), are localized. Although the spatiotemporal perinuclear colocalization of $cPLA_2\alpha$ and COXs has been proposed to be critical for their functional coupling leading to prostanoid production, definitive evidence for this paradigm has remained elusive. To circumstantiate this issue, we took advantage of a chimeric $cPLA_2\alpha$ mutant harboring the C2 domain of protein kinase Cα, which translocates to the plasma membrane following cell activation. Transfection analyses of the native or chimeric $cPLA_2\alpha$ in combination with COX-1 or COX-2 revealed that, even though the arachidonate-releasing capacities of native and mutant $cPLA_2\alpha$ were comparable, prostaglandin production by mutant $cPLA_2\alpha$ was markedly impaired as compared with that by native $cPLA_2\alpha$. We thus conclude that the perinuclear localization of $cPLA_2\alpha$ is preferential, even if not obligatory, for efficient coupling with COXs.

Key words: Phospholipase A2; Cyclooxygenase; Arachidonic acid; Prostaglandin; C2 domain

1. Introduction

Of a number of phospholipase A_2 (PLA_2) enzymes, including the cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$) and Ca^{2+}-independent PLA_2 ($iPLA_2$) families, group IVA $cPLA_2\alpha$ plays a central role in the stimulus-coupled release of arachidonic acid (AA), a precursor of eicosanoids including prostaglandins (PGs) and leukotrienes (LTs) [1,2]. $cPLA_2\alpha$ is the only PLA_2 enzyme that shows marked preference for the *sn*-2 AA over other fatty acids [1,2]. In activated cells, $cPLA_2\alpha$ undergoes Ca^{2+}-directed translocation from the cytosol to the perinuclear membrane and endoplasmic reticulum (ER) [3], in which downstream AA-metabolizing enzymes, including cyclooxygenases (COXs), 5-lipoxygenase (5-LO) and various terminal PG and LT synthases, are also located [4–6]. This Ca^{2+}-dependent translocation of $cPLA_2\alpha$ depends entirely on the N-terminal C2 domain [3]. In addition, phosphorylation of $cPLA_2\alpha$ by mitogen-activated protein kinases (MAPKs) and other kinases is also crucial for its optimal activation [7].

The C2 domains bind the membrane in a Ca^{2+}-dependent manner and thereby play an important role in Ca^{2+}-dependent membrane targeting of peripheral proteins. Most C2 domains have higher affinity for anionic phosphatidylserine than for zwitterionic phosphatidylcholine (PC), as exemplified by that of protein kinase Cα (PKCα) [8]. In contrast, the C2 domain of $cPLA_2\alpha$ preferentially binds to PC [9]. A recent study using PKCα/$cPLA_2\alpha$ chimeric mutants, in which their C2 domains were interchanged, has demonstrated that the plasma membrane translocation of PKCα and the perinuclear translocation of $cPLA_2\alpha$ in activated cells are crucially affected by lipid selectivity of their C2 domains [10]. Additionally, binding of the C2 domain of $cPLA_2\alpha$, but not that of PKCα, to vimentin, a component of the intermediate filament, may also influence the intracellular localization of $cPLA_2\alpha$ [11].

An emerging body of evidence suggests that spatiotemporal colocalization of eicosanoid-biosynthetic enzymes in the perinuclear region is critical for their effective functional coupling. This appears to be true for coupling between intermediate (COXs and 5-LO) and terminal enzymes in the AA cascade, in which the unstable intermediate substrates, PGH_2 and LTA_4, are transferred between these enzymes in proximity [12,13]. However, considering that exogenous AA can be converted to eicosanoids and that several $sPLA_2$s, which often release AA from the plasma membrane surface, can be functionally linked to COXs and 5-LO [14–17], it has still remained controversial whether the 'perinuclear' translocation of $cPLA_2\alpha$ is a prerequisite for its coupling with downstream enzymes.

To address this critical issue, we designed experiments employing the aforementioned $cPLA_2\alpha$ chimeric mutant possessing the PKCα C2 domain (PKC(C2)). By comparing the cellular AA-releasing and PGE_2-synthetic capacities of wild-type (WT) $cPLA_2\alpha$ and the PKC(C2)-$cPLA_2\alpha$ chimera, which translocate to the perinuclear and plasma membranes, respectively, upon cell activation [10], we provide evidence that the perinuclear localization of $cPLA_2\alpha$ is crucial, even if not essential, for its functional coupling with COX enzymes.

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Abbreviations: PLA_2, phospholipase A_2; $cPLA_2$, cytosolic phospholipase A_2; $sPLA_2$, secretory PLA_2; $iPLA_2$, Ca^{2+}-independent PLA_2; COX, cyclooxygenase; 5-LO, 5-lipoxygenase; AA, arachidonic acid; PG, prostaglandin; LT, leukotriene; PKC, protein kinase C; EGFP, enhanced green fluorescent protein; IL-1, interleukin-1; FCS, fetal calf serum; WT, wild-type; HEK, human embryonic kidney; PC, phosphatidylcholine

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2. Materials and methods

2.1. Materials

Human embryonic kidney (HEK) 293 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co.) containing 10% (v/v) fetal calf serum (FCS; Bioserum), as described previously [14]. cDNAs for cPLA$_2$α, COX-1 and COX-2 and HEK293 cells stably transfected with these enzymes were described previously [14]. The enzyme immunoassay kit for PGE_2 was purchased from Cayman Chemicals. Goat anti-human COX-1 and COX-2 antibodies and rabbit anti-human cPLA$_2$α antibody were purchased from Santa Cruz. A23187 was purchased from Calbiochem. Human interleukin-1β (IL-1) was purchased from Genzyme. Opti-MEM medium, Lipofectamine 2000 reagent, hygromycin and the mammalian expression vector pCDNA3.1/hyg(−) were purchased from Invitrogen. Horseradish peroxidase-conjugated anti-goat and anti-rabbit antibodies were purchased from Zymed. Rabbit anti-phospho-cPLA$_2$α antibody, which recognizes the MAPK-phosphorylated form of cPLA$_2$α, was purchased from Cell Signaling Technology.

2.2. Establishment of transfectants

Detailed procedures were described in our previous reports [14,15]. Briefly, enhanced green fluorescent protein (EGFP)-tagged PKC(C2)-cPLA$_2$ cDNA [10] was subcloned into pCDNA3.1/hyg(−) and transfected into COX-1- or COX-2-expressing HEK293 cells using Lipofectamine 2000 in Opti-MEM medium. The cells were cloned by limiting dilution in 96-well plates in cultured medium containing 7 μg/ml hygromycin. After culture for 3–4 weeks, wells containing a single colony were chosen and expanded. Clones expressing appropriate levels of each protein were screened by Western blotting and used in subsequent studies.

2.3. Activation of HEK293 Cells

[^{3}H]AA release and PGE_2 production by cells after stimulation for 30 min with 10 μM A23187 (immediate response) and for 4 h with IL-1 (delayed response) were assessed as described previously [14,15]. Briefly, cells were prelabeled with [^{3}H]AA (Amersham) (0.1 μCi/ml) overnight, washed with fresh medium, and then incubated with 10 μM A23187 in medium containing 1% FCS for 30 min or 1 ng/ml IL-1 in medium containing 10% FCS for 30 min or 4 h. The percentage [^{3}H]AA release was calculated using the formula $[S/(S+P)]\times 100$, where S and P are the radioactivity measured in the supernatant and cell pellet, respectively. The supernatants from replicate cells (without [^{3}H]AA prelabeling) were subjected to the PGE_2 enzyme immunoassay.

2.4. Western blotting

Lysates from 10^5 cells were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 7.5% gels under reducing conditions. Transfer to nitrocellulose membranes and subsequent Western blotting was performed as described previously [14]. Dilutions of the first antibodies used in Western blotting were as follows: anti-COX-1, 1:20 000; anti-COX-2, 1:2000; anti-cPLA$_2$α, 1:5000; and anti-phospho-cPLA$_2$α, 1:1000.

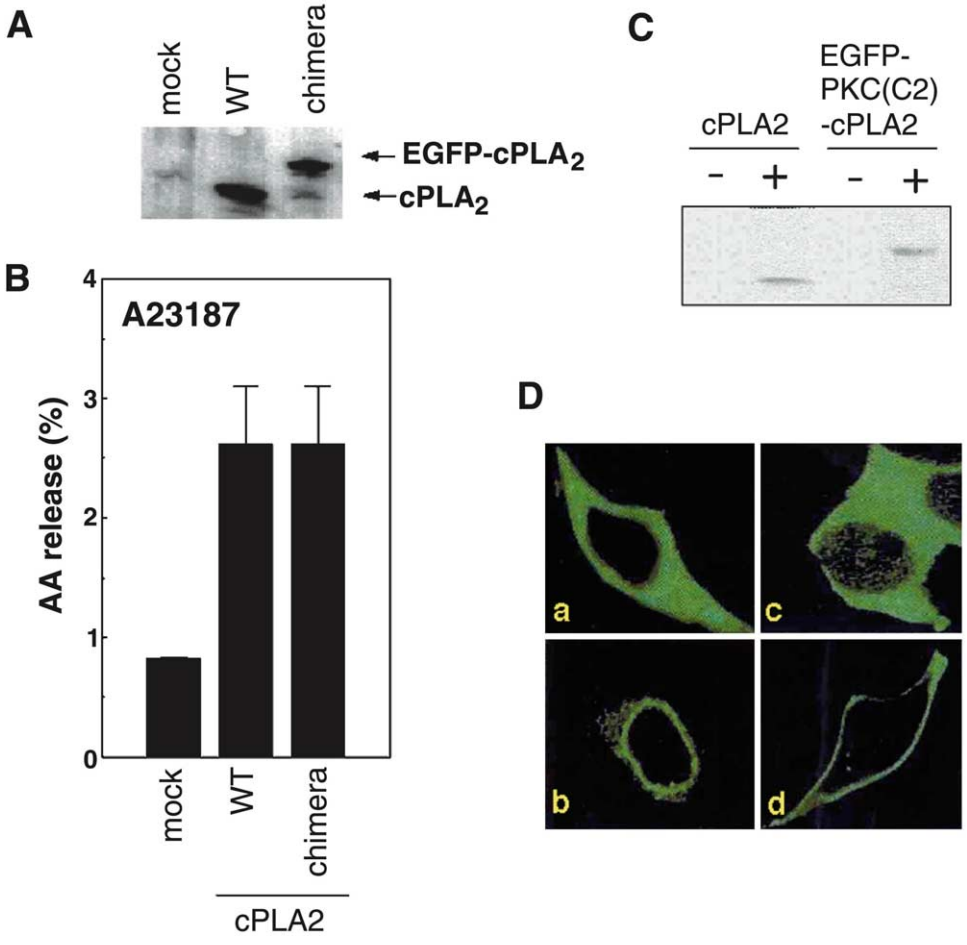

Fig. 1. AA release by WT and chimeric cPLA$_2$α. A: The expression of WT and chimeric cPLA$_2$α proteins in parental cells and transfectants, as assessed by Western blotting with an anti-cPLA$_2$α antibody. B: AA release by cells transfected with WT cPLA$_2$α or EGFP-PKC(C2)-cPLA$_2$α and control cells after stimulation for 30 min with 10 μM A23187. Values are means ± S.E.M. of three independent experiments. C: Detection of the phosphorylated forms of WT cPLA$_2$α and PKC(C2)-cPLA$_2$α before (−) and after (+) activation with A23187, as assessed by Western blotting with an anti-phospho-cPLA$_2$α antibody. D: Subcellular localization of the WT (panels a and b) and chimeric (panels c and d) cPLA$_2$α in the transfectants before and after activation with A23187.

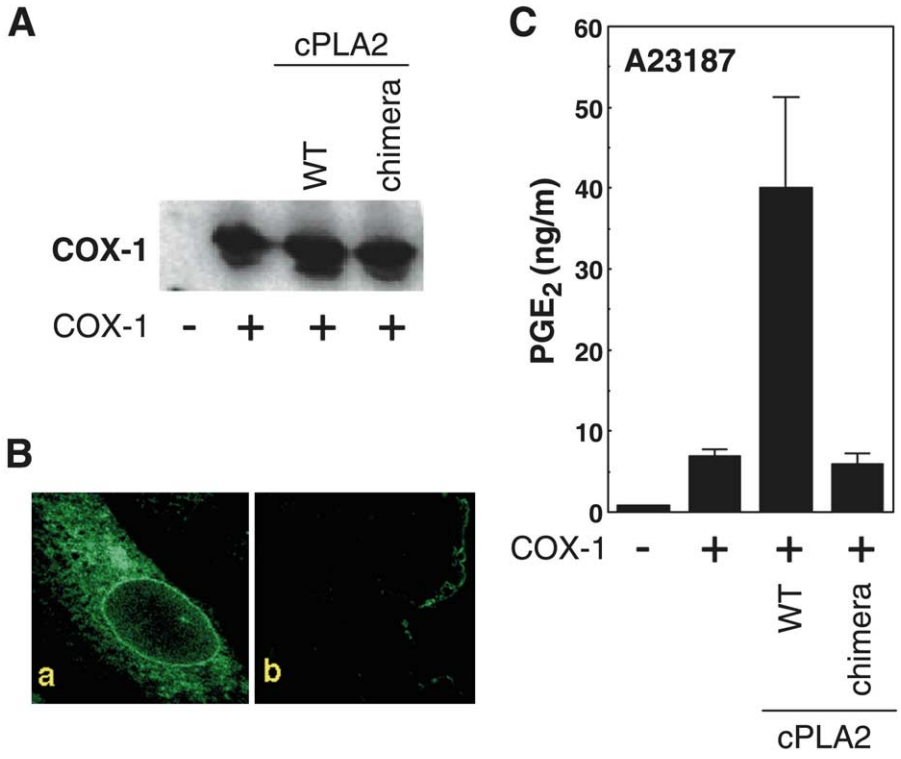

Fig. 2. COX-1-dependent PGE_2 production by WT and chimeric $cPLA_2\alpha$. A: The expression of COX-1 protein in parental cells and transfectants, as assessed by Western blotting with anti-COX-1 antibody. B: Subcellular localization of COX-1 in the COX-1-transfected (panel a) and -untransfected (panel b) cells. C: Cells were stimulated for 30 min with A23187 to assess immediate PGE_2 production. Values are means ± S.E.M. of three independent experiments.

2.5. Confocal laser microscopy

Cells grown on collagen-coated cover glasses (Iwaki Glass) were fixed with 3% paraformaldehyde for 30 min in phosphate-buffered saline (PBS). After three washes with PBS, the fixed cells were sequentially treated with 1% (w/v) bovine serum albumin (for blocking) and 1% (w/v) saponin (for permeabilization) in PBS for 1 h, with anti-$cPLA_2$, COX-1 or COX-2 antibody (1:200 dilution) for 1 h in PBS containing 1% albumin, and then with fluorescein isothiocyanate-goat anti-rabbit or anti-goat IgG (1:200 dilution) for 1 h in PBS containing 1% albumin. After six washes with PBS, the cells were mounted on glass slides using Perma Fluor (Japan Tanner), and the fluorescent signals were visualized using a laser scanning confocal microscope (IX70; Olympus), as described previously [13]. Localization of EGFP-tagged PKC(C2)-$cPLA_2$ was assessed by direct measurement of the EGFP fluorescence, as described [10].

3. Results

3.1. AA release

WT $cPLA_2\alpha$ and EGFP-fused PKC(C2)-$cPLA_2\alpha$ were each transfected into HEK293 cells. As reported previously, the N-terminal EGFP tag does not affect the function of $cPLA_2\alpha$ [10]. On immunoblotting with an anti-$cPLA_2\alpha$ antibody, the WT and chimera enzymes gave approximately 100- and 130-kDa protein bands, respectively, with a similar intensity (Fig. 1A). A weak 100-kDa band observed in control cells and EGFP-PKC(C2)-$cPLA_2\alpha$ chimera-transfected cells corresponded to $cPLA_2\alpha$ expressed endogenously in HEK293 cells. When these cells were stimulated with A23187 (Fig. 1B) or IL-1 (data not shown), the AA-releasing responses of both cells were almost equal, indicating that $cPLA_2\alpha$ is able to release AA from both the perinuclear and plasma membranes with the same potency. Consistent with this, phosphorylation levels of the WT and chimeric $cPLA_2\alpha$ after cell activation, as assessed by Western blotting using an antibody that recognizes a phosphorylated form of $cPLA_2\alpha$ on Ser^{505}, were comparable (Fig. 1C). The subcellular localization of the WT and chimeric $cPLA_2\alpha$ before and after cell activation, as assessed by confocal laser microscopy, is shown in Fig. 1D. Both the WT (panel a) and chimeric (panel c) enzymes were distributed in the cytosol before cell activation. After A23187 treatment, the majority of the WT (panel b) and chimeric (panel d) enzymes moved to the perinuclear envelope and the plasma membrane, respectively (Fig. 1D), as reported previously [10].

3.2. COX coupling

We then transfected COX-1 or COX-2 into cells expressing either WT $cPLA_2\alpha$ or PKC(C2)-$cPLA_2\alpha$ to assess their functional coupling. Comparable expressions of COX-1 (Fig. 2A) and COX-2 (Fig. 3A) in respective transfectants were verified by Western blotting. The expression levels of the WT and chimeric $cPLA_2\alpha$ as well as AA release were unaffected by cotransfection of COX enzymes in these cells (data not shown). Both COX-1 and COX-2 were located in the perinuclear envelope and the ER membrane in these transfectants, with COX-1 being dispersed into the cytoplasm along the ER membrane (Fig. 2B) and COX-2 being more enriched in the perinuclear area (Fig. 3B). These COX distributions are in agreement with previous studies [12,13].

Transfection of COX-1 alone modestly increased A23187-induced immediate PGE_2 generation (Fig. 2C), where AA is supplied by endogenous $cPLA_2\alpha$ [14]. Under the condition

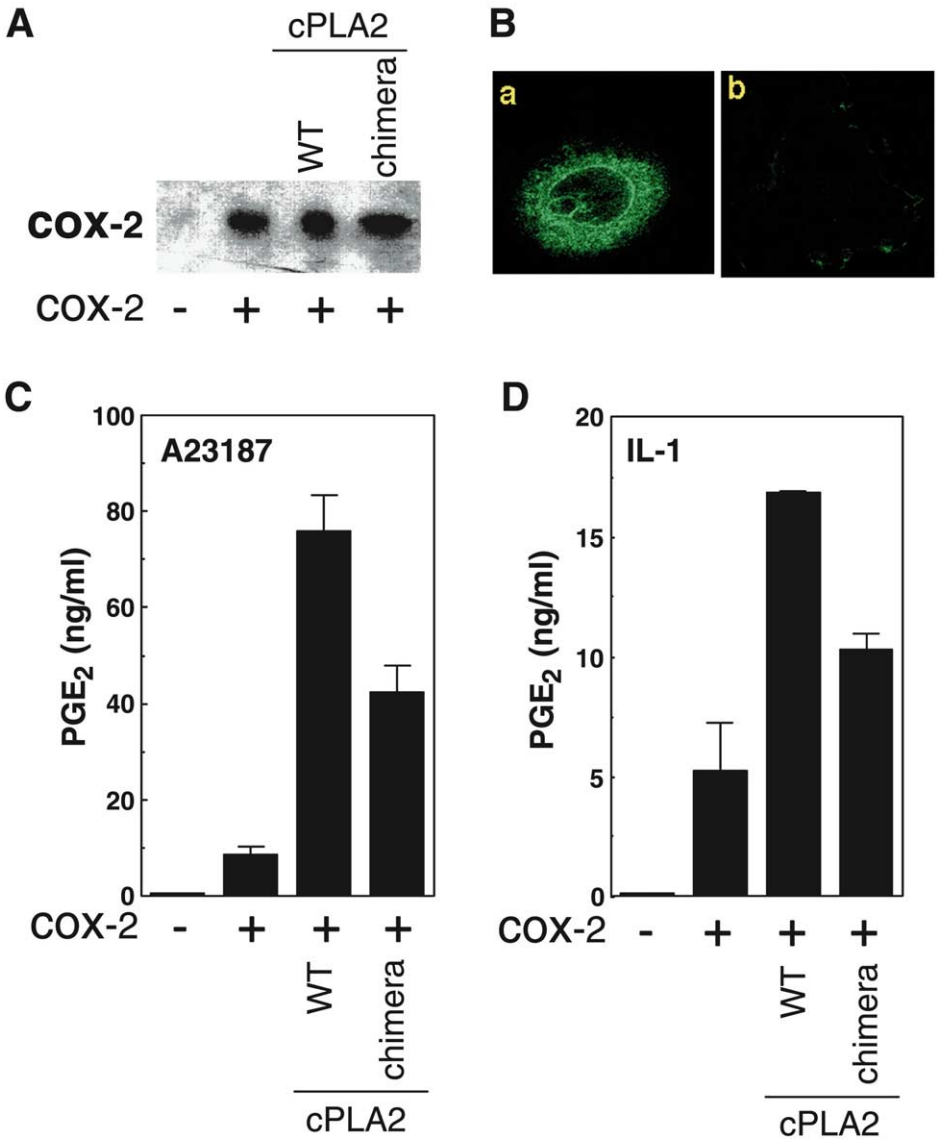

Fig. 3. COX-2-dependent PGE_2 production by WT and chimeric $cPLA_2\alpha$. A: The expression of COX-2 protein in parental cells and transfectants, as assessed by Western blotting with anti-COX-2 antibody. B: Subcellular localization of COX-2 in the COX-2-transfected (panel a) and -untransfected (panel b) cells. C,D: Cells were stimulated for 30 min with A23187 (C) or for 4 h with IL-1 (D) to assess immediate and delayed PGE_2 production, respectively. Values are means ± S.E.M. of three independent experiments.

where WT and chimera enzymes displayed comparable AA release as noted above, there was a marked increase in PGE_2 production in cells cotransfected with WT $cPLA_2\alpha$ and COX-1 relative to cells transfected with COX-1 alone, whereas the PKC(C2)-$cPLA_2\alpha$ chimera failed to augment COX-1-directed PGE_2 production (Fig. 2). This indicates that COX-1 is unable to utilize the AA released from the plasma membrane by PKC(C2)-$cPLA_2\alpha$ in this setting. No PGE_2 production occurred via COX-1 in the IL-1-induced delayed response, as previously reported [14,15].

Cells transfected with COX-2 alone modestly increased both A23187-induced immediate (Fig. 3C) and IL-1-stimulated (Fig. 3D) delayed PGE_2-biosynthetic responses, which were markedly augmented when WT $cPLA_2\alpha$ was cotransfected. Although cells cotransfected with PKC(C2)-$cPLA_2\alpha$ and COX-2 produced more PGE_2 than cells expressing COX-2 alone in both the A23187- and IL-1-stimulated responses, the amount of PGE_2 produced by PKC(C2)-$cPLA_2\alpha$/COX-2-cotransfected cells was significantly lower than that produced by WT $cPLA_2\alpha$/COX-2-cotransfected cells (Fig. 3). Thus, although COX-2-dependent PGE_2 production can occur even if AA is released at the plasma membrane, the perinuclear AA is more efficiently metabolized to PGE_2.

4. Discussion

Since many eicosanoid-biosynthetic enzymes, including COXs, 5-LO and several terminal PG and LT synthases, are localized in the perinuclear membrane and adjacent ER [4–6], the translocation of $cPLA_2\alpha$ into the same perinuclear compartment [3] seems reasonable in considering its role in supplying AA to the proximal downstream enzymes. Kim et al. [18] have recently shown that the AA released by $cPLA_2\alpha$ at the perinuclear membrane, but not the AA released by group V $sPLA_2$ at the plasma membrane, is metabolized to LTB_4 via

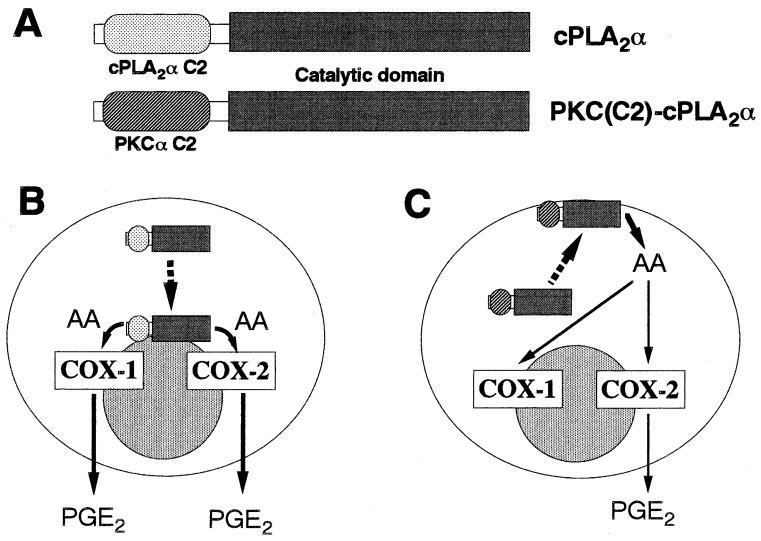

Fig. 4. Schematic model for coupling between WT or PKC(C2)-cPLA$_2\alpha$ and COXs. A: Structure of the WT cPLA$_2\alpha$ and the PKC(C2)-cPLA$_2\alpha$ chimera. B: The WT cPLA$_2\alpha$ translocates to the perinuclear membrane and the released AA is efficiently converted to PGE$_2$ via adjacent perinuclear COX enzymes. C: The chimeric PKC(C2)-cPLA$_2\alpha$ translocates to the plasma membrane and the released AA is diffused (or possibly delivered by putative carrier proteins) across the cytosol to reach the distal perinuclear COXs. Due to a difference in AA concentration sensitivity of the two COXs [12–14], only COX-2 can metabolize the diluted AA to PGE$_2$.

the perinuclear 5-LO in human neutrophils. Although this study has provided some insights into the proposal that perinuclear AA is favored over plasma membrane AA for eicosanoid biosynthesis, comparison of different types of PLA$_2$ makes it difficult to reach a definitive conclusion for this hypothesis. In this brief report, we performed analyses using a particular cPLA$_2\alpha$ mutant that translocates to the plasma membrane upon cell activation [10], which allowed us to substantiate the biological importance of the perinuclear translocation of cPLA$_2\alpha$ for the coupling with COXs. The results obtained from this study are summarized in Fig. 4.

We show that the PKC(C2)-cPLA$_2\alpha$ chimera, which translocates to the plasma membrane [10], releases AA as efficiently as does WT cPLA$_2\alpha$ in activated cells (Fig. 1). This is in line with a previous report that a chimera of cPLA$_2\alpha$ constitutively targeted to the plasma membrane by the N-terminal targeting sequence of the tyrosine kinase Lck leads to increased AA release [19]. Even though the phospholipid compositions of the plasma membrane and the perinuclear membrane may differ significantly, the ability of cPLA$_2\alpha$ to hydrolyze phospholipids without showing appreciable head group selectivity [20] may permit its equivalent action on both membranes.

Notably, distinct PGE$_2$ production (i.e. COX coupling) profiles between WT cPLA$_2\alpha$ and PKC(C2)-cPLA$_2\alpha$, relative to their comparable AA-releasing properties as noted above, argue that the chimeric cPLA$_2\alpha$ is less efficiently coupled with COXs than the WT enzyme. This is particularly evident when combined with COX-1, since no PGE$_2$ production by PKC(C2)-cPLA$_2\alpha$ via COX-1 occurred under the condition where WT cPLA$_2\alpha$ markedly increased PGE$_2$ production (Fig. 2). Unlike via COX-1, the PKC(C2)-cPLA$_2\alpha$ chimera increased PGE$_2$ production via COX-2 to some extent, yet it reached only about half the level of that by the WT enzyme (Fig. 3). These results collectively suggest that the perinuclear localization of cPLA$_2\alpha$ is required for its optimal COX coupling, even if not obligatory (particularly for COX-2 coupling). A likely explanation for the difference between COX-1 and COX-2 observed here is due to their different AA sensitivity in that COX-1 requires more AA than COX-2 to function properly in cells [12–14]. Assuming that the AA released at the plasma membrane is less accessible to the perinuclear COX enzymes, the local concentration of AA around the perinuclear area may reach a level allowed for COX-2 catalysis but insufficient for COX-1 action in this situation.

The notion obtained from this study can be applicable to cellular AA-releasing and eicosanoid-biosynthetic actions of other PLA$_2$ enzymes, such as sPLA$_2$s and iPLA$_2$s. The fact that the AA released from the plasma membrane can be metabolized to PGE$_2$, even though less efficiently than that released from the perinuclear membrane, is in agreement with the eicosanoid-biosynthetic function of the plasma membrane-acting sPLA$_2$s, such as group X sPLA$_2$ [15,17]. Nonetheless, although group X sPLA$_2$ has the most potent AA-releasing capability amongst the sPLA$_2$ family, group IIA and V sPLA$_2$s, which can be internalized through the heparanoid-shuttling mechanism and release AA around the perinuclear region, exhibit comparable or even more PGE$_2$-biosynthetic capacity than does group X sPLA$_2$ in HEK293 cells [17,21–23], an observation reminiscent of our present study. Relatively poor COX coupling of group VIA iPLA$_2$, despite its ability to markedly increase AA release, in HEK293 cells [14,24] may reflect that the membrane compartment on which iPLA$_2$ acts is distant from the perinuclear membrane.

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