

Clearance of group X secretory phospholipase A₂ via mouse phospholipase A₂ receptor

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Abstract Given the potent hydrolyzing activity toward phosphatidylcholine, group X secretory phospholipase A₂ (sPLA₂-X) elicits a marked release of arachidonic acid linked to the potent production of lipid mediators in various cell types. We have recently shown that sPLA₂-X can also act as a ligand for mouse phospholipase A₂ receptor (PLA₂R). Here, we found that sPLA₂-X was internalized and degraded via binding to PLA₂R associated with the diminished prostaglandin E₂ (PGE₂) formation in PLA₂R-expressing Chinese hamster ovary (CHO) cells compared to CHO cells. Indirect immunocytochemical analysis revealed that internalized sPLA₂-X was co-localized with PLA₂R in the punctate structures in PLA₂R-expressing CHO cells. Moreover, in mouse osteoblastic MC3T3-E₁ cells that endogenously express the PLA₂R, the internalized sPLA₂-X was localized in lysosomes. These findings demonstrate that PLA₂R acts as a clearance receptor for sPLA₂-X to suppress its strong enzymatic activity. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Secretory phospholipase A₂; Group X secretory phospholipase A₂; Phospholipase A₂ receptor; Internalization; Clearance receptor

1. Introduction

Secretory phospholipase A₂s (sPLA₂s) are a growing family of enzymes that hydrolyze the *sn*-2 fatty acid ester bonds of glycerophospholipids to produce free fatty acids and lyso-phospholipids [1,2]. sPLA₂s have several common characteristics including a relatively low molecular mass (13–18 kDa), the presence of 6–8 disulfide bridges, and an absolute catalytic requirement for millimolar concentrations of Ca²⁺ [3,4]. At present, mammalian sPLA₂s are classified into 10 different groups depending on the primary structure characterized by the number and positions of cysteine residues (IB, IIA, IIC,

IID, IIE, IIF, III, V, X and XII) [5–7]. Among them, group X sPLA₂ (sPLA₂-X) has 16 cysteine residues located at positions characteristic of the classical types of group IB and IIA sPLA₂s (sPLA₂-IB and sPLA₂-IIA), and also has an amino acid C-terminal extension that is typical of group II sPLA₂ subtypes [8]. We have recently shown that sPLA₂-X is one of the enzymes that possesses a potent hydrolyzing activity toward phosphatidylcholine, a major component of the extracellular face of the plasma membrane of mammalian cells [9]. In fact, sPLA₂-X can induce potent release of arachidonic acid leading to cyclooxygenase (COX)-dependent prostaglandin (PG) formation, as well as marked production of lyso-phosphatidylcholine in various cell types, including macrophages, spleen cells and colon cancer cells [10–12]. Given its high expression in macrophages and invasive colon cancers, sPLA₂-X is thought to play a crucial role in the progression of various disease states, including inflammation and colon tumorigenesis, via the production of lipid mediators.

In addition to its digestive function, sPLA₂-X has recently been identified as a high-affinity ligand for the phospholipase A₂ receptor (PLA₂R) in mice [11,13]. PLA₂R is a type I trans-membrane glycoprotein composed of a large extracellular portion including a characteristic tandem repeat of eight carbohydrate-recognition domains. Its overall molecular organization is related to unique members (subgroup VI) of the C-type animal lectin family, such as the macrophage mannose receptor and DEC-205 [14,15]. In the ligand–receptor relationship, there is strict species specificity, and sPLA₂-IB has been identified as a high-affinity ligand of PLA₂R in mice [16]. sPLA₂-IIA is a relatively weaker ligand and group IID sPLA₂ cannot bind to PLA₂R [16,17]. For the high-affinity receptor binding, proteolytic removal of the propeptide of sPLA₂-X is required, being similar to the case of sPLA₂-IB [13]. PLA₂R is abundantly expressed in type II alveolar epithelial cells in accordance with the expression of sPLA₂-IB and sPLA₂-X [18]. We have recently shown that PLA₂R plays a critical role in the production of pro-inflammatory cytokines during the progression of endotoxic shock by study of PLA₂R-deficient mice [19,20]. As for sPLA₂-IB, we have found that it elicits various biological responses via binding to PLA₂R, including lipid mediator production and cell proliferation [21]. In addition, PLA₂R is involved in the internalization and degradation of sPLA₂-IB [22] and exogenous ligands, such as snake venom sPLA₂ [23].

The powerful enzymatic activity of sPLA₂-X suggests that knowing about its PLA₂R-mediated responses would be indispensable for understanding its physiological and pathological functions. In the present study, we found that sPLA₂-X

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Abbreviations: PLA₂, phospholipase A₂; sPLA₂, secretory phospholipase A₂; sPLA₂-X, group X sPLA₂; sPLA₂-IB, group IB sPLA₂; sPLA₂-IIA, group IIA sPLA₂; COX, cyclooxygenase; PG, prostaglandin; PLA₂R, phospholipase A₂ receptor; Ab, antibody; CHO, Chinese hamster ovary; MEM, minimum essential medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; LAMP-2, lysosome-associated membrane protein type 2; IgG, immunoglobulin G

was rapidly internalized and degraded after PLA₂R binding, thus resulting in a decrease in PGE₂ production. Further analysis with indirect immunofluorescent and immunoelectron microscopy revealed the transport of sPLA₂-X into lysosomes after PLA₂R binding, which indicates that PLA₂R has a critical role as the clearance receptor for sPLA₂-X.

2. Materials and methods

2.1. Materials

Sodium [¹²⁵I]iodine (carrier-free, 3.7 GBq/ml) was purchased from Amersham Pharmacia Biotech. Purified recombinant mouse sPLA₂-X, rabbit anti-sPLA₂-X antibody (Ab), and rat monoclonal anti-PLA₂R Ab (912F) were prepared as described in our previous paper [18]. The specificity of rabbit anti-sPLA₂-X Ab was confirmed by its neutralizing effect on sPLA₂-X enzymatic activity with no inhibitory potency against other sPLA₂ enzymes [9]. The specificity of 912F for PLA₂R was also confirmed by a sandwich enzyme-linked immunosorbent assay (ELISA) system, in which the signal for circulating the soluble form of the receptor was absent in plasma prepared from PLA₂R-deficient mice [18].

2.2. Comparison of sPLA₂-X-induced PGE₂ production and the stability of sPLA₂-X between PLA₂R-CHO and CHO cells

Chinese hamster ovary (CHO) cells stably expressing mouse PLA₂R (PLA₂R-CHO cells) were prepared as described previously [11]. Human COX-2 cDNA and its expression plasmid were prepared [24] and transiently expressed in CHO and PLA₂R-CHO cells with Lipofect-Amine PLUS reagent (Life Technologies) according to the instructions of the manufacturer. Cells were grown in 96-well microplates in α -minimum essential medium (MEM), 10% fetal calf serum (FCS). After washing with phosphate-buffered saline (PBS), the cells were incubated with 25 nM mouse sPLA₂-X in α -MEM, 0.1% bovine serum albumin (BSA) at 37°C for various times. After incubation, the supernatant was collected following centrifugation at 1000 \times g for 5 min at 4°C. The released PGE₂ was quantified with PGE₂ EIA Monoclonal Kit (Cayman Chemicals Co.). In separate experiments, PLA₂R-CHO and CHO cells were incubated with mouse sPLA₂-X for various times at 37°C. The residual PLA₂ activity in the supernatant was determined by chromogenic assay, as described previously [11]. Briefly, the supernatant was incubated with mixed micelles consisting of 1 mM 1,2-bis(heptanoylthio)-1,2-dideoxy-*rac*-glycero-3-phosphocholine and 0.3 mM Triton X-100 in the assay buffer containing 25 mM Tris-HCl buffer (pH 7.5), 0.12 mM 5,5'-dithiobis(2-nitrobenzoic acid), 10 mM CaCl₂, 0.1 M KCl, and 1 mg/ml BSA. The reaction was monitored at the absorbance of 405 nm with a microplate reader, and the amount of the active form of sPLA₂-X in the supernatant was calculated from a standard curve using intact sPLA₂-X.

2.3. Kinetic studies on ligand internalization

Iodination of mouse sPLA₂-X was performed by the chloramine T method, as described previously [22], and the specific radioactivity of ¹²⁵I-sPLA₂-X was 1200 cpm/fmol. PLA₂R-CHO cells were grown in 24-well plates in α -MEM, 10% FCS. After washing with PBS, the cells were incubated with 1 nM ¹²⁵I-labeled mouse sPLA₂-X in binding medium (Hank's balanced salt solution containing 0.1% BSA) for 2 h at 4°C. The cells were washed with PBS, resuspended in binding medium, and then incubated at 37°C. At the end of the incubation, the supernatant was removed and the cells were treated with an acidic buffer (50 mM glycine, 0.1 M NaCl, pH 3.0) for 10 min at 4°C. The acidic buffer was harvested, and its radioactivity was determined. The residual cell-associated radioactivity was determined after solubilization of the cells with 1 N NaOH. The supernatant was precipitated with 10% trichloroacetic acid, and the trichloroacetic acid-soluble and -insoluble radioactivity was determined. The specific binding was calculated by subtracting the non-specific binding obtained with 100 nM mouse sPLA₂-X.

2.4. Analysis by indirect immunofluorescence microscopy

After incubation with mouse sPLA₂-X at 37°C, the cells were washed with PBS and fixed with 4% paraformaldehyde in Na-phosphate buffer (pH 7.2) for 4 h at room temperature. After washing with PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min. They were incubated with 1% skim milk in PBS for 15 min to

block non-specific binding followed by incubation with primary Abs, including rabbit anti-sPLA₂-X Ab (6 μ g/ml), rat anti-PLA₂R Ab (912F, 15 μ g/ml), or rat monoclonal Ab for lysosome-associated membrane protein type 2 (LAMP-2) (ABL-93 obtained from BD Pharmingen, 2.5 μ g/ml). After rinsing with PBS, the cells were incubated with fluorescent-labeled secondary Abs, such as Alexa Fluor 488-labeled goat anti-rabbit immunoglobulin G (IgG) Ab and/or Alexa Fluor 594-labeled goat anti-rat IgG Ab (Molecular Probes, 10 μ g/ml). After washing with PBS, the coverslips were mounted with Aqua Poly/Mount (Polysciences) and viewed under a laser confocal microscope (TCS NT; Leica). Green and red indicate the localization of signals of Alexa Fluor 488 and 594, respectively.

2.5. Analysis by immunoelectron microscopy

After incubation with 50 nM sPLA₂-X for 24 h, mouse osteoblastic MC3T3-E1 cells (ATCC) were washed and fixed in 4% paraformaldehyde in Na-phosphate buffer (pH 7.2) as described above. The cells were dehydrated in a graded series of ethanol and embedded in LR-White (London Resin). Ultrathin sections (70 nm) were mounted on collodion-treated nickel grids. The sections were allowed to react with anti-sPLA₂-X Ab followed by incubation with colloidal gold (5 nm)-conjugated goat anti-rabbit IgG Ab (E-Y Laboratories). After washing, the sections were counterstained with uranyl acetate and observed under a transmission electron microscope (JEOL, JEM-1200EX).

3. Results and discussion

3.1. Comparison of sPLA₂-X-induced PGE₂ production and the stability of sPLA₂-X between PLA₂R-CHO and CHO cells

In order to examine the involvement of PLA₂R in sPLA₂-X-induced responses, we first compared the potency of sPLA₂-X for PGE₂ production between PLA₂R-CHO cells and CHO cells. Since arachidonic acid released by sPLA₂-X can be efficiently converted to PGE₂ via COX-2 in various cell types [10,12], human COX-2 cDNA was transiently introduced into PLA₂R-CHO and CHO cells. Western blot analysis revealed that the expression level of COX-2 protein was the same between the two cell types (result not shown). In COX-2-expressing CHO cells, PGE₂ production elicited by exogenous arachidonic acid was markedly elevated compared to non-transfected cells and its level was comparable in the case with COX-2-expressing PLA₂R-CHO cells (data not shown). As shown in Fig. 1A, sPLA₂-X induced potent PGE₂ production in CHO cells for up to 5 h. In PLA₂R-CHO cells, however, PGE₂ production was slightly reduced at 1 h and markedly decreased after 2 h incubation compared to CHO cells, suggesting a negative role of PLA₂R on sPLA₂-X functions. We then examined the residual enzymatic activity in the culture medium after incubation of the cells with sPLA₂-X. As shown in Fig. 1B, active sPLA₂-X mostly remained in the supernatant during the incubation for up to 24 h in CHO cells, whereas it gradually diminished from 1 h and disappeared after 24 h incubation in PLA₂R-CHO cells. These findings suggest that PLA₂R plays a role in the clearance of sPLA₂-X resulting in the reduction of its enzymatic potency for the cell membranes.

3.2. Internalization and degradation of sPLA₂-X via PLA₂R

Next, we examined the internalization and degradation of ¹²⁵I-sPLA₂-X after binding to PLA₂R-CHO cells. After incubation with ¹²⁵I-sPLA₂-X for 2 h at 4°C, PLA₂R-CHO cells were washed, re-suspended in binding medium and incubated at 37°C for various times. After removing the supernatant, the cells were treated with acidic buffer at 4°C to dissociate the cell-surface ligand, and the residual cell-associated (acid-resis-

tant) radioactivity was determined as the intracellular ligand. As shown in Fig. 2A, most of the cell-bound ligand was removed by the acid wash before the incubation at 37°C. The amount of cell-surface ligand gradually decreased upon warming, while the intracellular radioactivity rises concomitantly, reaching a plateau after 45 min. The fate of internalized ^{125}I -sPLA₂-X was also examined as follows. The incubation medium after warming was subjected to trichloroacetic acid precipitation to determine the amount of native (trichloroacetic acid-insoluble) and degraded (trichloroacetic acid-soluble) ligand. As shown in Fig. 2B, about 43% of the pre-bound ligand was dissociated from the cell surface as an intact form

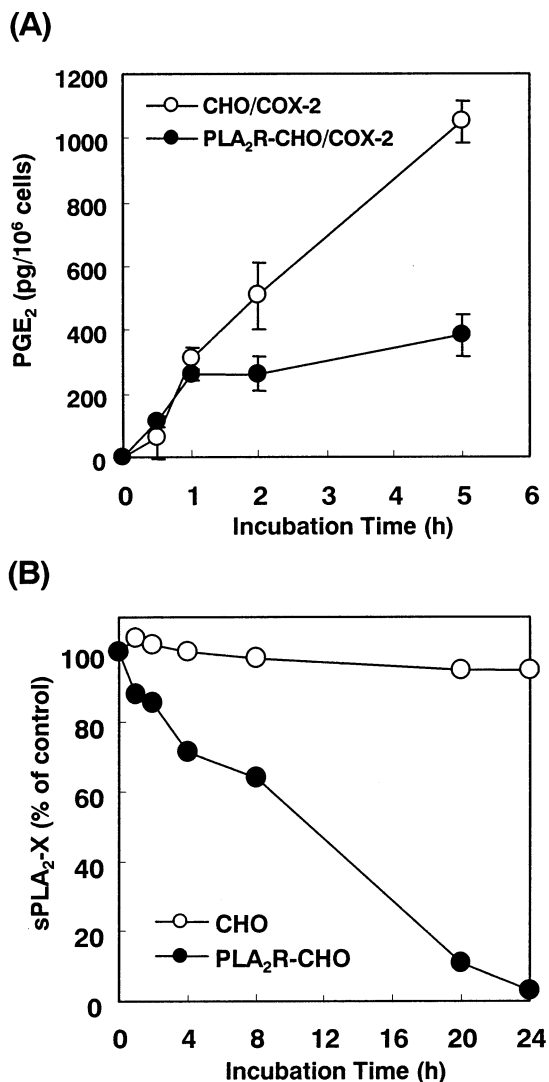


Fig. 1. Comparison of sPLA₂-X-induced PGE₂ production and the stability of sPLA₂-X between PLA₂R-CHO and CHO cells. A: After transient transfection with human COX-2 cDNA, PLA₂R-CHO and CHO cells were incubated with 10 nM sPLA₂-X for various times at 37°C, and the PGE₂ amount in the supernatant was quantified. The results were expressed after subtracting the values obtained from the incubation in the absence of sPLA₂-X at each time point. B: After incubation with sPLA₂-X for the indicated times, the residual PLA₂ activity in the supernatant was measured by chromogenic assay and the amount of the active form of sPLA₂-X was calculated by a standard curve using intact sPLA₂-X. The results are expressed as a percentage of the amount of sPLA₂-X originally added. Each point represents the mean ± S.E.M. of triplicate measurements. The data are representative of three experiments.

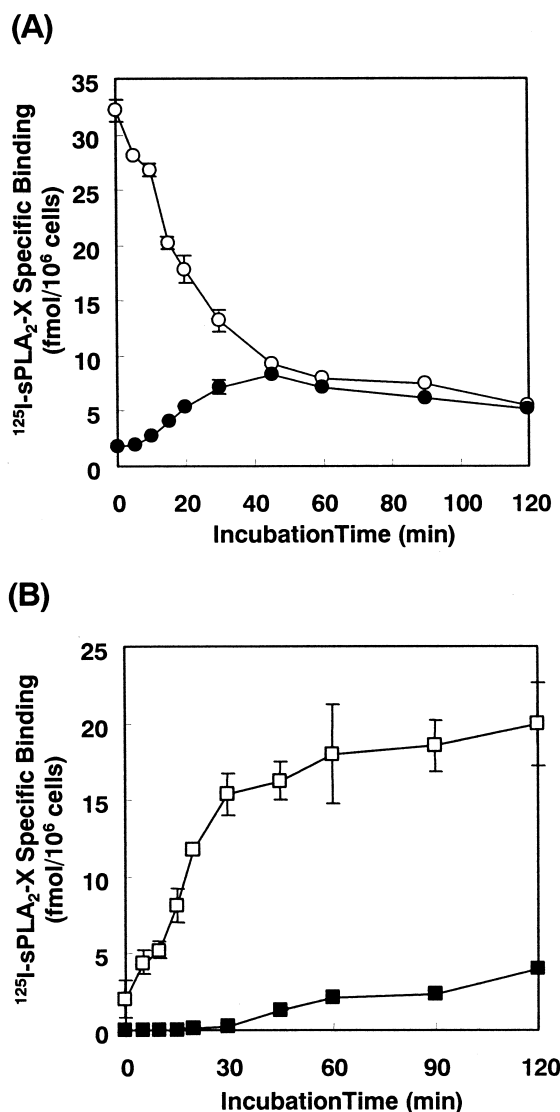


Fig. 2. Internalization and degradation of sPLA₂-X in PLA₂R-CHO cells. After incubation with 2 nM ^{125}I -sPLA₂-X for 2 h at 4°C, PLA₂R-CHO cells were washed, re-suspended in binding medium and incubated at 37°C for the indicated times. After incubation, the supernatant was removed and the cells were treated with acidic buffer for 10 min at 4°C. A: The acidic buffer was harvested, and its radioactivity was determined (○). The residual cell-associated radioactivity (●) was determined after solubilization of the cells with 1 N NaOH. B: The supernatant was precipitated with 10% trichloroacetic acid, and the trichloroacetic acid-soluble (■) and insoluble (□) radioactivities were determined. The specific binding was calculated by subtracting the non-specific binding obtained with 100 nM mouse sPLA₂-X from each point. Each point represents the mean ± S.E.M. of triplicate measurements. The data are representative of three experiments.

during the first 30 min of warming, which probably was due to the reversible ligand binding to the receptor. After 45 min of warming, the degraded ligands began to appear in the medium and then gradually increased. These findings demonstrate that PLA₂R can internalize sPLA₂-X and promote its degradation.

3.3. Co-localization of internalized sPLA₂-X with PLA₂R

In order to determine the subcellular localization of internalized sPLA₂-X and PLA₂R, we performed confocal immu-

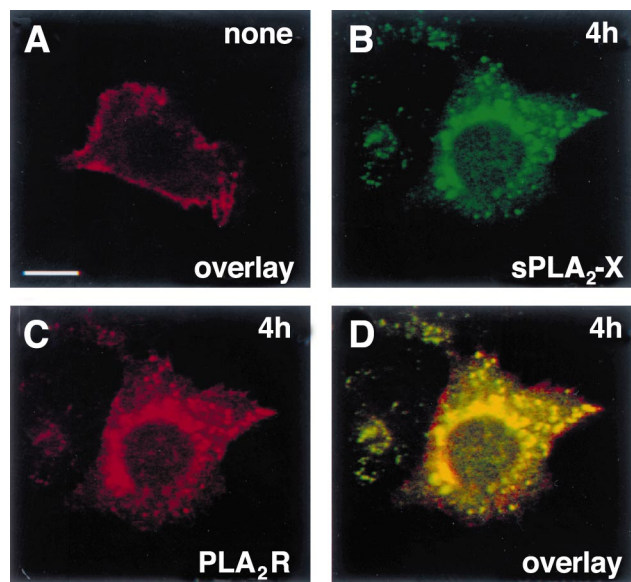


Fig. 3. Confocal microscopic analysis of subcellular localization of internalized PLA₂R and sPLA₂-X. PLA₂R-CHO cells were incubated without (A) or with (B–D) 50 nM sPLA₂-X for 4 h at 37°C. The cells were subjected to indirect immunofluorescent microscopy as described in Section 2. The red and green areas indicate the staining signals for sPLA₂-X (B) and PLA₂R (C), respectively, and the yellow areas indicate the regions where PLA₂R and sPLA₂-X are co-localized (D). Each photograph has the same magnification. The bar indicates 10 μm.

no fluorescent microscopic analysis. After incubation with sPLA₂-X for 4 h at 37°C, PLA₂R-CHO cells were fixed and stained with anti-sPLA₂-X Ab and anti-PLA₂R Ab. Before the incubation, PLA₂R was mostly detected in the plasma membranes with a small portion at the intracellular punctate structures (Fig. 3A). After the incubation, sPLA₂-X was internalized (Fig. 3B) and PLA₂R was translocated from the cell surface to the intracellular punctate structures (Fig. 3C). The overlay of each signal revealed their co-localization in the dense perinuclear structures and the punctate cytoplasmic structures that represent the endosome, lysosomes or recycle compartment (Fig. 3D). In contrast, there were no detectable signals with both Abs under any conditions in control CHO cells (results not shown).

3.4. Clearance of sPLA₂-X in MC3T3-E₁ cells

In order to verify the clearance function of native PLA₂R, we examined the stability of sPLA₂-X in mouse osteoblastic MC3T3-E₁ cells that endogenously express high levels of PLA₂R [25]. In this experiment, we used polyclonal anti-PLA₂R Ab that can block the association of sPLA₂-X with mouse PLA₂R [18]. As shown in Fig. 4, sPLA₂-X activity in the culture medium gradually decreased during the incubation with MC3T3-E₁ cells, with the activity diminishing to 50% after 24 h-incubation. Pretreatment with anti-PLA₂R Ab resulted in almost complete protection for the decline of enzymatic potency in the medium, suggesting that native PLA₂R also acts as a clearance receptor for sPLA₂-X.

To determine the compartmentalization of internalized sPLA₂-X via native PLA₂R, MC3T3-E₁ cells were incubated with sPLA₂-X at 37°C for 24 h, and then stained with anti-sPLA₂-X Ab (Fig. 5A) or Ab against LAMP-2 (Fig. 5B), a known lysosomal marker. The overlay of each signal obtained

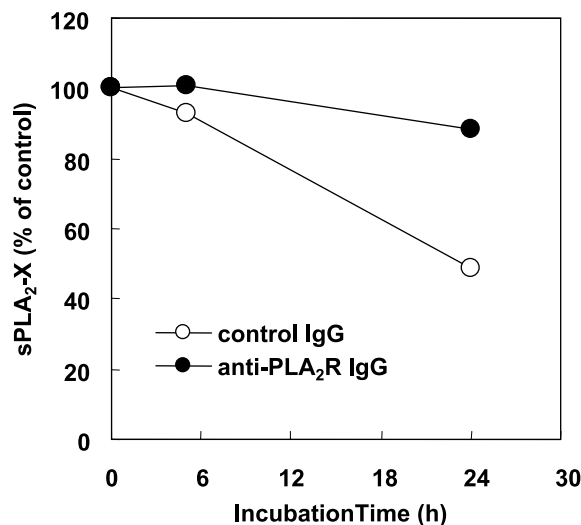


Fig. 4. Clearance of sPLA₂-X in MC3T3-E₁ cells. MC3T3-E₁ cells were pre-incubated with anti-PLA₂R Ab or control rabbit IgG for 1 h at 37°C, and then treated with 10 nM sPLA₂-X for the indicated times at 37°C. The residual PLA₂ activity in the supernatant was measured and the amount of sPLA₂-X was calculated. The results are expressed as the percentage of the amount of sPLA₂-X originally added. Each point represents the mean ± S.E.M. of triplicate measurements. The data are representative of three experiments.

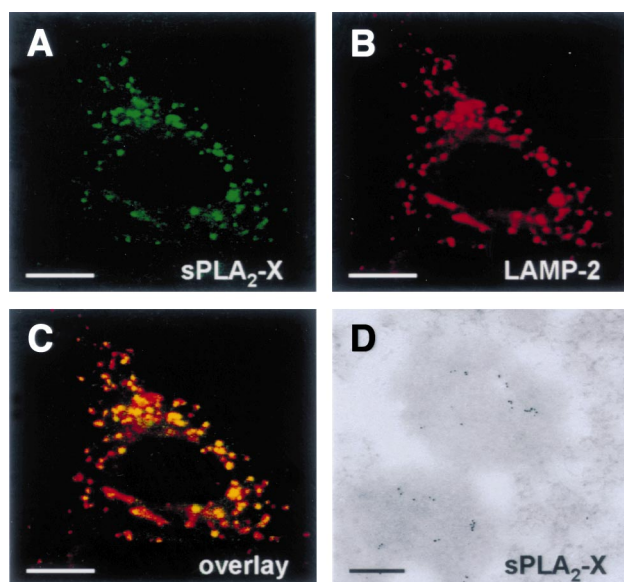


Fig. 5. Localization of internalized sPLA₂-X in lysosomes. A–C: MC3T3-E₁ cells were incubated with 50 nM sPLA₂-X for 24 h and then subjected to indirect immunofluorescent microscopy as described in Section 2. The red and green areas indicate the staining signals of sPLA₂-X (A) and LAMP-2 (B), respectively. The overlay (C) shows co-localization of sPLA₂-X and LAMP-2, and yellow areas indicate the regions where sPLA₂-X is co-localized with LAMP-2. Each bar indicates 10 μm. D: MC3T3-E₁ cells were incubated with 50 nM sPLA₂-X for 24 h. After fixation of the cells, ultrathin sections of the cells were reacted with anti-sPLA₂-X Ab followed by incubation with colloidal gold (5 nm)-conjugated goat anti-rabbit IgG Ab. After washing, the sections were counterstained with uranyl acetate. The bar indicates 200 nm.

with confocal microscopy clearly demonstrates that the internalized ligand is transported into the lysosomes (Fig. 5C). Furthermore, analysis by immunoelectron microscopy also revealed that the colloidal gold representative of internalized sPLA₂-X was localized at the lysosome-like structures (Fig. 5D). In contrast, there were no detectable signals of anti-sPLA₂-X Ab in MC3T3-E1 cells without incubation of sPLA₂-X (results not shown).

Endocytosis is one of the innate host defense systems against microorganisms and toxic molecules [26]. It has been noted that sPLA₂-X possesses a potent hydrolyzing activity toward intact cell membranes leading to marked production of various types of lipid mediators [9]. In the present study, PGE₂ production by sPLA₂-X was diminished by clearance of the active form of sPLA₂-X in PLA₂R-CHO cells (Fig. 1). Since sPLA₂-X is expressed in the lung and spleen where PLA₂R is co-localized in type II alveolar epithelial cells and is present in the neighboring splenic lymphocytes [18], PLA₂R might act as part of the endogenous defense system by withdrawing sPLA₂-X from the extracellular fluid. This could be of crucial importance in various disease states when high levels of sPLA₂-X are detected, such as invasive colon cancers [12]. Although PLA₂R-mediated endocytosis has been observed with other sPLA₂ ligands, including sPLA₂-IB and snake venom sPLA₂ [11,13], the compartmentalization of these ligands and receptor following internalization has not been studied because of the unavailability of their specific Abs. In the present study, we identified the localization of internalized sPLA₂-X and PLA₂R in the vesicular structures representative for endosome, lysosomes or recycling compartment in CHO cells by confocal immunofluorescent microscopy. In the absence of sPLA₂-X, PLA₂R was also located at the intracellular punctate structures (Fig. 3A), suggesting a ligand-independent intracellular trafficking of the receptor as proposed for rabbit PLA₂R [23]. In fact, the internalization rate constant of PLA₂R for sPLA₂-X ($k_e = 0.023 \text{ min}^{-1}$), calculated from kinetic studies (Fig. 3), is comparable with the rates of other constitutively recycling receptors, including its structural homolog, the mannose receptor [23,27]. In MC3T3-E1 cells that express native PLA₂R, the internalized sPLA₂-X was transported into the lysosomes where the ligand could be degraded (Fig. 4).

In conclusion, we have demonstrated that PLA₂R plays a critical role in the clearance of sPLA₂-X. With respect to sPLA₂-IB, PLA₂R can mediate various biological responses after recognition of this ligand, including cell proliferation and lipid mediator production [21]. Recent studies have also shown that sPLA₂-IB is targeted to the nucleus after its binding to the cell surface [28]. These findings suggest that sPLA₂-X binding to PLA₂R might be linked to some signal transduction systems leading to the induction of biological responses. Alternatively, the endosomal vesicles may serve as a vehicle delivering sPLA₂-X to specific intracellular compartments where the enzyme can work before being degraded in the lysosomes. Further studies on the PLA₂R-mediated endocytosis and signaling events of sPLA₂-X in various tissues and

species should lead to further understanding of the physiological and pathological functions of sPLA₂-X.

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