

Identification of group X secretory phospholipase A₂ as a natural ligand for mouse phospholipase A₂ receptor

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Abstract Phospholipase A₂ receptor (PLA₂R) mediates various biological responses elicited by group IB secretory phospholipase A₂ (sPLA₂-IB). The recently cloned group X sPLA₂ (sPLA₂-X) possesses several structural features characteristic of sPLA₂-IB. Here, we detected a specific binding site of sPLA₂-X in mouse osteoblastic MC3T3-E₁ cells. Cross-linking experiments demonstrated its molecular weight (180 kDa) to be similar to that of PLA₂R. In fact, sPLA₂-X was found to bind the recombinant PLA₂R expressed in COS-7 cells, and its specific binding detected in mouse lung membranes was abolished by the deficiency of PLA₂R. These findings demonstrate sPLA₂-X to be one of the high-affinity ligands for mouse PLA₂R. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Phospholipase A₂; Secretory phospholipase A₂; Group X secretory phospholipase A₂; Phospholipase A₂ receptor; Lipid mediator

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 lysophospholipids [1,2]. sPLA₂s have several common characteristics including a relatively low molecular mass (13–18 kDa), the presence of 6 to 8 disulfide bridges and an absolute catalytic requirement for millimolar concentrations of Ca²⁺ [3,4]. At present, mammalian sPLA₂s are classified into nine different groups depending on the primary structure characterized by the number and positions of cysteine residues [3,5–9]. Among them, the group II subfamily (IIA, IID, IIE and V) is thought to play a role in the production of several lipid mediators especially in the delayed phase of the cell activation process, because their expression levels are up-regulated under various inflammatory conditions [6,7,10,11]. In contrast, group IB sPLA₂ (sPLA₂-IB) has long been thought to be a digestive enzyme, given its abundance in the pancreas. However, the discovery of the PLA₂ receptor (PLA₂R) has expanded our concept of sPLA₂-IB [12]. In addition to its digestive function, sPLA₂-IB exerts various biological responses,

including cell proliferation and lipid mediator releases, through binding to PLA₂R [13]. The PLA₂R is composed of a large extracellular portion including a characteristic tandem repeat of eight carbohydrate recognition domains, and its overall molecular organization is related to a unique member of the C-type animal lectin family, including the macrophage mannose receptor [14]. Our recent studies with PLA₂R-deficient mice have demonstrated its critical role in the production of pro-inflammatory cytokines during the progression of endotoxic shock [15,16]. In the ligand–receptor relationship, there is a strict species specificity and sPLA₂-IB was identified as an endogenous ligand of PLA₂R, at least in rats and mice [17]. Group IIA sPLA₂ (sPLA₂-IIA) can also bind PLA₂R with about 10-fold lower affinity compared to sPLA₂-IB [17], whereas group IID sPLA₂ can not act as a ligand in spite of its structural similarities with sPLA₂-IIA [18].

The recently cloned group X sPLA₂ (sPLA₂-X) possesses several structural features characteristic of other sPLA₂s [5]. It has 16 cysteine residues located at positions characteristic of both sPLA₂-IB and sPLA₂-IIA, an amino acid C-terminal extension that is typical of sPLA₂-IIA, and a propeptide sequence attached at NH₂-terminals similar to sPLA₂-IB. We and other groups have recently shown that sPLA₂-X possesses a powerful hydrolyzing activity toward phosphatidylcholine and elicits a marked release of arachidonic acid from several intact cell membranes [19,20]. Considering the structural similarities of sPLA₂-X with sPLA₂-IB and IIA, knowing about its potency as a ligand for PLA₂R is indispensable for understanding its physiological and pathological functions. In the present study, we purified mouse sPLA₂-X and examined its binding characteristics in mouse osteoblastic MC3T3-E₁ cells that possess high levels of native PLA₂R [21]. Further binding studies in recombinant PLA₂R-expressing cells and in PLA₂R-deficient mice also demonstrated that sPLA₂-X is one of the high-affinity ligands for murine PLA₂R.

2. Materials and methods

2.1. Materials

Sodium [¹²⁵I]iodine (carrier-free, 3.7 GBq/ml) was purchased from Amersham Pharmacia Biotech. Purified recombinant human sPLA₂-IB, IIA and X were prepared as described previously [19]. Porcine pancreatic sPLA₂-IB was obtained from Boehringer Mannheim. Disuccinidyl suberate (DSS) was purchased from Pierce Chemical Co. Indoxam was synthesized at Shionogi Research Laboratories [22].

2.2. Recombinant expression and purification of mouse pro- and mature sPLA₂-X

Mouse sPLA₂-X cDNA was isolated from mouse spleen by polymerase chain reaction based on the human sPLA₂-X sequence [5], which was then inserted into pcDNA3.1(+) (Invitrogen) to construct the expression plasmid. Transfection into Chinese hamster ovary cells,

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Abbreviations: PLA₂, phospholipase A₂; sPLA₂, secretory phospholipase A₂; sPLA₂-IB, group IB sPLA₂; PLA₂R, phospholipase A₂ receptor; sPLA₂-IIA, group IIA sPLA₂; sPLA₂-X, group X sPLA₂; DSS, disuccinidyl suberate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline

selection of stably expressing clones, collection of the conditioned medium and purification with an antibody-coupled affinity column were performed as described for human sPLA₂-X [19], based on the cross-reactivity of anti-human sPLA₂-X antibody with mouse enzyme. Analysis of the final reverse-phase high performance liquid chromatography preparations by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) revealed the existence of two species of sPLA₂-X, and NH₂-terminal sequence analysis with an Applied Biosystems Procise Sequencer revealed them to be a mature form of sPLA₂-X (13 kDa) and its pro-form (14 kDa) having a propeptide sequence at the NH₂ terminus.

2.3. Binding experiments

Iodination of mouse sPLA₂-X and porcine sPLA₂-IB was performed by the chloramine T method as described previously [23] and the specific radioactivity of ¹²⁵I-sPLA₂-X and ¹²⁵I-sPLA₂-IB were 1200 and 1100 cpm/fmol, respectively. MC3T3-E1 cells (ATCC) were cultured in 24-well plates in 10% FCS/DMEM. At confluence, the cells were washed three times with phosphate-buffered saline (PBS) and incubated with various concentrations of mouse ¹²⁵I-sPLA₂-X in 0.4 ml of the binding buffer (Hanks' balanced salt solution, pH 7.4, containing 0.1% BSA) for 2 h at 4°C. After the incubation, cells were washed with PBS three times, and the cell-bound radioactivity was measured. Specific binding was determined as the differences between the presence and absence of 100 nM unlabelled mouse sPLA₂-X. Displacement experiments were performed by incubating with 1.5 nM mouse ¹²⁵I-sPLA₂-X in the presence of various concentrations of sPLA₂ proteins under the same assay conditions as described above. The IC₅₀ value was evaluated from the inhibition curves as the concentration that inhibits half of the specific binding.

In separate experiments, the binding potency of sPLA₂-X to mouse recombinant PLA₂R was examined as follows. The cDNA encoding mouse PLA₂R was transiently introduced into COS-7 cells with FuGene 6 reagent (Roche Diagnostics), and the binding activity of ¹²⁵I-sPLA₂-X was evaluated as described above. The inhibitory effect of indoxam on sPLA₂-X binding to PLA₂R-expressing COS-7 cells was examined by incubating with 2 nM ¹²⁵I-sPLA₂-X in the presence of various concentrations of indoxam.

The generation of PLA₂R-deficient mice was described in our previous paper [15] and they were backcrossed to C57BL/6J mice more than eleven times. C57BL/6J mice matched for gender and age were used as wild-type mice. Preparation of the crude lung membranes and their binding assay with ¹²⁵I-sPLA₂-X (2 nM) was performed according to the same protocol as the sPLA₂-IB binding study described in the previous report [15]. Specific binding was determined as the differences between the presence and absence of unlabeled 100 nM mouse PLA₂-X.

2.4. Affinity cross-linking experiment

Confluent MC3T3-E1 cells grown in 10-cm-diameter dishes were incubated in 6 ml of binding medium containing 2 nM ¹²⁵I-sPLA₂-X with or without 100 nM unlabelled mouse sPLA₂-X for 2 h at 4°C. In separate experiments, the cells were incubated with 2 nM ¹²⁵I-sPLA₂-IB with or without 500 nM unlabelled porcine sPLA₂-IB. After washing three times with Hanks' medium, pH 7.6, cells were incubated in 8 ml of Hanks' medium containing 0.25 mM DSS for 30 min at room temperature. The reaction was stopped by the addition of Tris/HCl (final 50 mM, pH 7.4). The cells were harvested and collected by centrifugation. They were lysed by adding 50 µl of 1% Triton X-100 containing a mixture of protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 mM EDTA and 20 µg/ml leupeptin). After centrifugation at 10 000 × g for 15 min, the resultant supernatant was separated by SDS–PAGE with a 4–20% gradient SDS–polyacrylamide gel (Daiichi Pure Chemical, Tokyo). After electrophoresis, the gel was dried and exposed to the imaging plate and read with a Fujix BAS2000 bio-image analyzer.

3. Results

3.1. ¹²⁵I-sPLA₂-X binding to MC3T3-E1 cells

We prepared a highly active radioligand of mouse sPLA₂-X and examined its binding characteristics in MC3T3-E1 cells that possess high levels of native PLA₂R on the cell surface [21]. As shown in Fig. 1A, a saturable and specific binding of

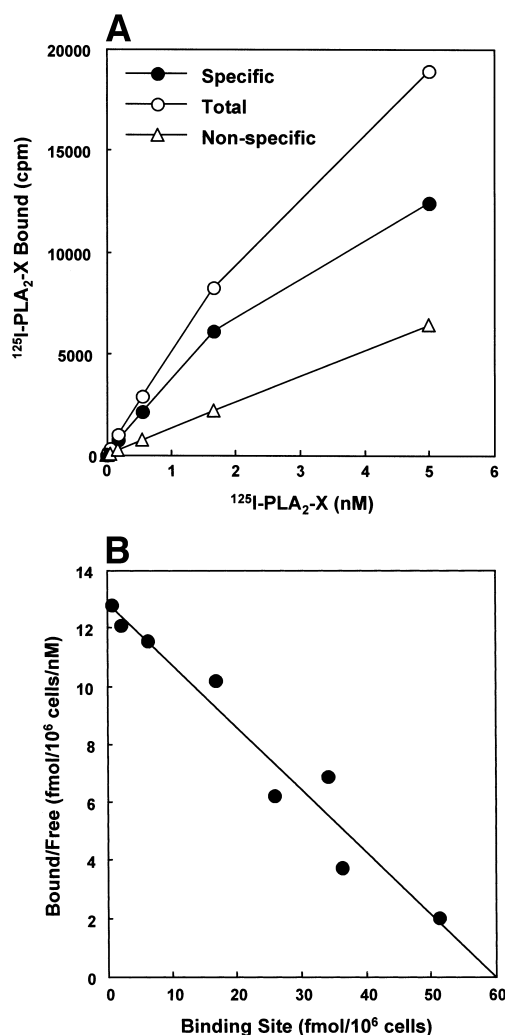


Fig. 1. Equilibrium binding of ¹²⁵I-sPLA₂-X to MC3T3-E1 cells. A: Saturation isotherm of ¹²⁵I-sPLA₂-X binding to MC3T3-E1 cells. Specific binding was calculated by subtracting the non-specific binding from the total binding. B: Scatchard plot of the specific ¹²⁵I-sPLA₂-X binding. Each point represents the mean of duplicate measurements. The data are representative of three experiments.

mouse ¹²⁵I-sPLA₂-X was detected. Scatchard analysis of these data (Fig. 1B) demonstrated the existence of a single class of binding sites with an equilibrium dissociation constant (K_d) of 4.6 nM and a maximum binding capacity (B_{max}) of 59.7 fmol/10⁶ cells (about 36 000 binding sites/cell), which coincided with the B_{max} value reported in the porcine sPLA₂-IB binding to the PLA₂R in MC3T3-E1 cells [21]. Next, the specificity of the sPLA₂-X binding site was examined by evaluating the relative inhibitory potencies of various sPLA₂ proteins. As for the mouse sPLA₂-X species, pro-sPLA₂-X blocked the binding with about 10-fold weaker potency than the mature form of sPLA₂-X (Fig. 2). Porcine and human sPLA₂-IB also strongly blocked the sPLA₂-X binding with IC₅₀ values of 1.14 and 1.71 nM, respectively. Inversely, mouse sPLA₂-X blocked the porcine sPLA₂-IB binding to the PLA₂R in MC3T3-E1 cells (data not shown). In both cases, human sPLA₂-IIA showed a much weaker suppressive effect.

In order to characterize a high-affinity binding site of ¹²⁵I-sPLA₂-X, we performed the affinity labeling experiments using a homobifunctional cross-link reagent, DSS. As shown in

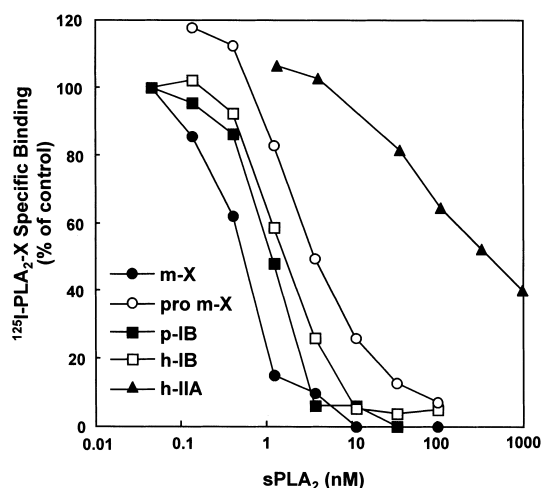


Fig. 2. Displacement of ^{125}I -sPLA₂-X binding by sPLA₂ proteins in MC3T3-E1 cells. MC3T3-E1 cells were incubated with 1.5 nM ^{125}I -sPLA₂-X in the presence of various concentrations of mouse mature sPLA₂-X (m-X), pro-sPLA₂-X (pro m-X), porcine sPLA₂-IB (p-IB), human sPLA₂-IB (h-IB), and human sPLA₂-IIA (h-IIA) and the specific binding activity was measured. The results are expressed as the percentage of ^{125}I -sPLA₂-X specific binding in the absence of these sPLA₂s. Each point represents the mean of duplicate measurements. The data are representative of three experiments.

Fig. 3, the ^{125}I -sPLA₂-X binding complex was specifically detected at an apparent molecular mass of 200 kDa only in the treatment with DSS (lanes 4 and 6), which was completely abolished in the presence of an excess amount of unlabeled sPLA₂-X protein (lane 3) as well as porcine sPLA₂-IB (data not shown). In a similar cross-linking experiment, the cross-

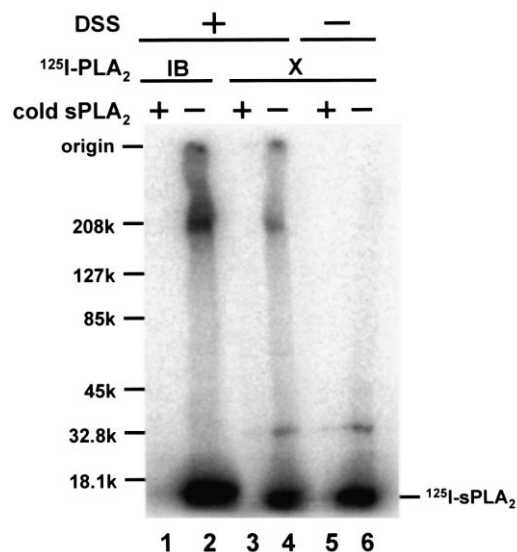


Fig. 3. Characterization of the sPLA₂-X binding site by affinity cross-linking. Binding assays were carried out at 4°C for 2 h by incubating MC3T3-E1 cells with 2 nM ^{125}I -porcine sPLA₂-IB in the presence (lane 1) or absence (lane 2) of 500 nM of unlabeled porcine sPLA₂-IB, or with ^{125}I -mouse sPLA₂-X in the presence (lanes 3 and 5) or absence (lanes 4 and 6) of 100 nM of unlabeled mouse sPLA₂-X. At the end of incubation, the cells were washed and treated (lanes 1–4) or untreated (lanes 5 and 6) with 0.25 mM DSS for 30 min and then analyzed as described in Section 2 under the reduced conditions. Molecular weight markers are indicated on the left.

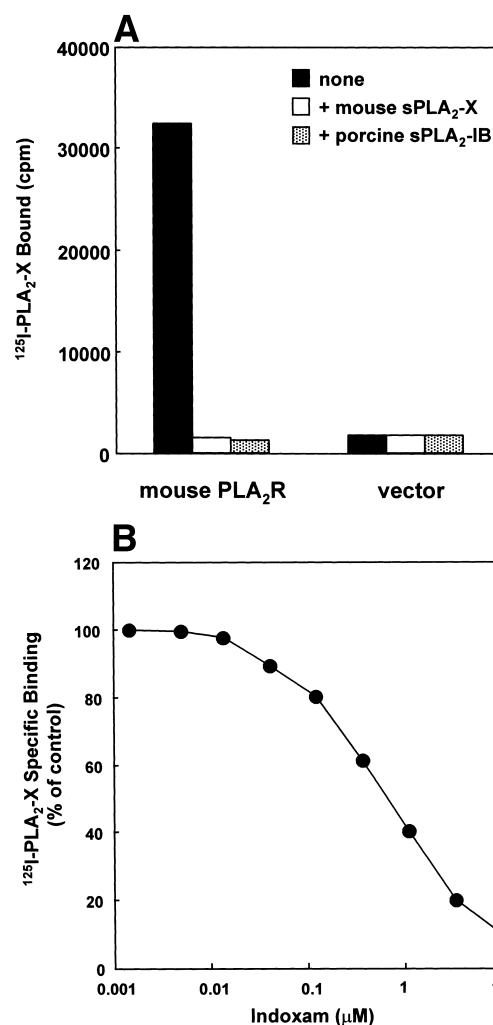


Fig. 4. ^{125}I -mouse sPLA₂-X binding to mouse PLA₂R expressed in COS-7 cells. A: COS-7 cells transfected with PLA₂R cDNA or vector alone were incubated with 2 nM ^{125}I -mouse sPLA₂-X in the absence (none) or presence of 300 nM of unlabeled mouse sPLA₂-X or porcine sPLA₂-IB for 2 h at 4°C, and the binding activity was determined. B: PLA₂R-expressing COS-7 cells were incubated with 1.5 nM ^{125}I -sPLA₂-X in the presence of various concentrations of indoxam, and the specific binding activity was measured. The results are expressed as the percentage of ^{125}I -sPLA₂-X specific binding in the absence of indoxam. Each point represents the mean of duplicate measurements. The data are representative of three experiments.

linked complex composed of sPLA₂-IB (14 kDa) and PLA₂R (180 kDa) showed the same molecular weight in MC3T3-E1 cells (lanes 1 and 2). Thus, the binding specificity and biochemical features of the sPLA₂-X binding site strongly suggest its relation to PLA₂R.

3.2. ^{125}I -sPLA₂-X binding to recombinant PLA₂R

In order to verify the identity of the sPLA₂-X binding protein, we examined the binding potency of mouse sPLA₂-X to the recombinant mouse PLA₂R transiently expressed in COS-7 cells. As shown in Fig. 4A, a high amount of specific sPLA₂-X binding was detected in the PLA₂R-expressing COS-7 cells in contrast to its absence in the mock-transfected cells. The sPLA₂-X binding was also blocked by porcine sPLA₂-IB and the specific sPLA₂-IB binding detected in the PLA₂R-express-

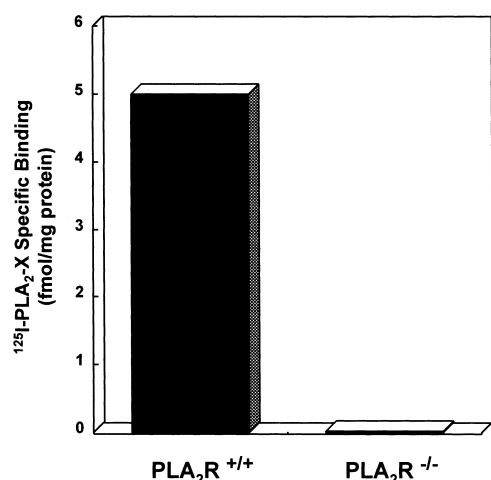


Fig. 5. sPLA₂-X binding activity in mouse lung membrane fractions. Specific binding activity of ¹²⁵I-sPLA₂-X in crude lung membranes prepared from wild-type (+/+) or PLA₂R-deficient mice (-/-) was examined as described in Section 2. Data represent the mean value for five mice.

ing cells was completely blocked by mouse sPLA₂-X (data not shown). As shown in Fig. 4B, a sPLA₂ specific inhibitor indoxam [22] suppressed the sPLA₂-X binding to mouse PLA₂R in a dose-dependent manner with an IC₅₀ value of 670 nM. Thus, these findings demonstrate that mouse sPLA₂-X is one of the natural ligands of mouse PLA₂R.

3.3. ¹²⁵I-sPLA₂-X binding to mouse lung membranes

To check for the existence of other binding components of sPLA₂-X in mice, we examined the binding activity of ¹²⁵I-sPLA₂-X in the crude lung membrane fractions and found a considerable amount of its specific binding (Fig. 5). The binding was saturable, specific for sPLA₂-X and displaceable by sPLA₂-IB (data not shown). In PLA₂R-deficient mice that we have recently generated [15], there was no significant binding in the prepared lung membranes, demonstrating that PLA₂R represents the specific binding site for sPLA₂-X in mice.

4. Discussion

Since the discovery of PLA₂R, sPLA₂s have been thought to act as signaling molecules for various biological responses in addition to their enzymatic activities [12,13]. In the present study, sPLA₂-X was identified as a natural ligand of mouse PLA₂R in native and recombinant receptors. Since there is a strict species specificity in the ligand–receptor relationship, the binding affinity of sPLA₂-X for mouse PLA₂R cannot be directly compared with those of sPLA₂-IB and IIA due to the lack of these mouse enzymes. In previous binding studies with snake venom *Oxyuranus scutellatus* toxin 1 as a radioligand, mouse sPLA₂-IB showed a similar binding affinity for mouse PLA₂R with human sPLA₂-IB, whereas mouse sPLA₂-IIA had about 6- to 10-fold lower affinity [17]. In the present work, mouse sPLA₂-X showed a three-fold higher affinity compared to human sPLA₂-IB in MC3T3E1 cells (Fig. 2), suggesting that sPLA₂-X is one of the high-affinity ligands for mouse PLA₂R. Pro-sPLA₂-X had a weaker binding potency than its mature form, indicating that proteolytic removal of the propeptide is required for the high-affinity receptor binding, as in the case of sPLA₂-IB [24]. Because some inbred

mouse strains have a natural mutation in the sPLA₂-IIA gene [25,26], the contribution of sPLA₂-IIA as a PLA₂R ligand could be much lower in mice.

We have previously shown a pivotal role of PLA₂R in the promotion of pro-inflammatory cytokine synthesis during murine endotoxic shock, based on the findings of reduced cytokine production in PLA₂R-deficient mice as well as in indoxam-administered mice [15,16]. In the present study, the sPLA₂-X specific binding was completely abrogated by the deficiency in PLA₂R (Fig. 5) and by the addition of indoxam (Fig. 4B). Furthermore, our recent immunohistochemical analysis with sPLA₂-IIA-deficient mice revealed co-localization of sPLA₂-X and PLA₂R in alveolar type II pneumocytes, which are known to play a role in the production of various cytokines and lipid mediators (Ikeda et al., unpublished observation). These findings suggest a potential involvement of sPLA₂-X in the receptor-mediated regulation of cytokine synthesis under inflammatory conditions. Since sPLA₂-X induces a potent release of fatty acids leading to eicosanoid formation in several inflammatory cells [19,20], the potential role of PLA₂R in this process should be examined in future work. In particular, PLA₂R might play a role in the internalization and degradation of sPLA₂-X to suppress its powerful enzymatic activity, as reported for snake venom sPLA₂s [27]. Finally, further elucidation of PLA₂R-mediated biological responses should enable us to assign more precise biological roles to the growing family of mammalian sPLA₂s.

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