Differential effects of annexins I, II, III, and V on cytosolic phospholipase A2 activity: specific interaction model

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Abstract Annexins (ANXs) are a family of proteins with calcium-dependent phospholipid binding properties. Although inhibition of phospholipase A2 (PLA2) by ANX-I has been reported, the mechanism is still controversial. Previously we proposed a 'specific interaction' model for the mechanism of cytosolic PLA2 (cPLA2) inhibition by ANX-I [Kim et al., FEBS Lett. 343 (1994) 251–255]. Here we have studied the cPLA2 inhibition mechanism using ANX-I, N-terminally deleted ANX-I (ΔANX-I), ANX-II, ANX-II$_2$P11$_2$, ANX-III, and ANX-V. Under the conditions for the specific interaction model, ANX-I, ΔANX-I, and ANX-II$_2$P11$_2$ inhibited cPLA2, whereas inhibition by ANX-II and ANX-III was negligible. Inhibition by ANX-V was much smaller than that by ANX-I. The protein–protein interactions between cPLA2 and ANX-I, ΔANX-I, and ANX-II$_2$P11$_2$ were verified by immunoprecipitation. We can therefore conclude that inhibition of cPLA2 by specific interaction is not a general function of all ANXs, and is rather a specific function of ANX-I. The results are consistent with the specific interaction model.

Key words: Annexin; cPLA2 inhibition; Specific interaction; Substrate depletion

1. Introduction

Annexins (ANXs) (also called lipocortins) are a family of intracellular proteins that bind phospholipids and membranes in a Ca^{2+}-dependent manner [1]. Several members of the ANX family have been implicated in the regulation of cellular processes including membrane fusion, inflammation, proliferation, and differentiation [2–5]. However, despite many functional possibilities, well-characterized biological functions or relationships between structure and function have not been determined for any of the ANXs.

The structure of ANXs consists of an N-terminal tail and a C-terminal core domain. The core domain consists of four or eight repeats of a highly homologous 70 amino acid sequence, which is known to be a common primary structure for the binding of Ca^{2+}, phospholipids, and ATP [1,6,7]. The N-terminal domains of the ANXs have great diversity in both length and sequence. Since these domains contain the sites for phosphorylation and selective proteolytic cleavage, they are considered to be the regulatory region of each protein and seem to be important for the specific cellular functions of each ANX [8].

ANX-I has been shown to inhibit phospholipase A2 (PLA2); however, the mechanism of the inhibition is still controversial [9]. PLA2 represents a growing family of enzymes with a common function of catalyzing the release of fatty acid from the *sn*-2 position of membrane phospholipids, thereby leading to production of lipid metabolites including arachidonic acid, leukotrienes, and lysophospholipids [10,11]. There are at least three types of PLA2s, secretary PLA2 (sPLA2), cytosolic PLA2 (cPLA2), and Ca^{2+}-independent PLA2 (iPLA2) [10]. Detailed enzymatic studies have shown that ANX-I inhibits cPLA2, a 100 kDa protein of submicromolar Ca^{2+} requirement, by 'specific interaction' [12]. Other studies using sPLA2, a 14 kDa protein of millimolar Ca^{2+} requirement, have demonstrated that the inhibition is due to the ability of ANX-I to bind to the substrate, which results in substrate depletion and apparent inhibition [13,14]. Increasing reports suggest that cPLA2 is a key enzyme responsible for signal transduction in inflammation, cytotoxicity and mitogenesis [10,11]. ANX-I suppresses cPLA2 activity not only in the in vitro system but also in cultured cells [15,16]. Thus ANX-I may function as an endogenous negative regulator of cPLA2.

Since ANXs share most of their functions, probably through the homology in the core domain, it is important to know whether the inhibition of cPLA2 is a general function of all ANXs or a specific function of ANX-I. If the substrate depletion mechanism is correct, all ANXs should display similar inhibition patterns towards different types of PLA2s. Also it is important to know whether the inhibitory activity is in the N-terminal or in the core domain of ANX-I. Herein we have studied the inhibition of cPLA2 using various forms of ANXs, including ANX-I and N-terminally deleted ANX-I (ΔANX-I), in which amino acids 1–32 were deleted. Effects of ANX-II, ANX-II$_2$P11$_2$ tetramer, ANX-III, and ANX-V on the activity of cPLA2 were also investigated.

2. Materials and methods

2.1. Chemicals and reagents

1-Stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine (2-AA-PC) (56.0 mCi/mmol) and 1-acyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphoethanolamine (2-AA-PE) (57.0 mCi/mmol) were purchased from Amersham (Buckinghamshire, UK) and used as substrates. Unlabeled 2-AA-PC and 2-AA-PE were purchased from Sigma (St.

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Louis, MO, USA). Scintillation fluid (Aquasol-2) was from NEN Probe (Eugene, OR, USA). All other reagents were of analytical reagent grade or better.

Rabbit antiserum was raised against human ANX-I produced in *Escherichia coli*. Mouse anti-ANX-I antibody was purchased from Transduction Laboratories (Lexington, KY, USA). Mouse anti-ANX-II monoclonal antibody was purchased from Zymed (USA). Rabbit anti-ANX-V antibody and mouse anti-cPLA2 antibody were from Santa Cruz Biotechnology Inc. (USA). Anti-rabbit HRP (horseradish peroxidase) and anti-mouse HRP were purchased from Caltag Laboratories (USA).

2.2. Preparation of ANXs

Recombinant ANX-I and ΔANX-I (in which amino acids 1–32 were deleted) were produced in *E. coli* and purified as described previously [17]. ANX-II and ANX-II_2P11_2 were the kind gifts of Dr. Waisman of University of Calgary, Canada [18]. ANX-III was produced in *E. coli* as a GST fusion protein using the expression plasmid which was kindly provided by Dr. Russo-Marie of INSERM, Paris, France [19]. ANX-III was purified after cutting off the GST tail. ANX-V was purchased from Sigma (St. Louis, MO, USA).

2.3. Preparation of cPLA2 and sPLA2

Bee venom sPLA2 was purchased from Sigma (St. Louis, MO, USA). A 100 kDa cPLA2 was partially purified from porcine spleen essentially according to the methods described previously [20]. Since purification of cPLA2 from porcine spleen is laborious and time consuming, we cloned the cPLA2 cDNA in a baculovirus vector pFast Bac HT (Gibco-BRL, USA) and produced cPLA2 in sf9 cells (details will be published elsewhere). Cells from 50 ml culture were lysed in 1 ml of buffer (20 mM Tris/HCl pH 7.4, 10% glycerol, 1% NP-40, 0.1% BSA, 0.1 mM PMSF, Complete™EDTA free tablet, 0.1 M Ca^{2+}) and used as the source of sf9-cPLA2.

2.4. Standard assay for PLA2 activity

PLA2 activity was assayed using sonicated liposomes prepared as described previously [12,19]. A stock solution of the substrate was prepared as follows: 10–20 nmol of the substrate was dried under nitrogen, then suspended in 0.5–1.0 ml of distilled water by sonication (10 s×3) in a bath-type sonicator (Ultrasonik 300, NEY). The standard reaction mixture (200 μl) for PLA2 assay contained 0.33 nmol (1.65 μM) of radioactive substrate (approximately 39 000 cpm), 200 μg of fatty acid-free bovine serum albumin (BSA) and 10 ng of PLA2 in 75 mM Tris/HCl (pH 7.5). The reaction was started by adding the enzyme to the reaction mixture and was incubated at 37°C for 1 h. Non-esterified fatty acid was extracted as follows. First, 0.55 ml of water was added and the sample was vortex-mixed and centrifuged at 5600×*g* for 5 min. Then 0.75 ml of the upper phase was transferred to a new tube, to which 100 mg of silica gel and 0.75 ml of *n*-heptane were added. The samples were vortex-mixed and centrifuged again for 5 min. The supernatant was dried with a Speed Vac freeze drier and the lipid was resuspended in chloroform/methanol (1:1, v/v) which contained unlabeled arachidonic acid (1 μg/μl) in methanol. Phospholipid and neutral lipid were separated by migration on layers of silica gel 60 F254 (Merck, Darmstadt, Germany) plate in petroleum ether/ethyl ether/acetic acid (80:20:1, v/v/v). After drying, the plates were subjected to iodine vapor and lipids were identified by their co-migration with unlabeled arachidonic acids. Products were quantified by scraping their corresponding spots into counting vials containing 2 ml of Aquasol-2. Radioactivity was determined using a Packard Tri-Carb scintillation spectrophotometer. For experiments in which the substrate concentration dependence was determined, unlabeled phospholipid was added to labeled phospholipid to give a designated final concentration. For accurate control of Ca^{2+} concentration, a $CaCl_2$/EGTA buffering system was used [21]. In all analyses, samples were tested in triplicates and were adjusted for non-specific release by subtracting a control value in which preparation of the enzyme was omitted. For inhibition assays, 20–100 nM of ANXs were added to the reaction mixture.

2.5. Immunoprecipitation of cPLA2•ANX complexes with ANX antibodies

The lysate of the sf9 cells over-expressing cPLA2 was used in this experiment. 50 μl of the sf9-cPLA2 lysate was incubated at 4°C for 2 h with or without 2 μg each of ANX and 200 μg of BSA in 200 μl of buffer (75 mM Tris/HCl pH 7.4, 0.1 μM Ca^{2+}). Anti-ANX antibody corresponding to each ANX was added and the samples were incubated further for 1 h, then immune complexes were precipitated with Protein-A agarose (Santa Cruz Biotechnology Inc, CA, USA). The cPLA2 activity in the precipitate or in the supernatant was determined in the standard buffer containing 5 mM $CaCl_2$.

The cPLA2 in the pellet was also analyzed by Western blot. The pellet was boiled in the gel loading buffer and proteins were separated by 10% SDS–PAGE, and then transferred to a nitrocellulose membrane (Schleicher and Schuel, Keene, NH, USA). The membrane was probed with monoclonal antibody to cPLA2 and visualized using the ECL system (Amersham, Buckinghamshire, UK).

2.6. Expression of data

All determinations were carried out in triplicate, and experiments were repeated at least three times. All data are shown as mean ± S.E.M. Effects of ANXs were presented by the percentage of cPLA2 activity compared to the control value.

3. Results

3.1. Effect of calcium on the activity of PLA2s from different sources

In the previous experiments of cPLA2 inhibition by ANX-I, cPLA2 from porcine spleen was used [12]. To characterize the recombinant cPLA2 from sf9 cells, the enzymatic activity was measured under various Ca^{2+} ion concentrations. The sf9-cPLA2 was active under Ca^{2+} concentrations as low as 0.1 μM and showed nearly identical activity with porcine spleen cPLA2 under all Ca^{2+} concentrations (Fig. 1). On the other hand at least 0.1 mM Ca^{2+} was necessary for the sPLA2 activity. Therefore sf9-cPLA2 was used in all other experiments.

3.2. Calcium dependence of the cPLA2 inhibition by ANXs

In order to investigate the mechanism of cPLA2 inhibition by ANXs in detail, assays were carried out under various concentrations of ANXs and Ca^{2+} ion. ANX-I, ΔANX-I, ANX-II, ANX-II_2P11_2, ANX-III, and ANX-V were used. Inhibition of cPLA2 by ANXs was determined using 2-AA-PC as a substrate at 0.1, 1, 10, 100 μM, and 1 mM Ca^{2+} ion concentrations. The substrate concentration was 1.65 μM,

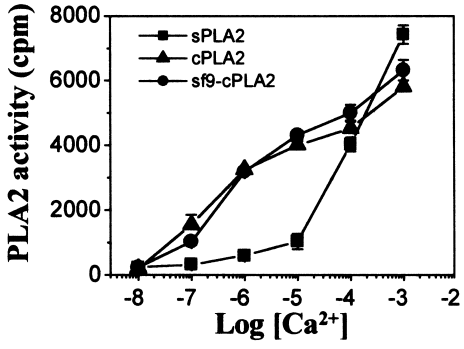

Fig. 1. Calcium dependence of the PLA2 activity. PLA2s from different sources were used. PLA2 activity was determined using 1-stearoyl-2-[1-^{14}C]-*sn*-glycero-phosphocholine (2-AA-PC, approximately 39 000 cpm) as a substrate in 75 mM Tris–HCl (pH 7.5) in the presence of various concentrations of free Ca^{2+} as described in Section 2. ●, Recombinant cPLA2 produced in sf9 cells harboring the recombinant baculovirus (sf9-cPLA2). ▲, Partially purified porcine spleen cPLA2. ■, Bee venom secretory PLA2 (sPLA2) purchased from Sigma. Data represent mean ± S.E.M. of triplicate experiments and the figure is a representative of three independent experiments.

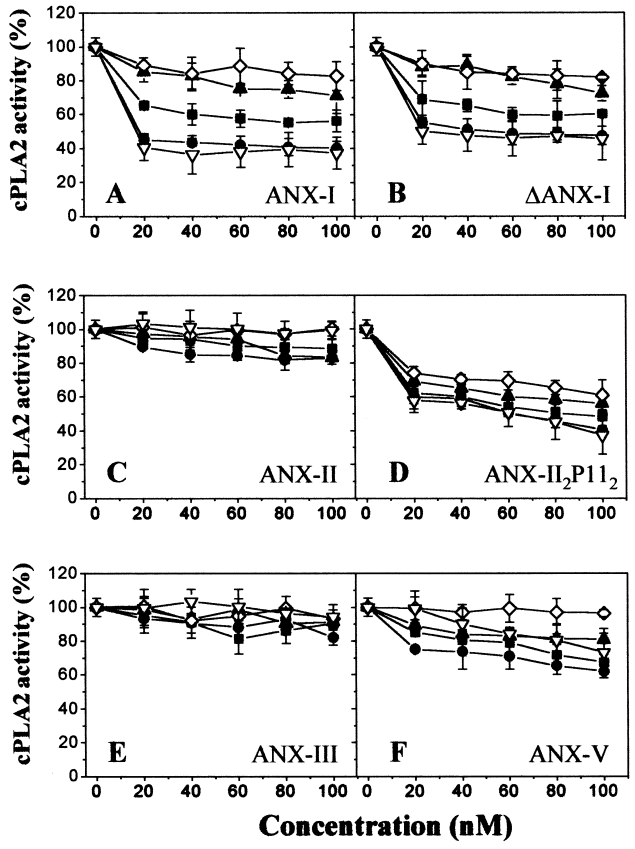

Fig. 2. Inhibition of cPLA2 by ANXs. Inhibition of cPLA2 activity by various ANXs was determined using 2-AA-PC as a substrate under various Ca^{2+} concentrations. The sf9-cPLA2 was used in this experiment. Concentrations of cPLA2 and the substrate were approximately 0.5 nM (10 ng/200 μl) and 1.65 μM, respectively. Concentrations of ANXs were varied from 20 nM to 100 nM. cPLA2 activity with or without ANXs was determined and the percentage of the activity in the presence of ANXs was calculated. ▽, 0.1 μM Ca^{2+}; ●, 1 μM Ca^{2+}; ■, 10 μM Ca^{2+}; ▲, 100 μM Ca^{2+}; ◇, 1 mM Ca^{2+}. Data represent mean ± S.E.M. of triplicate experiments and the figure is a representative of three independent experiments.

which was a large excess over the enzyme (0.5 nM) and inhibitor (20–100 nM) concentrations. Fig. 2 shows the percentage of the remaining 2-AA-PC hydrolyzing activity in presence of ANXs. In presence of ANX-I the PLA2 activity decreased, suggesting inhibition of the enzymatic activity (Fig. 2A). The inhibition reached a plateau at 20 nM ANX-I, the lowest concentration used in this experiment. Percent remaining activity was smallest, and therefore, percent inhibition was largest at 0.1 μM or 1 μM Ca^{2+} concentration. It decreased with increasing Ca^{2+} concentration, and became negligible at 1 mM Ca^{2+} concentration. These results are consistent with the following interpretation. Binding of ANX-I to the substrate is Ca^{2+} dependent. At Ca^{2+} concentration of 0.1 μM or 1 μM, substrate binding by ANX-I is minimal, and therefore, ANX-I is available for PLA2 inhibition. Substrate binding by ANX-I increases with increasing calcium concentration, which results in the reduction of free ANX-I for cPLA2 inhibition. At 1 mM Ca^{2+} concentration, ANX-I is depleted by binding to the substrate and unavailable for cPLA2 inhibition.

To determine the importance of the N-terminal sequence for the inhibitory activity, ΔANX-I was used as an inhibitor. As shown in Fig. 2B, the pattern of the inhibition was similar to that of ANX-I. Even though the inhibition by ΔANX-I was slightly less than that of ANX-I, it was within the day to day variation range. Therefore the N-terminal sequence is not likely to be very important for the inhibition. Inhibition by ANX-II was very small compared to that of ANX-I. Interestingly ANX-II$_2$P11$_2$ showed a marked inhibition, whereas ANX-III had virtually no effect on cPLA2 activity. Inhibitory effect of ANX-V on the cPLA2 activity was much smaller compared to that of ANX-I. At 1 μM calcium where the inhibition was the maximum, the inhibition by ANX-I reached to a near maximum value (55%) at 20 nM (Fig. 2A), whereas the inhibition by ANX-V reached to 40% at 100 nM (Fig. 2F). To give 40% inhibition, 10 nM of ANX-I was needed (data not shown).

Fig. 3 shows a replotting of data in Fig. 2. The remaining cPLA2 activity in the presence of ANXs was plotted against the logarithmic value of the Ca^{2+} concentration. In all cases the plot was essentially linear except for the values at 0.1 μM calcium. In case of ANX-I, ΔANX-I, and ANX-II$_2$P11$_2$, inhibition at 0.1 μM calcium and at 1 μM calcium was very similar. Inhibition by ANX-V at 1 μM was larger than that at 0.1 μM calcium; however, experiments at 0.1 μM calcium were prone to more error due to the lower value of the cPLA2 assay and the difference was insignificant (Fig. 1). The slope for ANX-I is the largest and that for ANX-II$_2$P11$_2$ is the smallest. Therefore the inhibition by ANX-I mostly depends on the Ca^{2+} concentration and ANX-II$_2$P11$_2$ is the least affected by this.

3.3. Substrate concentration dependence of the cPLA2 inhibition by ANXs

To rule out the possibility that the cPLA2 inhibition by ANXs is due to substrate depletion, the substrate concentration was varied from 1.65 μM to 33 μM while holding other

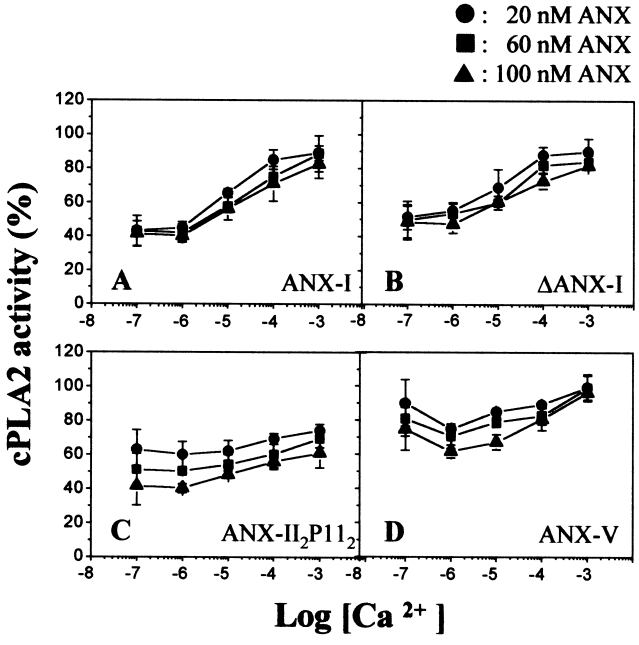

Fig. 3. Ca^{2+} concentration dependence of the cPLA2 inhibition by ANXs. Data in Fig. 2 are replotted against Ca^{2+} concentration. ●, 20 nM; ■, 60 nM; ▲, 100 nM of ANXs. Other details were as in Fig. 2.

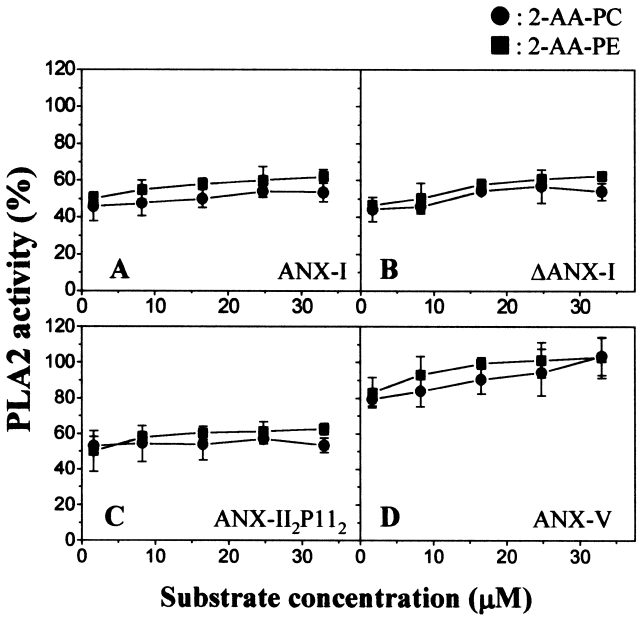

Fig. 4. Inhibition of cPLA2 by ANXs under higher substrate concentrations. The concentrations of Ca^{2+} and ANXs were 1 μM and 20 nM, respectively. The substrate concentrations were varied from 1.65 μM to 33.0 μM. ●, 2-AA-PC; ■, 2-AA-PE. Other details were as in Fig. 2.

components constant. Both 2-AA-PC and 2-AA-PE were used as substrates. As shown in Fig. 4, inhibition of cPLA2 by ANX-I, ΔANX-I, and ANX-II_2P11_2 was essentially independent of substrate concentration. On the other hand ANX-V showed some inhibitory activity only at the lowest substrate concentration (1.65 μM) and virtually had no activity at higher substrate concentrations. If the mechanism was substrate depletion, increasing the substrate concentration would abolish the inhibition [13]. Therefore the results in Fig. 4 demonstrate that the mechanism of cPLA2 inhibition by ANX-I is specific interaction and not substrate depletion. It is likely that the inhibitory effect of ANX-V is mainly due to substrate depletion.

3.4. Immunoprecipitation of cPLA2•ANX complex by anti-ANX antibody

To demonstrate that cPLA2 inhibition by ANX-I, ΔANX-I, and ANX-II_2P11_2 is due to direct interaction between cPLA2 and ANXs, anti-ANX antibodies were used to investigate the stability and co-precipitability of cPLA2 and ANX complexes. Since inhibition by specific interaction was observed under intracellular calcium concentration 0.1 μM (Figs. 2, 3), the immunoprecipitation was performed under 0.1 μM calcium concentration. The binding mixture contained cPLA2, each ANX, and the specific antibody to each ANX; for example in the mixture for ANX-I, anti-ANX-I antibody was included. The results are shown in Table 1. In case of ANX-I or ΔANX-I, the cPLA2 activity was mostly in the pellet, suggesting that the cPLA2•ANX-I or cPLA2•ΔANX-I complex was precipitated with the anti-ANX-I antibody. The cPLA2•ANX-II_2P11_2 complex was also precipitated with anti-ANX-II antibody. On the other hand when precipitated with anti-ANX-V antibody cPLA2 activity was mostly in the supernatant indicating the lack of cPLA2•ANX-V complexes in the pellet. To further verify the precipitation of cPLA2•ANX complexes by ANX antibodies, Western blot analysis was performed. The pellet obtained as in Table 1 was dissolved in the gel loading buffer, separated on a 10% SDS–PAGE gel, and analyzed by Western blot using anti-cPLA2 antibody. As shown in Fig. 5, cPLA2 was present in the precipitates of ANX-I, ΔANX-I, and ANX-II_2P11_2. In case of ANX-V, the interaction with cPLA2 was very weak. The result in Fig. 5 is consistent with the data in Table 1 as well as the cPLA2 inhibition by ANXs at 0.1 μM calcium concentration as shown in Figs. 2 and 3.

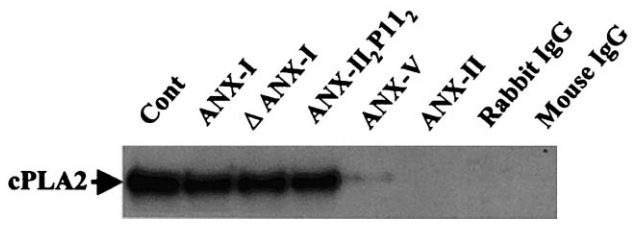

Fig. 5. Immunoprecipitation of the cPLA2•ANX complex with ANX antibodies. The sf9-cPLA2 cells were grown and the cell lysate was used in this experiment as described in Section 2. The incubation mixture contained 50 μl of sf9-cPLA2 lysate, 200 μg of BSA, 2 μg of each ANX, and specific antibody to each ANX in 200 μl of buffer (75 mM Tris–HCl pH 7.4, 0.1 μM Ca^{2+}). The cPLA2•ANX complexes were immunoprecipitated with Protein-A agarose and analyzed by Western blot as described in Section 2. The blot was probed with anti-cPLA2 antibody, and visualized using HRP-conjugated anti-rabbit antibody and the ECL system (Amersham, UK). Controls: Cont, cPLA2 only; Rabbit IgG and Mouse IgG, preimmune.

4. Discussion

The mechanism by which ANX-I inhibits cPLA2 activity is still a controversial issue. In this contribution we tried to address this issue by determining the effects of ANX-I as well as other ANXs on cPLA2 activity. We have demon-

Table 1
cPLA2 activity in the supernatant or pellet after immunoprecipitation with anti-ANX antibodies

Immunoprecipitation mixture	cPLA2 activity (%)	
	Pellet	Supernatant
cPLA2+BSA+anti-PLA2 antibody	98.2 ± 2.5	1.2 ± 0.5
cPLA2+BSA+anti-ANX-I antibody	1.2 ± 0.1	99.1 ± 4.1
cPLA2+ANX-I+anti-ANX-I antibody	75.1 ± 3.9	20.4 ± 3.1
cPLA2+ΔANX-I+anti-ANX-I antibody	70.6 ± 8.3	23.5 ± 2.3
cPLA2+ANX-II_2P11_2+anti-ANX-II antibody	51.5 ± 3.7	26.1 ± 7.9
cPLA2+ANX-V+anti-ANX-V antibody	11.7 ± 3.5	85.9 ± 7.1

The immunoprecipitation mixture contained sf9-cPLA2 cell lysate, each ANX, and specific antibody to each ANX. cPLA2 activity in the supernatant or pellet after immunoprecipitation was determined. Data represent mean ± S.E.M. of triplicate experiments.

strated that (1) cPLA2 is specifically inhibited by ANX-I under the conditions of specific interaction, (2) deletion of the N-terminal tail has little effect on the inhibitory activity, (3) while ANX-II does not inhibit cPLA2, ANX-II_2P11_2 does, (4) ANX-III does not inhibit cPLA2, and (5) ANX-V has much less inhibitory activity. In addition, immunoprecipitation studies have revealed that cPLA2 forms complexes with ANX-I, ΔANX-I, and ANX-II_2P11_2, but not with ANX-II and ANX-III, and much less efficiently with ANX-V, which are in good agreement with the enzymatic studies.

Inhibition of cPLA2 by various members of the ANX family has been reported. However, most of the data were from the experiments performed under the conditions where the substrate depletion mechanism dominates [22–24]. Even though there have been no detailed enzymatic studies on the mechanism of cPLA2 inhibition by ANXs other than ANX-I, most articles in the literature describe the mechanism as 'substrate depletion'. There is only one report, in fact by us, that concludes the inhibition mechanism is by 'specific interaction' [12]. If the mechanism is 'substrate depletion', all ANXs should display similar inhibition patterns because this mechanism is due to the ability of ANXs to bind to phospholipids. Fig. 2 clearly demonstrates that this is not the case. Therefore the differential effects of ANXs on the cPLA2 inhibition provide more supportive evidence for the 'specific interaction' model. The inhibitory effect of ANX-I is independent of the substrate concentration, whereas that of ANX-V was abolished with increasing substrate concentration (Fig. 4D). This result provides additional support that the inhibition mechanism of ANX-I, but not of ANX-V, is specific interaction.

Since the N-terminal region is specific to each ANX, we asked whether this region is important for the cPLA2 inhibition by ANX-I. To our surprise, deleting the N-terminal region had little effect on the cPLA2 inhibitory activity of ANX-I. This indicates that even though the core domain sequences are highly conserved, there are enough differences to differentiate the cPLA2 binding properties. This phenomenon is supported by the fact that anti-ANX-I antibody derived from human ANX-I specifically recognizes all ANX-I across the species from mold to human, while it does not recognize any other types of ANXs from any source including human cells (unpublished observation). In view of the specificity of the N-terminal for each ANX, the N-terminal of ANX-I would be related to other specific functions of ANX-I, such as mediation of anti-inflammatory function of glucocorticoids [25–27], and translocation from the cytosol to the nucleus by epidermal growth factor or stress signals [5,28–30].

As shown in Figs. 2, 5, and Table 1, ANX-II does not inhibit cPLA2, while ANX-II_2P11_2 does. This result is consistent with the previously reported observation by Wu and others [31] that P11 but not ANX-II binds and inhibits cPLA2. Two immediate questions are (1) whether ANX-I and P11 have any homology, and (2) whether they share the same binding site on cPLA2. Comparison of ANX-I and P11 sequences shows no significant homology. Results from the preliminary experiments suggest that the binding sites are different (unpublished observation). Another point to be noted is that the Ca^{2+} concentration dependence of the cPLA2 inhibition by ANX-II_2P11_2 is less than that by ANX-I or ΔANX-I (Fig. 3). This seems to be due to the fact that there is no Ca^{2+} binding site on P11, the effector subunit of cPLA2 inhibition [31]. This may also be the reason for the results that the cPLA2 activity in the supernatant and in the pellet does not add up to near 100%, representing the inhibition of cPLA2 under 5 mM Ca^{2+} concentration used in the assay (Table 1).

Whether the cPLA2 inhibition by ANX-I occurs in vivo is still an open question. Inhibition of cPLA2 under physiological calcium concentrations suggests that the inhibition may occur in cells and is related to the regulation of cPLA2 activity. However since the inhibition at 0.1 μM calcium (resting state) and at 1 μM calcium (activated state) is similar, it is unlikely that this is a major mechanism for cPLA2 regulation in cells. It may rather be related to other mechanisms of cPLA2 regulation such as phosphorylation. In conclusion, the mechanism of cPLA2 inhibition by ANX-I is consistent with the 'specific interaction' model and the patterns of cPLA2 inhibition by ANXs support this mechanism. The cPLA2 inhibition is a specific function of ANX-I and is not a general function of all ANXs. The N-terminal region may not be very important for cPLA2 inhibition. The results presented here are potentially important since ANX-I specifically inhibited cPLA2 near intracellular Ca^{2+} concentration. ANX-I may regulate several biological processes through regulation of cPLA2 activity.

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