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Binding and Inhibition Studies on Lipocortins Using Phosphatidylcholine Vesicles and Phospholipase A₂ from Snake Venom, Pancreas, and a Macrophage-like Cell Line*

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Studies are reported on the inhibition of phospholipase A₂ (PLA₂) from porcine pancreas, cobra (*Naja naja*) venom, and the P388D₁ macrophage-like cell line by human recombinant lipocortin I and bovine lung calpactin I. Membrane vesicles prepared from 1-stearoyl,2-arachidonoyl phosphatidylcholine (PC) and other PCs were utilized as substrate. Binding studies using sucrose flotation gradients showed that both lipocortin I and calpactin I bind to these vesicles although less tightly than to vesicles prepared from anionic phospholipids or fatty acids. Binding to PC was somewhat enhanced by Ca²⁺. Inhibition of cobra venom PLA₂ was not observed when PC vesicles were used as substrate but was when dipalmitoyl phosphatidylethanolamine was used. Both the pancreatic and macrophage enzymes were inhibited when acting on PC. Interestingly, the inhibition of the macrophage enzyme toward PC depended on the fatty acid attached to the *sn*-2 position of PC with arachidonate > oleate > palmitate. Inhibition was also highest at low [PC]; these inhibition results can be explained by the "substrate depletion model" (Davidson, F. F., Dennis, E. A., Powell, M., and Glenney, J. (1987) *J. Biol. Chem.* 262, 1698-1705). Experimental and theoretical considerations suggest that the *in vitro* inhibition by lipocortins of this macrophage PLA₂ from a cell that makes lipocortin and is active in prostaglandin production is due to effects on substrate availability rather than direct inhibition.

Recent progress in the structural characterization of lipocortins I-VI (1) has shown that the species equivalents of lipocortin I and calpactin I (lipocortin II) are present in rat lymphoid organs and are expressed in murine macrophage cell lines including J774 and P388P.1. Following our previous studies on "substrate depletion" (2), we have now investigated the ability of human recombinant lipocortin I and bovine lung calpactin I to inhibit phospholipase A₂ (PLA₂)¹ (3) from

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¹ The abbreviations used are: PLA₂, phospholipase A₂; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SDS, sodium dodecyl sulfate; EGTA, [ethylenedis(oxyethylenenitrilo)]tetraacetic acid.

porcine pancreas, cobra venom (*Naja naja*), and a partially purified PLA₂ from the mouse macrophage-like cell line P388D₁ (4), using phosphatidylcholine (PC) vesicles as substrate. The ability of calpactin I and lipocortin I to bind to PC and to fatty acid was also investigated.

Studies of the effects of "lipocortins" on porcine pancreatic PLA₂ hydrolysis of *Escherichia coli* phospholipids or PC in deoxycholate mixtures have indicated that PLA₂ inhibition in those systems may be accounted for by depletion by the inhibitor of either substrate, or the cofactor of the enzyme, and may be complicated by inhibitor-induced phase changes in the substrate (2, 5). However, there have also been reports of inhibition of the pancreatic PLA₂ by various lipocortins when PC is used as substrate (6-10) or with PE using several different PLA₂s (11). Although the physical form of the phospholipid was not specified in any of these studies, neither detergents nor sonication was mentioned, and it can therefore be assumed that the phospholipids were in some kind of multilayer structure. In one case (9), the essential cofactor Ca²⁺ was not included in the assay, making it likely that the lipocortin inhibited solely by binding up the trace amounts of cofactor. However, the other reports were intriguing since calpactin I and lipocortin I have been reported not to bind PC (12, 13).

Lipocortins are proposed to be important in *in vivo* anti-inflammatory reactions due to the fact that purified proteins in this family added to prelabeled cells and tissues appear to decrease the release of [³H]arachidonic acid or thromboxane in response to stimuli (14, 15). The mechanism of PLA₂ inhibition by these proteins in the only other commonly used *in vitro* assay system (phospholipid vesicles) therefore warranted further study. In particular, if it could be ascertained whether *in vitro* inhibition always involves depletion effects or sometimes works by a different mechanism then perhaps more progress could be made toward quantitating the effect and determining the likely relevance of these proteins as inhibitors of PLA₂s as discussed in a recent commentary (16).

In the present studies, in all cases in which inhibition was observed, the bulk concentration of substrate was very low, confirming our earlier findings with *E. coli* substrate (2) and subsequent studies by Aarsman *et al.* (11). Furthermore, the potency of inhibition of the macrophage enzyme depended on which type of long chain PC was employed as substrate, and the inhibition could be overcome by raising the PC concentration. Inhibition of the *N. naja* PLA₂ toward PC, its preferred substrate, was not seen although the pancreatic enzyme was inhibited. However, when other substrates were used on which the *N. naja* PLA₂ exhibits lower rates of hydrolysis, inhibition by calpactin I and lipocortin I was achieved. The results indicate that inhibition by lipocortin on this substrate

involves competition for binding of PC. A preliminary report has appeared (17).

EXPERIMENTAL PROCEDURES

Materials—1,2-Dipalmitoyl-*sn*-glycerol phosphorylcholine (dipalmitoyl PC), 1,2-dipalmitoyl-*sn*-glycerol phosphoylethanolamine (dipalmitoyl PE), 1-stearoyl-2-arachidonoyl-*sn*-glycerol phosphorylcholine (1-stearoyl,2-arachidonoyl PC), and 1-palmitoyl-2-oleoyl-*sn*-glycerol phosphorylcholine (1-palmitoyl,2-oleoyl PC) were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). 1-Palmitoyl,2-[1-¹⁴C]palmitoyl PC (54 mCi/mmol), 1-palmitoyl,2-[1-¹⁴C]oleoyl PC (57 mCi/mmol), 1-stearoyl,2-[1-¹⁴C]arachidonoyl PC (60 mCi/mmol), 1-palmitoyl,2-[1-¹⁴C]palmitoyl PE (110 mCi/mmol), and [³H]palmitic acid (208 mCi/mg) were purchased from Amersham Corp. Thin layer chromatography plates (250- μ m layer of Silica Gel G) were purchased from Analtech (Newark, DE). Scintillation fluid (Safety-Solve) was purchased from Research Products International (Mount Prospect, IL). All other reagents were analytical reagent grade or better.

Calpactin I was purified from bovine lung according to procedures published previously (18) and showed one 36-kDa band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis by staining with Coomassie Blue. Amino-terminal sequence analysis showed a unique sequence corresponding to the amino terminus of the calpactin I light chain as expected for a pure sample of calpactin I (the heavy chain of which has a blocked amino terminus), indicating no significant proteolysis. Human recombinant lipocortin I was the generous gift of Dr. R. B. Pepinsky (Biogen Corp.). Porcine pancreatic PLA₂ was from Boehringer Mannheim. Cobra venom (*N. naja*, Pakistan) PLA₂ was purified according to procedures published previously (19) from lyophilized venom obtained from the Miami Serpentarium (Miami, FL).

P388D₁ Phospholipase A₂ Preparation—The purification of the PLA₂ from the P388D₁ macrophage-like cell line is discussed in detail elsewhere (4). The enzyme preparation used herein was that obtained after the second of two butanol extractions, referred to as BE-II. The PLA₂ in the BE-II preparation is about 60-fold purified, and the preparation is free of phospholipases A₁, C, and D (4). The enzyme is stored as a lyophilized powder and is stable in this state for at least several months. Lyophilized BE-II was dissolved in enzyme buffer (50 mM Tris-Cl, pH 8.0) to a concentration of either 0.32 or 0.64 mg/ml. Freshly dissolved aliquots were used for the kinetic studies.

PC- and Fatty Acid-binding Assays—Binding of calpactin I and lipocortin I to PC and free fatty acid was done using sucrose flotation gradients essentially as described previously (2). The lipid was dried *in vacuo* and then resuspended by probe sonication in 10 mM imidazole, pH 6.8, 40 mM KCl to a final concentration of 33 mM in a total volume of 0.5 ml. The PC used was 1-stearoyl,2-[1-¹⁴C]arachidonoyl PC (1.12 μ Ci) mixed with unlabeled 1-stearoyl,2-arachidonoyl PC to achieve the desired concentration. [³H]Palmitic acid (10 μ Ci) was likewise mixed with unlabeled palmitic acid to achieve the same concentration and volume. Aliquots of 25 μ l of the lipid sonicates were combined with 5 μ g of inhibitor protein and concentrated buffer to achieve a final volume of 47 μ l containing 10 mM imidazole, pH 6.8, 40 mM KCl, 2 mM MgCl₂, and the desired concentration of CaCl₂. For zero free Ca²⁺, 10 mM EGTA was added. For 2 and 10 mM Ca²⁺, no EGTA was added, only CaCl₂. The mixtures were allowed to remain at room temperature for 35 min; 78 μ l of 80% sucrose solution in the same buffer was then added to each sample, and 100 μ l of each of these mixtures was placed on the bottom of a centrifuge tube (Beckman Ultra-Clear, 8 \times 20 mm). This layer was overlaid with 150 μ l each of 40, 30, and 20% sucrose in the same buffer and finally with 150 μ l of buffer alone. The gradients were centrifuged for 5.5 h at 50,000 rpm in a Beckman SW Ti-55 rotor at 4 °C and allowed to come to a halt without using a brake (which took approximately 30 min). Each gradient was quickly frozen by immersion in liquid N₂ and then sliced with a razor into eight equal fractions that were allowed to thaw in microcentrifuge tubes. After thawing, the liquid was spun to the bottoms of the tubes and two 15- μ l aliquots removed for scintillation counting and SDS-polyacrylamide gel electrophoresis. Each sample for SDS-polyacrylamide gel electrophoresis was combined with 5 μ l of standard 4 \times reducing sample buffer plus an additional 1 μ l of 10% SDS. Samples were run on 14% gels at 30 mA for 4 h after which they were fixed and silver stained according to the procedure of Oakley *et al.* (20). Developed gels were subjected to densitometry using a Bio-Rad densitometer.

Assay of Porcine and Cobra Venom PLA₂—Assays were carried out in a total volume of 0.2 ml in 50 mM Tris-HCl, pH 8.0, containing 10

mM CaCl₂ and 10 μ M sonicated PC in vesicles. If an inhibitor protein was included, it was added to the assay solution and allowed to equilibrate with the substrate for 10 min at 40 °C before beginning the assay by the addition of 10 μ l of PLA₂ stock solution. The final concentration of both PLA₂s was 0.1 ng/200- μ l assay. With the porcine pancreatic PLA₂, the assay was allowed to proceed for 60 min at 40 °C, and hydrolysis of 1-stearoyl,2-[1-¹⁴C]arachidonoyl PC in that time was approximately 6% above the background (1% in control samples without calpactin I and not more than 8% under any conditions. With the cobra venom PLA₂, the reaction was allowed to proceed for 2 min at 40 °C, and hydrolysis was 2–4% above background (<1%). All reactions were stopped by the addition of 300 μ l of chloroform/methanol/acetic acid (157:114:29, v/v), and the chloroform layers containing substrate and products were removed, dried *in vacuo*, and subjected to thin layer chromatography as described below.

Assays using sonicated PE were carried out in a total volume of 0.2 ml in 20 mM Tris-HCl, pH 8.0, containing 5 mM CaCl₂ and 1.3 μ M PE. The [¹⁴C]dipalmitoyl PE was used undiluted with cold PE, and a concentrated stock solution was made by drying the [¹⁴C]PE *in vacuo* and resuspending by sonication in the assay buffer. The PE was difficult to resuspend; so after sonication for 5 min, an aliquot was subjected to scintillation counting to determine the true concentration in the stock solution from which dilutions were made. Calpactin I (5.4 μ g/200- μ l assay) was added 10 min prior to starting the assay with 5 ng of PLA₂. Duplicate reactions were stopped by the addition of 1.32 ml of modified Dole reagent. Reactions were terminated after 15 min in the case of cobra venom PLA₂ and 30 min in the case of pancreatic PLA₂ and extracted by a modified Dole procedure. In 30 min, the pancreatic enzyme hydrolyzed about 14% of the PE above background (which was 4%). In 15 min, the cobra enzyme hydrolyzed roughly 8% above the background, indicating similar activities of both enzymes on the substrate.

PLA₂ activities on ³H-labeled autoclaved *E. coli* cells were assayed as described previously (2). The assays were started by the addition of ³H-labeled *E. coli* cells and stopped after 5 min at 0 °C by the addition of bovine serum albumin (20 mg/ml) and 2 N HCl. The supernatants were removed for scintillation counting after spinning down the cells, and the average of duplicates, corrected for blanks, is reported.

Assay of BE-II PLA₂—The standard assay conditions included 2 mM CaCl₂, 20 mM Tris-HCl, pH 8.0, and 10 μ M PC containing about 100,000 cpm of *sn*-2-1-¹⁴C-fatty acyl PC. PC was prepared as vesicles by sonicating the phospholipid in buffer and Ca²⁺ using an MSE model 100-watt probe sonicator until the lipid suspension clarified (about 5 min). The reaction was started by the addition of the enzyme solution (16 μ g of protein) to give a final volume of 0.5 ml. The assays were then incubated at 40 °C for 90 min.

For experiments in which the substrate concentration dependence was determined, it was necessary to vary the time of incubation in order to have hydrolysis of less than 5% of total substrate, where the enzymatic rate was found previously to be linear with time (21). The time was varied from 15 min (low substrate) to 150 min (high substrate). At the lowest substrate concentrations tested, radiolabeled phospholipid could not be diluted with unlabeled phospholipid, and samples contained 20,000–30,000 cpm. All times were adjusted to generate at least 1,000 cpm of product to ensure accuracy.

Enzymatic reactions were stopped by the addition of 0.5 ml of chloroform/methanol/acetic acid (2:4:1, v/v). The entire sample was spotted onto a lane (2-cm width) of a 10 \times 20-cm thin layer chromatography plate, and the lipid components were separated over the 10-cm length by elution with chloroform/methanol/water (65:25:4, v/v). The lipids were visualized by exposure to I₂ vapor, and the zones corresponding to fatty acid and PC were scraped directly into scintillation vials to which 6 ml of scintillation fluid was added. Blanks were obtained by substituting enzyme buffer for the freshly prepared enzyme solution and incubating for the duration of the assay. The background hydrolysis was found to be less than 0.5%. Averages of duplicate assays are reported with blanks subtracted.

RESULTS

Binding to Phosphatidylcholine and Fatty Acid—The binding of calpactin I and lipocortin I to sonicated PC vesicles and to free fatty acid was tested using sucrose flotation gradients similar to those described previously (2) for PS/PE liposomes. However, the PC vesicles were not as buoyant as

PS/PE liposomes, as might be the result of the lower propensity of PC to form very large fused phases. Therefore, it was necessary to centrifuge the PC-containing gradients for longer times (6 h instead of 2 h) in order to obtain all of the phospholipid in the top several fractions of the tubes. Calpactin I and lipocortin I stayed in the bottom two or three fractions of the gradients in the absence of lipid (data not shown). In pilot assays, calpactin I was tested for binding to PC at a phospholipid concentration equal to that used with PS/PE liposomes in previous experiments (2) (1.2 mM, 50 μg of total phospholipid/gradient). However, under conditions in which calpactin I would have been expected to bind tightly to PS/PE liposomes, PC caused smearing of the protein throughout the gradient (data not shown). This is indicative of a looser binding phenomenon than was seen with PS. The concentration of PC was therefore raised, and at a 10-fold higher amount of PC (Fig. 1), both calpactin I and lipocortin I were found in discrete zones in the gradients, with approximately 30% of the protein associated with PC and the remainder at the bottom of the tube.

The effect of $[\text{Ca}^{2+}]$ on the binding of calpactin I to 1-stearoyl,2-arachidonoyl PC was also investigated. When EGTA was present, very little protein was bound to PC (Fig. 2A). There appeared to be a weak dependence on $[\text{Ca}^{2+}]$ because as it was increased (B and C), so too was the binding of calpactin to a maximum of about 30% at 10 mM Ca^{2+} . In all the binding experiments, the preparation of the gels for densitometry was done by silver staining. All the fractions of a single gradient were run together in one gel in order to assess their relative intensities because the extent of development of the silver stain could vary in different preparations, and the absolute intensities of bands containing the same amount of protein sometimes differed in gels developed at different times. Interestingly, the light chain of calpactin I always developed color much more quickly than did the heavy chain (the light chain is not stained at all by Coomassie Blue). Therefore, if the amount of heavy chain was very low in a

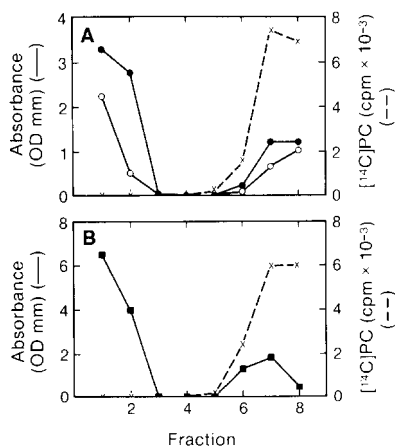


FIG. 1. **Binding of calpactin I and lipocortin I to phosphatidylcholine vesicles.** Calpactin I (A) and lipocortin I (B) (4 μg /gradient) were preincubated with sonicated phosphatidylcholine vesicles (535 μg /gradient) in 10 mM imidazole, pH 6.8, 40 mM KCl, 2 mM MgCl_2 , and 10 mM CaCl_2 before being adjusted to 50% sucrose, overlaid with a 40–0% sucrose gradient, and subjected to ultracentrifugation for 6 h at 4 $^{\circ}\text{C}$. 1-Stearoyl,2-[1- ^{14}C]arachidonoyl phosphatidylcholine (x) floated to the top of the gradients, and calpactin I heavy chain (●), light chain (○), and lipocortin I (■) partitioned between the bottom of the tubes (fractions 1–3) and the phosphatidylcholine-containing fractions at the top. The phospholipid and relative protein contents of each fraction were determined by liquid scintillation counting and SDS-polyacrylamide gel electrophoresis, silver staining, and densitometry, respectively.

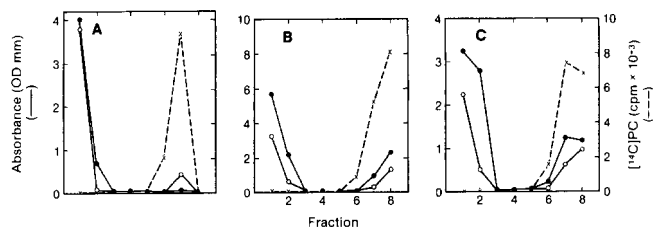


FIG. 2. **Binding of calpactin I to phosphatidylcholine vesicles at different $[\text{Ca}^{2+}]$.** Preincubation of calpactin I with 1-stearoyl,2-[1- ^{14}C]arachidonoyl PC, sucrose flotation gradients, and analysis of fractions was carried out as described in Fig. 1 except that the buffers contained A, 10 mM EGTA; B, 2 mM Ca^{2+} ; or C, 10 mM Ca^{2+} . x, ^{14}C phosphatidylcholine; ●, calpactin I heavy chain; ○, calpactin I light chain.

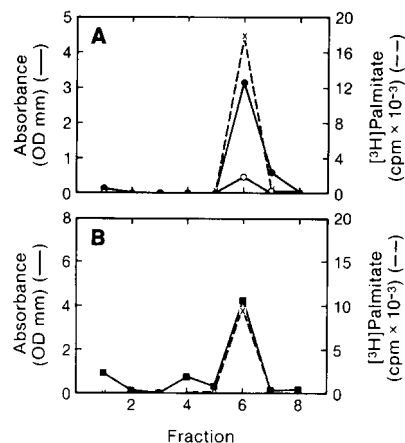


FIG. 3. **Binding of calpactin I and lipocortin I to fatty acid.** Calpactin I (A) and lipocortin I (B) (4 μg /gradient) were preincubated with free palmitate at a molar concentration equal to the phosphatidylcholine used in Figs. 1 and 2 but totaling 200 μg (due to its smaller molecular weight). All other conditions were as in Fig. 1. $[\text{Ca}^{2+}]$ was 10 mM in all buffers, and therefore much of the fatty acid was probably in the form of calcium palmitate. x, ^3H palmitate; ●, calpactin I heavy chain; ○, calpactin I light chain; ■, lipocortin I.

given lane, it may not have been possible to visualize it before overdevelopment of the rest of the gel became imminent, and the reaction was stopped. This may account for some of the apparent variations in heavy to light chain ratios as the $[\text{Ca}^{2+}]$ was raised in the gradients, and more protein became associated with the upper phosphatidylcholine-containing fractions.

In marked contrast to the extent of binding of both proteins to phosphatidylcholine, binding of calpactin I and lipocortin I to free fatty acid under the same conditions (Fig. 3) was complete. In the experiment shown in Fig. 3, palmitic acid was used at a molar concentration equal to that of the phosphatidylcholine used in the previous experiments. Oleic acid gave similar results, but arachidonic acid was retained at the bottom of the gradients with calpactin I (data not shown). Although binding of calpactin I and lipocortin I to palmitic acid at 10 mM Ca^{2+} was more extensive than to PC, it was probably not as efficient as to PS/PE liposomes, judging from preliminary experiments at lower concentrations of fatty acid.

Lipocortin Effects on the Hydrolysis of Sonicated Phosphatidylcholine Vesicles by the Secreted Phospholipases A_2 —In Fig. 4, dose-response curves are shown for calpactin I inhibition of pancreatic and cobra venom (*N. naja*) PLA_2 toward 10 μM 1-stearoyl,2-arachidonoyl PC vesicles. Initial activation followed by inhibition was seen with the pancreatic enzyme. However, the cobra venom PLA_2 was not inhibited at all under the conditions used and was, if anything, slightly activated. The apparent IC_{50} for the pancreatic PLA_2 was approximately 10 $\mu\text{g}/\text{ml}$ or 0.1 μM calpactin I holoprotein, and the

enzymes were each present at 0.5 ng/ml or 0.94 nM. For the pancreatic PLA₂, reactions were allowed to proceed for 60 min at 40 °C, resulting in 5.8 ± 0.2% hydrolysis in the PLA₂ controls. The cobra venom PLA₂ is much more active than the pancreatic enzyme on PC, and after 2 min, hydrolysis in the controls was 1.8 ± 0.3%. This would indicate a difference in rate of approximately 10-fold, consistent with the different rates for these enzymes typically observed with PC under other conditions (e.g. Ref. 22). It should be noted, however, that under the conditions used here, control time courses of hydrolysis by the cobra venom enzyme were not linear but curved over, reaching a maximum of about 7.5% hydrolysis in about 7.5 min. The pancreatic PLA₂ time courses showed a brief lag period and then were roughly linear until a little over 6% hydrolysis after which they too curved over.

Although the calpactin I did not inhibit the cobra venom PLA₂ toward phosphatidylcholine, it was able to inhibit this enzyme when other substrates were used (Table I). When sonicated dipalmitoyl PE was used as the substrate, under conditions in which the cobra venom and pancreatic PLA₂s gave approximately equal rates of hydrolysis, a 39% inhibition of the cobra venom enzyme was seen (Table I), whereas the pancreatic PLA₂ was 54% inhibited (data not shown). The cobra venom PLA₂ was also inhibited by calpactin I and lipocortin I when ³H-labeled *E. coli* cells were used as substrate. Therefore, the lack of inhibition of the cobra venom PLA₂ in Fig. 4 cannot be taken to imply a unique specificity of the calpactin I for the pancreatic enzyme but rather shows that the inhibition depends on which substrate is used for a given PLA₂. Notably, the cobra venom enzyme was not inhibited toward the substrate on which it normally gives its best rates, whereas the pancreatic enzyme, for which PC is a poor

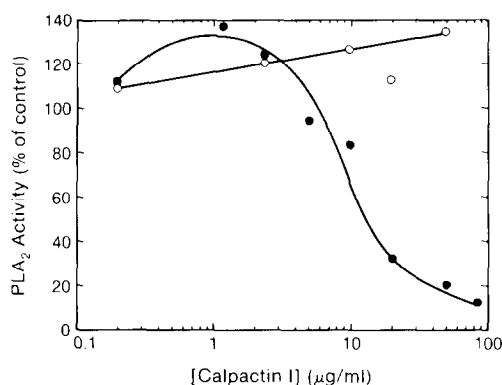


FIG. 4. Dose-response curves showing the effect of calpactin I on pancreatic and cobra venom PLA₂s acting on sonicated PC vesicles. The activity of each PLA₂ (0.1 ng/200 μl) was measured in the presence of increasing amounts of calpactin I at 40 °C with 10 μM 1-stearoyl,2-[¹⁴C]arachidonoyl PC in standard buffer. The activity is expressed as percent of controls containing no calpactin I for ●, porcine pancreatic PLA₂ and ○, cobra venom (*N. naja*) PLA₂. The apparent IC₅₀ (relative to the control in the absence of calpactin I) for the pancreatic PLA₂ was approximately 10.3 μg of calpactin I/ml, which corresponds to 0.1 μM holoprotein or 0.2 μM heavy chain.

substrate, was inhibited. Switching to a substrate on which the cobra venom PLA₂ also acts poorly (PE or *E. coli*) allows inhibition of both enzymes to be observed under identical conditions. These results are similar to those of Rothut *et al.* (14), who found that *N. naja* PLA₂ could be inhibited by a 32-kDa lipocortin toward phosphatidic acid but not phosphatidylcholine, and those of Aarsman *et al.* (11), who saw inhibition of several PLA₂s, including pancreatic, toward PE.

Effects on PLA₂ from P388D₁ Cells—Both lipocortin I and calpactin I were found to inhibit a partially purified PLA₂ from the mouse macrophage-like cell line P388D₁. The substrate used in Fig. 5 was 10 μM sonicated 1-stearoyl,2-arachidonoyl PC, and the approximate IC₅₀ of both inhibitors was 5 μg/ml (approximately 0.1 μM in 38-kDa chains). Fig. 6 shows the effect on the inhibition of the macrophage PLA₂ by calpactin I when different PCs were used as substrates. At the highest concentration of calpactin I used, less than 20% inhibition was seen using dipalmitoyl PC as substrate. About 80% inhibition was seen with that same amount of calpactin I and at that same concentration of substrate when 1-palmitoyl,2-oleoyl PC and 1-stearoyl,2-arachidonoyl PC were used. However, the IC₅₀ in the presence of oleoyl-containing PC was higher than that observed with the more unsaturated, longer chain arachidonoyl-containing phosphatidylcholine: 13 μg/ml (0.3 μM 38-kDa chain) and 3.3 μg/ml (0.09 μM), respectively.

The dependence of the inhibition on substrate concentration was examined for PLA₂ hydrolysis of 1-stearoyl,2-arachidonoyl PC at three different concentrations of calpactin I, and the results are shown in Fig. 7. In this experiment, the concentration of calpactin I was held constant as the concentration of substrate was raised. In a previous report (21) it was shown that the macrophage enzyme exhibits different types of kinetics depending on the range of substrate concentrations used. In the concentration range used here (<10 μM), the PLA₂ activity was shown previously to give hyperbolic velocity *versus* S curves, and Michaelis-Menten kinetics were assumed. At concentrations above 10 μM substrate, the velocity *versus* S curves indicated cooperativity of some sort. In the experiment shown in Fig. 7, the actual velocity curves are shown in *panel A* and are replotted as the double reciprocals in *panel B*. Within the accuracy of the assay and given the uncertainties of the macrophage PLA₂ mechanism, it is not possible to distinguish whether or not the inhibited samples give genuinely linear double-reciprocal plots that would fit better to Michaelis-Menten kinetics than other models (see "Discussion"). Similar substrate dependence of the inhibition by calpactin I can also be seen when 1-palmitoyl-2-oleoyl PC is used as substrate, as shown in Fig. 8. At 1 μM substrate, about 60% inhibition is observed, and this is reduced to about 15% when the substrate concentration is raised to 5 μM.

DISCUSSION

Binding to PC—Although it had been reported previously that calpactin I and lipocortin I do not bind to PC under

TABLE I
Inhibition by lipocortins of cobra venom PLA₂ acting toward different substrates

Inhibitor	Substrate	[Phospholipid]	[Inhibitor]	[PLA ₂]	Inhibition
			M		%
Calpactin I	Dipalmitoyl PE	1 × 10 ⁻⁶	3 × 10 ⁻⁷	2 × 10 ⁻⁹	39 ± 15
Calpactin I	[³ H] <i>E. coli</i> cells ^a	8 × 10 ⁻⁶	5 × 10 ⁻⁷	1 × 10 ⁻⁷	68 ± 10
Lipocortin I	[³ H] <i>E. coli</i> cells ^b	1 × 10 ⁻⁶	2 × 10 ⁻⁷	3 × 10 ⁻⁸	62 ± 10

^a The buffer was 100 mM Tris-HCl, pH 8.5, containing 10 mM CaCl₂.

^b The buffer was 350 mM Tris-HCl, pH 8.5, containing 30 mM CaCl₂.

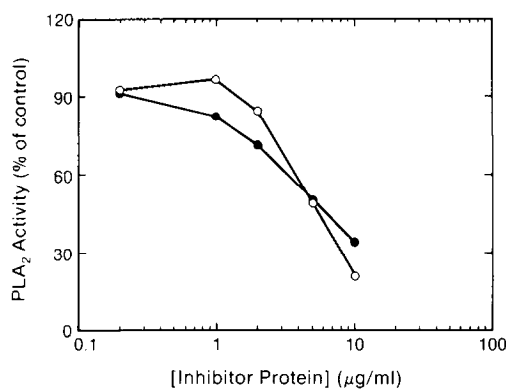


FIG. 5. Dose-response curve showing the effect of calpactin I (●) and lipocortin I (○) on macrophage phospholipase A_2 activity toward 1-stearoyl,2-arachidonoyl PC under standard assay conditions.

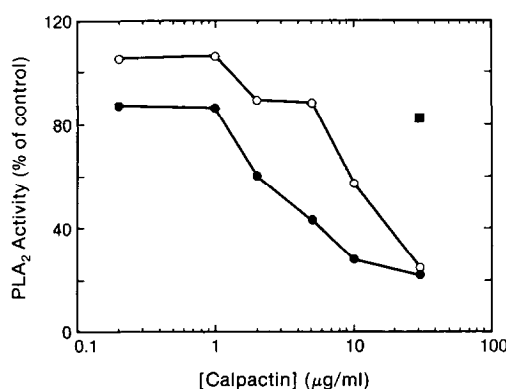


FIG. 6. Dose-response curve showing the effect of calpactin I on macrophage phospholipase A_2 activity toward ■, dipalmitoyl PC; ●, 1-palmitoyl-2-oleoyl PC; and ○, 1-stearoyl-2-arachidonoyl PC. Standard assay conditions were used.

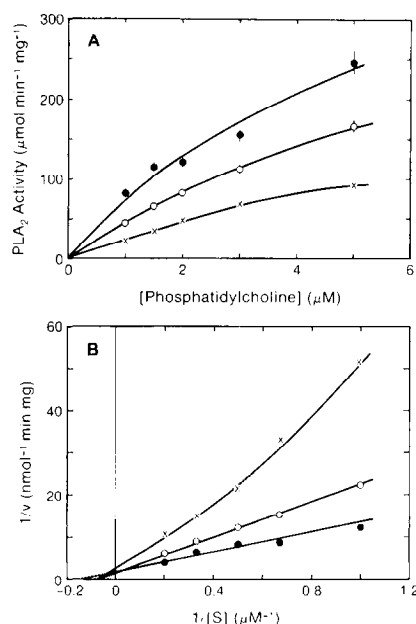


FIG. 7. Velocity versus S plot of macrophage phospholipase A_2 activity with 1-stearoyl-2-arachidonoyl PC. A, the activity of the enzyme was measured in the presence of ●, 0 $\mu\text{g/ml}$; ○, 1 $\mu\text{g/ml}$; and ×, 5 $\mu\text{g/ml}$ calpactin I. Standard assay conditions were employed. B, double-reciprocal plots of the data shown in A.

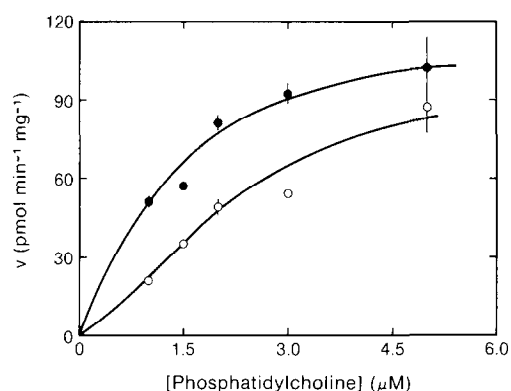


FIG. 8. Velocity versus S plot of phospholipase A_2 activity as a function of 1-palmitoyl-2-oleoyl PC concentration in the absence (●) and the presence (○) of 5 $\mu\text{g/ml}$ calpactin I.

conditions in which PS was bound (13, 14), we have found that the proteins do bind to PC albeit with a lower affinity than is exhibited for PS. At a 10-fold greater concentration of PC than was used with PS and using a 5-fold lower concentration of protein and much higher $[\text{Ca}^{2+}]$, 30% of the protein was bound to lipid compared with 100% when PS/PE liposomes were used (2). The results obtained are consistent with those of Schlaepfer and Haigler (12), who looked for but did not find binding of ^{125}I -lipocortin I to large thin walled PC vesicles using sedimentation. At the concentration of PC and Ca^{2+} used by these authors, significant binding would not have been detected with sonicated PC vesicles in the sucrose flotation assay either, although it should be noted that the calpactins may have different affinities for PC in different structures. These results imply that the binding of lipocortin I and calpactin I to PC-sonicated vesicles is weaker than to anionic lipids. A dependence for binding on the concentration of Ca^{2+} was also seen with PC but over a millimolar rather than micromolar range. The Ca^{2+} requirement of calpactin I has been shown to be dependent on the concentration of PS/PE liposomes (13), and at high enough lipid (about half of the concentration of PC used in Fig. 1), no Ca^{2+} is required for binding to that phospholipid. It has also been observed that the Ca^{2+} affinity of lipocortin I is increased by PS but not by PC (12). Under conditions in which PS increases the affinity of the calpactin for Ca^{2+} and vice versa, PS/ Ca^{2+} complexes are probably formed. PC, as a zwitterion, does not bind Ca^{2+} to its surfaces as tightly or as extensively as does PS, nor does Ca^{2+} induce phase changes of PC. This difference may be at the root of the lower affinity of the lipocortin protein for PC as well as the broader Ca^{2+} concentration dependence for binding.

It is possible that the nature of the calpactin-phospholipid complex differs for PC and PS or else is the same but harder to assume with PC. Calpactin I and lipocortin I cause aggregation of PS/PE liposomes (13, 18), resulting in an extended protein/phospholipid phase that sediments easily from aqueous solution upon centrifugation and floats in sucrose solutions. The aggregation phenomenon is not surprising since negatively charged phospholipids have a natural tendency to form nonbilayer phases such as hexagonal and cubic phases under a variety of conditions. Sonicated PC vesicles, on the other hand, are generally small, far less easily aggregated, and cannot be quantitatively sedimented in the absence of protein even by ultracentrifugation. It is presumably because of their smaller size, resulting in higher relative densities, that they float less easily than PS in sucrose gradients. Binding of calpactin to sonicated PC vesicles may not induce

aggregation of the vesicles, in which case binding might not be detectable by either light scattering or sedimentation from aqueous solution, and this could account for the results of previous studies in which those detection methods were used (13). Also, if no significant aggregation occurs, the protein-PC complex may tend to be more dense than allowable for flotation of both components. Thus, as the centrifugal force displaces the light lipid phase upward in the gradient, the normal on/off equilibrium of the protein bound to lipid might be shifted more to "off," as the buoyancy of the lipid is increased upon dissociation of the protein. Thus, with PC it may be necessary to have a higher total ratio of phospholipid to bound protein than is the case with PS in order to prevent dissociation of the protein as it rises through the gradient. This could explain the smearing of protein at lower PC:calpactin I ratios. For reasons such as these, another assay for PC binding is needed in order to obtain K_D values.

Fatty Acid Effects—The ability of calpactin I and lipocortin I to bind to free fatty acid may influence the kinetic results of PLA₂ assays. Therefore, inhibition of porcine pancreatic PLA₂ by lipocortin binding of fatty acid, which is the enzyme's activator on PC, was considered but seemed unlikely in the assays performed here because of the low percent hydrolysis in all assays. The macrophage PLA₂ is inhibited by free fatty acid not activated by it (23), and it was also inhibited by lipocortin. However, the potential for fatty acid binding must be considered at the higher percentages of hydrolysis which are frequently reported for control PLA₂ samples. Furthermore, the ability to bind fatty acids and their metabolites may impinge on the choice of techniques for the measurement of levels of these molecules in any system in which lipocortins are present.

Substrate Dependence of the Inhibition by Lipocortins—The inhibition reported herein of pancreatic and macrophage PLA₂ hydrolysis of PC vesicles supports previous reports of inhibition of pancreatic PLA₂s by calpactin I and uteroglobin on PC substrates (6, 10). However, it is evident from the results with pancreatic, cobra venom, and the macrophage PLA₂s that the degree of inhibition depends on which substrate is used and its concentration. The following data indicate that inhibition involves competitive substrate-binding constants: (i) the lack of inhibition on a substrate that *N. naja* PLA₂ hydrolyzes efficiently, although inhibition was observed on a poorer substrate of the enzyme; (ii) the different IC₅₀ values obtained with the macrophage PLA₂ and different PCs even though this PLA₂ has similar K_m values on all three PCs used (21); and (iii) the dependence of the inhibition on substrate concentration, an effect that has been observed previously for these inhibitors with other PLA₂s and other substrates (2, 11). What is not clear in the case of PC is whether there is competition between the inhibitor and substrate for the active site of the PLA₂ (classical competitive inhibition, K_s versus K_I), competition between the inhibitor and enzyme for substrate (substrate depletion, K_s versus K_0), or whether one is actually dealing with a partition coefficient (K) of the inhibitor between solution and bilayers, with the result that the inhibitor in the surface confers a physical effect on the phospholipid phase or change in T_m which consequently changes the enzyme's K_s or k_{cat} on the substrate.

Competitive and substrate depletion inhibition can only be distinguished kinetically under certain conditions, as illustrated by Fig. 9. The velocity equation of a system in which substrate depletion is occurring is

$$\frac{v}{V_{max}} = \frac{[S]}{K_s + [S]}$$

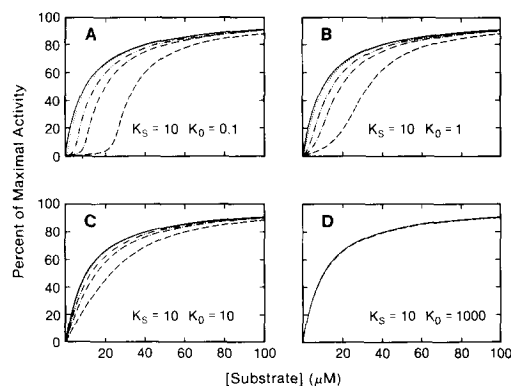


FIG. 9. Theoretical velocity versus S plots generated using the equations for simple substrate depletion kinetics (see "Discussion"). K_s , the binding constant of a protein inhibitor to substrate phospholipid, is given the hypothetical values of A, 0.1; B, 1; C, 10; and D, 100. K_0 , the binding constant of an enzyme to its substrate, is given the hypothetical value of 10; and I , the inhibitor concentration, is varied from 0 to 25 μM as the curves proceed from left to right: none (—), 1 μM (· · · · ·), 5 μM (· - · - ·), 10 μM (- - -), and 25 μM (- - -).

where S represents the concentration of free substrate available for combination with the enzyme (assuming one inhibitor molecule binds to one substrate molecule, no cooperative effects are involved, and the enzyme displays simple Michaelis-Menten kinetics on the substrate). The [S] can be calculated for any total substrate ($[S]_t$) and total inhibitor ($[I]_t$) concentrations using the following equation.

$$[S] = \frac{\sqrt{([I]_t - [S]_t + K_0)^2 + 4 K_0 [S]_t} - ([I]_t - [S]_t + K_0)}{2}$$

Therefore, if K_0 , K_s , and V_{max} are established, then the velocity curve as a function of substrate concentration can be calculated. In Fig. 9, we have held the V_{max} and K_s constant at arbitrarily chosen values and plotted the velocity curves for four different relative K_0 values in order to illustrate the effect of these ratios on the shapes of the inhibition curves as a function of substrate concentration. As can be seen, as the inhibitor's affinity for substrate (K_0) approaches the enzyme's K_s for substrate, the potency of the inhibitor decreases, and the shape of the inhibited curve becomes less and less sigmoidal, to the point where the plots resemble competitive inhibition. If K_0 is sufficiently high on a given substrate relative to the enzyme's K_s , then inhibition may not be observed unless massive quantities of inhibitor are used. Such phenomena could explain the kinetic plots obtained for inhibition of the macrophage PLA₂ toward different PCs.

On the other hand, in the PC assay system, in which there is less affinity of the lipocortins for the substrate and in which more lipocortin or calpactin must be used in order to obtain inhibition than was the case with anionic lipids, competitive protein binding could be important. The ultracentrifugation data of Ahn *et al.* (24), which show lipocortin K_D values of about 10^{-5} M for pancreatic PLA₂, and other studies reporting IC₅₀ values of 10^{-5} M for uteroglobin (10) to 10^{-7} M for calpactin I (6) with pancreatic PLA₂s on PC bilayers could all be consistent with such a hypothesis. However, even though the shape of the kinetic curves in Fig. 7 may be consistent with active site competitive inhibition, they are also consistent with a substrate depletion mode of inhibition in the case in which $K_0 \approx K_s$, as just described, particularly given the error of the assays for the PLA₂s and limitations on the amounts of inhibitor protein available. Interestingly, the K_m values of the macrophage PLA₂ on all the PCs used herein are on the order of 10^{-5} M (21).

Observations that support the hypothesis of a direct phase effect on substrate by calpactin I and lipocortin I include the following. First, association of these proteins with PC has now been demonstrated under some conditions. Furthermore, significant binding under assay conditions would require a K_D of only about 10^{-6} M, which is conceivable by comparison with other's estimations of K_D values for PS (25) and the relative affinities observed here. In addition, lipocortin by itself has been reported to have some surfactant properties (cited in Ref. 1 as a personal communication). If this is so, then it is logical to assume that partitioning into lipid bilayers is possible. If that happened, then as the PC:lipocortin ratio was raised, the protein would become diluted in the surface, and localized phase effects could diminish. Surfactant-like effects could also explain the often observed activation of PLA₂ by lipocortin I and calpactin I in some concentration regimes. That is, phase changes have the potential either to activate or to inhibit PLA₂s depending upon the enzyme and substrate under study (26–29).

Another line of evidence supportive of lipocortin-induced substrate effects concerns the stoichiometries of protein to phospholipid when inhibition is achieved. The distinction between dissociation constants and stoichiometries should first be noted, however. In competitive inhibition, it is the concentrations of all three components S, E, and I, and the values of K_s and K_I that determine the extent of inhibition. Similarly, in substrate depletion inhibition, it is the concentrations of the three components and K_s and K_0 . In neither case is stoichiometry the *main* operative factor, but rather it is the absolute concentrations relative to the appropriate dissociation constants that are definitive. Second, it must be noted that the concentration of phospholipid added to an assay is not the concentration that the enzyme initially "sees" unless a solubilizing detergent is added. The maximum amount of surface phospholipid in vesicles will be as much as 60% of the total if small single-walled vesicles are used or as low as 10% or less of the total if multimellar vesicles (liposomes) are used. Thus, if concentrations are to be compared from one assay to the next, it is important to state the method of preparation of the phospholipid and what kind of vesicles were obtained. (The PC used in this study was sonicated and therefore is expected to be a mixture of small multilamellar and unilamellar vesicles.) Given these caveats, it appears that in all the PC systems of which we know in which a purified lipocortin-type protein was used and Ca²⁺ was in excess (this study and Refs. 6 and 10), the maximum probable surface PC/total inhibitor molar ratio at the IC₅₀ was as low as stoichiometric and not higher than 8:1. In making this calculation, the extreme case was assumed in which 60% of the lipid was on the surface. This recurring range of stoichiometries at the IC₅₀ values lends weight to the argument that inhibition may be due to a direct effect on substrate structure.

Considering the large size of these proteins compared with lipid, molecular weight 38,000 *versus* about 300–800, it is not unreasonable to assume that the binding of one lipocortin I or calpactin I molecule could effectively cover many more than one lipid headgroup in the surface at any given instant. However, the stoichiometries just cited would suggest substrate depletion as opposed to phase effects *only* if the K_0 for the substrate were low enough to have enough inhibitor bound to the surface to cover a sufficient number of phospholipid headgroups so as to coat the surface. On the other hand, a partition coefficient that favors insertion of most of the inhibitor protein into the outermost phospholipid layer at micromolar PC would also be consistent with the observed stoichiometries if the insertion of protein caused appropriate

perturbations of the phospholipid phase so as to inactivate the phospholipase A₂. To distinguish these, more sensitive techniques for quantifying lipid binding are necessary. At present, the results obtained herein could be explained equally well by competitive or substrate depletion mechanisms or by surface-active partitioning of the inhibitor. However, if the inhibition appears to be competitive, the next question is whether the apparent dissociation constants indicate a probability that the inhibitor would be a significant one *in vivo*. So far, we have no evidence of a tight association of a lipocortin protein with any phospholipase A₂. The natural corollary is the question of which PLA₂ is appropriate to test (as discussed in more detail elsewhere (16)). We have employed herein a partially purified phospholipase A₂ from macrophage-like cells but see no indication of specificity. Other phospholipases A₂ do occur in these cells and might also be tested once they are further purified and characterized. No matter which enzymes or inhibitors are investigated, however, the experience of our laboratory and others shows that substrate dependencies must be examined under conditions in which the time courses of reactions are observed. (The need for time courses is dictated by the complexity of phospholipase A₂ action toward many substrates.) If there were no dependence of the inhibition on substrate concentration, then direct protein binding by inhibitor would seem likely. However, such behavior has not yet been documented for any of the lipocortins.

Conclusion—It appears now that all the biochemical investigations into the effects of lipocortins on the activities of various phospholipases A₂ could be explained by substrate depletion or surfactant effects. These inhibitors/activators appear to bind only weakly to PLA₂s, yet the inhibition that arises from their effects on membranes in *in vitro* assays may be a reflection of intrinsic properties that give rise to membrane-modulating effects *in vivo*. In this case, there would be the potential to inhibit many more physiological components than just PLA₂. We must stress, however, that there is as yet no evidence for such a link and the membranes of cells are more complicated than PC or even PS bilayers. In summary, it seems that the most intense research in the mode of action of the proposed members of the lipocortin family should now center on the biological studies and the basic questions of which, if any, lipocortins are induced and where; whether a PLA₂ or some other enzyme involved in eicosanoid biosynthesis is being affected; and whether the apparent antiinflammatory effect of some of these proteins is due to a more generalized mechanism such as an effect on Ca²⁺ transport.

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