Inhibition of Phospholipase A$_2$ by “Lipocortins” and Calpactins

AN EFFECT OF BINDING TO SUBSTRATE PHOSPHOLIPIDS*

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The “lipocortins” are a group of proteins that have been reported to inhibit phospholipase A$_2$ by direct interaction with enzyme. Two proteins which have been identified as lipocortin on the basis of inhibition of phospholipase A$_2$ activity have recently been cloned and sequenced. These have been shown to be identical to the calpactins, which are membrane cytoskeletal proteins serving as major substrates of the tyrosine protein kinases. We have now found that two forms of calpactin (I and II) inhibit porcine pancreatic phospholipase A$_2$ in an assay using Escherichia coli cells or extracted phospholipid vesicles as substrate, but only when the substrate concentration is very low. Both calpactins, as well as another, 73-kDa inhibitory protein, were found to bind purified phospholipids and E. coli membrane directly. Kinetic studies show that the inhibition of phospholipase A$_2$ by calpactin can be overcome by high phospholipid substrate concentrations, whether E. coli cells or isolated phospholipid vesicles are used. For example, in the presence of 5 $\times$ 10$^{-4}$ M phospholipase A$_2$ and 1 $\times$ 10$^{-3}$ M calpactin, the inhibition decreases from 100 to 0% as phospholipid in vesicles is raised from 2 to 8 $\mu$M. The evidence reported here strongly suggests that in vitro inhibition of phospholipase A$_2$ by lipocortin is due to sequestering of the phospholipid substrate by the inhibitor protein, rather than a direct interaction with the phospholipase. These results raise questions about the physiological significance of the inhibition of phospholipases by calpactins.

The lipocortins are defined as anti-inflammatory proteins which are inducible by steroids, secreted by cells, and thought to act prior to the cyclooxygenase pathway by inhibition of a phospholipase A$_2$. In the search for the purified form of such an inhibitor protein, several 36-kDa proteins have been isolated, using as an assay their ability to inhibit phospholipase A$_2$ in vitro. While there was initial evidence for smaller molecular weight proteins possessing lipocortin-like activity, these proteins are now thought to be proteolytic fragments of a 38-kDa parent form. The first 36-kDa purified protein to be assigned the name lipocortin is now known as lipocortin I and is thought to be a potent phospholipase A$_2$ inhibitor regulated in vivo by phosphorylation (for reviews, see Refs. 1 and 2). Recently, sequence analysis has united the study of lipocortin with another line of research, that involving membrane-associated cytoskeletal proteins that serve as substrates of viral and growth factor receptor tyrosine kinases. These membrane cytoskeletal proteins have been termed calpactins I and II to denote their calcium-dependent phospholipid and actin binding properties and are also known as p36 and p35. Cloning and sequencing of the cDNA for calpactin I has revealed the surprising result that it is greater than 50% homologous to lipocortin I (3, 4). Further sequence results have shown that calpactin II is equivalent to lipocortin I (5), and a second form of “lipocortin” is equivalent to calpactin I (6). In other words, “calpactin I” equals “lipocortin II” and “calpactin II” equals “lipocortin I.” For simplicity, we will refer to these proteins as calpactins.

Calpactin I as isolated from intestine, lung, or lymphocytes can occur as a 38-kDa monomer or as a heterologous tetramer of two 38-kDa subunits and two 11-kDa subunits, the latter bearing homology to the S100 proteins (7–9). Calpactin II occurs only as a 35–37-kDa monomer and was originally identified in A431 cells on the basis of shared functional and antigenic properties with calpactin I. Further investigation revealed it to be the same protein as the 35-kDa epidermal growth factor receptor substrate described by Fava and Cohen (10, 11). One of the early controversies as to the identity of lipocortin I in different cell types may have its roots in the coexistence in many tissues (3) of the multisubunit antigenically related calpactin I, which in addition to its native subunits has also a protease-sensitive region separating a 33-kDa core from a 5-kDa tail (7). Sequence homology between calpactins I and II is greater than 50% in this central core region (3, 4), where Ca$^{2+}$ and phospholipid are known to bind (12, 13) and less than 20% in the amino-terminal tail which in calpactin I contains the site of tyrosine phosphorylation (7) and the binding site for the 11-kDa light chain (14).

In an effort to elucidate the functions of these related proteins, we have initiated studies on the nature of the inhibition of phospholipase A$_2$ by calpactins. We and others have developed kinetic approaches for analyzing phospholipase A$_2$ action (15–20), but due to the insoluble nature of the substrate, these analyses can be complex (21). Different extracellular phospholipase A$_2$s show variations in specificity for different polar head groups (22–24), so that caution must be applied when analyzing even simple substrate analogues (25), and the ability of an inhibitor to partition between aqueous and lipid phases or otherwise affect the aggregated state of the substrate must always be considered. Although it has been suggested (26) that the mechanism of lipocortin action is by direct binding to phospholipase A$_2$ to inactivate the enzyme, no experimental evidence has been given to support this claim.
Furthermore, calpactin I is known to interact with the cytoskeletal proteins spectrin and actin in the presence of high Ca"²⁺ and with phospholipids at micromolar concentrations of free Ca"²⁺ (12). Thus, we questioned whether direct binding of calpactin to the phospholipid membrane might not be responsible for phospholipase A₂ inhibition in vitro. We now report that calpactin I and the 38-kDa subunit of calpactin I both bind phospholipid in a Ca"²⁺-dependent manner and that both calpactins are equally potent in inhibiting phospholipase A₂ under certain assay conditions. A related 73-kDa protein is even more potent at inhibiting phospholipase A₂ and also binds to phospholipids. We show that calpactin inhibition of phospholipase A₂ is dependent on substrate concentration and is most probably due to substrate depletion by direct substrate/inhibitor complexation not involving a specific interaction with the enzyme.

**EXPERIMENTAL PROCEDURES**

**Isolation of Proteins—Calpactins I and II and the related 73-kDa protein were isolated from bovine lung by a modification of the previously described method (12), the details of which are provided elsewhere (5). The proteins were characterized by two-dimensional gel electrophoresis and shown to be the same protein (10) and, by Western blotting using Cohen's anti-p38 (27) and our anti-technical calpactin I (12) antibodies. By these criteria there was no detectable cross-contamination of the two calpactins. Peptide mapping of the calpactins was performed as described previously (10).

**Phospholipid Binding by Calpactins and the 73-kDa Protein—** Phospholipid binding by calpactin was monitored by sucrose flotation gradients at a defined free Ca"²⁺ concentration. Calpactin I, calpactin II, the 73-kDa protein, or cytochrome c (25 μg each) were mixed with phospholipid vesicles (50 μg) prepared by sonication in 10 mM imidazole, 40 mM KCl, 2 mM MgCl₂, 10 μM EDTA, and either 5 mM CaCl₂ (1 μM free Ca"²⁺) or 8.5 mM CaCl₂ (10 μM free Ca"²⁺) at pH 6.8. Vesicles were prepared by mixing 2 mg of phosphatidylserine (PS) with 2 mg of phosphatidylethanolamine (PE) (both from Sigma) and 1 μg (0.5 mg) of 1,2-dioleoyl-3-phosphatidyl-1-[14C]serine ([14C]PS) (Amerham Corp.), drying under vacuum, and sonication into 1 ml of 10 mM Tris-HCl, 50 mM NaCl, pH 8.0. The calpactin plus lipid solution was then added to 50% sucrose in the same buffer using an 80% sucrose stock solution. This was overlaid with a gradient of sucrose consisting of 150 pl each of 40% sucrose, 30% sucrose, 20% sucrose, and buffer only in a 5 ml ultracentrifuge tube and centrifuged at 50,000 rpm (Beckman SW 50.1 rotor) for 2 h. After centrifugation, the gradients were immersed in liquid N₂ and 8 fractions prepared by cutting into sections with a razor blade. The fractions were added to SDS-sample buffer, and equal amounts of each fraction were analyzed by SDS-PAGE, Coomasie Blue Staining, and quantitation by densitometry. The distribution of phospholipid in each fraction was determined by scintillation counting.

**Preparation of "H-Labeled E. coli—** Radiolabeled bacteria were obtained by a modified version of the procedure of Patricia et al. (28). E. coli (K-12C600) were grown to log phase in L-broth (400 ml), harvested by centrifugation at 50,000 rpm (Beckman SW 50.1 rotor) for 2 h. After centrifugation, the gradients were immersed in liquid N₂ and 8 fractions prepared by cutting into sections with a razor blade. The fractions were added to SDS-sample buffer, and equal amounts of each fraction were analyzed by SDS-PAGE, Coomasie Blue Staining, and quantitation by densitometry.

**Isolation of E. coli Phospholipid and Determination of Specific Radioactivity—** Cells (8 ml of the 40-ml suspension described above) were pelleted, resuspended in 1.6 ml of buffer, and extracted according to the method of Bligh and Dyer (30). The extract (0.7 μmol) was resuspended in CHCl₃, quantitated on the basis of inorganic phosphate (32, 33), and stored under N₂ at -20 °C. Extraction was 96% complete based on radioactive yield. The specific radioactivity of this lipid was 2.0 × 10⁶ cpm/nmol of P₃. Extragation of the original cell suspension by the procedure indicated that 1.8 × 10⁷ cells contained approximately 10 nmoi of phospholipid, in reasonable agreement with previously reported results (34).
Phospholipase A₂ Activity Toward [³H]Phospholipid Vesicles—To prepare stock solutions of E. coli-derived vesicles, phospholipid was extracted as described above, dried under N₂, and in vacuo, resuspended in 100 mM Tris-HCl, pH 8.5, 1 mM CaCl₂, and sonicated for 5 min, on ice, just before use. Assays were carried out with appropriate amounts of phospholipase A₂ and calpactin I or I₁ in 100 mM Tris-HCl, pH 8.5, 1 mM CaCl₂ in a total volume of 150 μl at 0 °C. Reactions were started with the addition of 1 ng of phospholipase A₂ and stopped by the addition of 663 μl of CHCl₃/MeOH, 1:2, containing 4 mM EDTA followed by standard Bligh and Dyer (30) extraction. Time points were chosen to achieve six to eight points per assay within a (linear) hydrolysis range of 1-5% (background hydrolysis was <1%).

Chloroform containing phospholipase reaction products were subjected to one-dimensional thin layer chromatography (34) on Silica Gel G plates in petroleum ether/ether/acetic acid, 80:20:1. The fatty acid and diacylglycerol plus phospholipid spots were retrieved and subjected to scintillation counting. Triacylglycerol radioactivity was found to be negligible. Percent hydrolysis was calculated as cpm of fatty acid divided by (cpm of fatty acid + cpm of phospholipid). For each assay condition, percent hydrolysis was plotted as a function of time and subjected to linear least squares analysis (N = 6-8). The slope thus obtained was used to calculate the hydrolysis rate (nmol min⁻¹ mg⁻¹). Nonzero slopes all gave P₀ (|r| > r₀) < 0.05, and the average P₀ (|r| > r₀) was less than 0.02. Error bars represent standard deviations from the calculated least squares fit. In experiments where there was no apparent phospholipase A₂ activity, least squares analysis also showed no statistical correlation for hydrolysis versus time (P₀ > 0.05), and slopes of zero were assigned. The standard deviation from the mean percent hydrolysis was always less than 0.10 in these cases.

Binding of Inhibitory Proteins to E. coli—To monitor the binding of phospholipase inhibitory proteins to bacteria, E. coli were grown in the absence of [³H]oleic acid followed by autoclaving and washing as above. Bacteria (20 μg of protein) and inhibitors (20 μg) were adjusted to 100 μl with 100 mM Tris-HCl, 10 mM CaCl₂, and incubated for 10 min on ice. The solutions were centrifuged at 13,000 × g for 5 min, and supernatant and pellet fractions were analyzed by SDS-PAGE and Coomassie Blue staining. Controls included a protein which does not bind lipid (cytochrome c) and solutions in which EGTA was omitted instead of Ca⁺² with 1% SDS.

RESULTS

Characterization of Calpactins—Calpactins I and II were purified from bovine lung and were homogeneous as assayed by high-resolution one- and two-dimensional SDS-polyacrylamide gel electrophoresis. Peptide mapping (Fig. 1) demonstrates that calpactins I and II are distinct from each other, whereas the mass of bovine calpactin II is strikingly similar to the peptide map of human calpactin II (p85) from A431 adenocarcinoma cells. Furthermore, amino acid sequence analysis of a truncated form of this molecule has revealed a 90% sequence identity to human lipocortin (5). Thus, the calpactin II used in this study is most probably the bovine equivalent of p85 or lipocortin. Although calpactin I was isolated as a complex of light and heavy chains, we removed the light chain subunit by gel filtration in 6 M guanidine followed by renaturation. This was done in order to compare calpactin I to calpactin II, which exists only as a monomer. All experiments in this report in which calpactin I was used were performed with this renatured preparation, although phospholipase A₂ inhibition was also observed with the complex of heavy and light chains (not shown).

Inhibition of Phospholipase A₂ by Calpactins and 73-kDa Protein—When calpactins I and II were used in any of the standard phospholipase A₂ kinetic assay systems employed in our laboratory on radioactive (35) or thiol-labeled substrate (16), containing 0.5-10 mM PE, PC, PS, or PI, or vesicles or micelles with 1-10 mM Ca⁺² and concentrations of calpactin up to 10 times greater than the enzyme (10⁻⁴-10⁻⁵ M), no inhibition was observed (data not shown). In striking contrast, when the substrate used was E. coli cells in amounts consistent with previously published work (29, 34), all three proteins displayed an apparent dose-dependent inhibition of phospholipase A₂ (Fig. 2). As shown in Fig. 2, increasing amounts of calpactin lead to a progressive decrease in the amount of free fatty acid released into the assay supernatant by phospholipase A₂, and the two calpactins display an identical inhibitory activity over a broad concentration range. Surprisingly, the 73-kDa protein is a more potent inhibitor of phospholipase A₂ in this system than the calpactins. Since this protein is approximately twice the molecular weight of calpactin, the half-maximal inhibition observed at an approximately 3-fold lower concentration by weight represents a 6-fold lower molar concentration when compared to calpactin.

Because the E. coli assay measures only BSA-extractable radioactivity, the inhibition could potentially be an artifact if calpactin caused free fatty acid to remain bound to the E. coli membrane. This was found not to be the case. The distribution of free fatty acid label in E. coli lipids following phospholipase A₂ treatment of the cells was measured by quantitative extraction and TLC. As shown in Table I, this method of analysis confirms that the presence of calpactin in the assay results in an actual decrease in hydrolysis of cellular phospholipids by phospholipase A₂. The distribution of radioactivity in E. coli lipids before phospholipase A₂ treatment, with or without calpactin, is similar to previously reported results (28). When the cells are reacted with phospholipase A₂, the distribution remains the same except for a decrease corresponding to 20% of total radioactivity from the PE/PG in the E. coli lipid spot and an equivalent increase in free fatty acid. This represents 40% hydrolysis of the PE and PG. Inclusion of 7.5 μg of calpactin in the assay results in reversion to the original distribution, that is, 100% inhibition of phospholipase A₂ activity. By comparison, 93% inhibition was observed under identical assay conditions using BSA extraction and centrifugation to analyze [³H]oleic acid release (data not shown).

Interaction of Calpactins and 73-kDa Protein with Phospholipid Vesicles—Calpactin I is known to bind to acidic phospholipids, and this property has been used to identify calpactins I and II in A431 cells. Since the ability to bind to phospholipids may be important for calpactin's phospholipase inhibitory activity, we tested calpactins I and II and the 73-kDa protein for this property. The proteins were mixed with phospholipid vesicles (using [¹⁴C]PS as tracer), adjusted to 60% sucrose, and centrifuged through a sucrose flotation gradient. As shown in Fig. 3, the extent of association of calpactins with vesicles is dependent on the free Ca⁺² in the solution. At 1 μM free Ca⁺², <25% of the calpactin is associated with the lipid, whereas at 10 μM free Ca⁺², >95% of the calpactin is vesicle bound. Both calpactin I and II behave identically in this assay. In addition, the 73-kDa protein is also tightly associated with vesicles at 10 μM free Ca⁺², whereas the control protein, cytochrome C, is not.

Binding of Calpactins to Substrate Rather than Enzyme in E. coli Assay—An obvious question was whether calpactins I and II and the 73-kDa protein bind directly to the E. coli which are used as substrate in the phospholipase assay. The interaction of calpactin I and II and the 73-kDa protein with the E. coli membrane was monitored using a simple centrifugation method. Bacteria with bound protein were pelleted
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A

electrophoresis

Fig. 1. Peptide maps of calpactins I and II. Bovine lung calpactin I (A) and calpactin II (B) were resolved by SDS-PAGE and stained with Coomassie Blue. Bands were excised, labeled with \(^{131}I\), and digested with chymotrypsin. Soluble peptides were separated by electrophoresis and chromatography and visualized by autoradiography. For comparison, the peptide map of p35 from A431 cells is shown (C). Arrows in panels B and C indicate some of the peptides in common between bovine calpactin II and human calpactin II (p35 or lipocortin).

![Peptide maps of calpactins I and II.](image)

B

by low speed centrifugation, and aliquots of the supernatant and pellet fractions were analyzed by SDS-PAGE and Coomassie Blue staining. As shown in Fig. 4, all 3 of the proteins which inhibit phospholipase A\(_2\) also bind to the bacteria. Consistent with the 73-kDa protein being a more potent inhibitor of phospholipase, a higher percentage of 73-kDa protein binds to the bacteria than is the case with calpactin. When the bacteria or Ca\(^{2+}\) are omitted, negligible amounts of inhibitory protein are found in the pellet fraction. Cytochrome c, which did not inhibit the phospholipase A\(_2\), also does not bind to the bacteria (under conditions of high Ca\(^{2+}\)).

To assess whether calpactin II binds to phospholipase A\(_2\), a solution of calpactin in the same buffer used for phospholipase A\(_2\) assays was applied to a column of phospholipase A\(_2\) immobilized on Sepharose. Although phospholipase A\(_2\) on the column was present in greater than 100-fold molar excess to calpactin, more than 95% of the calpactin passed through unretarded, and no further protein was eluted with EGTA or 1% SDS (data not shown).

**Effects of Preincubation with Enzyme or Substrate**—Typically, when *E. coli* are used to assay lipocortin inhibition, there is a preincubation period of 10 min for inhibitor protein plus phospholipase A\(_2\) before the reaction is started. However, we found that preincubation of calpactin I with phospholipase A\(_2\) up to 60 min does not affect the inhibition when concentrations of enzyme, inhibitor, and substrate are constant (data not shown). On the other hand, since calpactin binds tightly to phosphatidylserine (PS), we asked whether preincubation of calpactin with unlabeled PS has any effect on phospholipase A\(_2\) inhibition by calpactin. As shown in Fig. 5, when PS vesicles alone are added to the phospholipase assay mix, the amount of \(^{3}H\) label released from the *E. coli* is reduced by 40-70%, probably due to competition with and dilution of *E. coli* lipids. When the same levels of PS are added to the assay in

| Table I |
| --- |
| Distribution of \(^{3}H\) label in *E. coli* lipids |

| Addition* | Fatty acid | PE + PG | Lyso phospholipid | Cardiolipin, phosphatidic acid | Diacylglycerol | Triacylglycerol |
| --- | --- | --- | --- | --- | --- | --- |
| None | 6 | 61 | 2 | 22 | 10 | 1 |
| Calpactin I | 8 | 59 | 2 | 18 | 11 | 2 |
| Phospholipase A\(_2\) | 27 | 43 | 2 | 20 | 7 | 1 |
| Phospholipase A\(_2\) + calpactin I | 4 | 66 | 2 | 20 | 7 | 1 |

*Proteins were incubated in 150 \(\mu\) of 100 mM Tris-HCl buffer, pH 8.5, containing 10 mM CaCl\(_2\) for 10 min at 0°C. Then \(^{3}H\)-labeled *E. coli* cells were added (2 \(\mu\)g of protein, 0.32 nmol of phospholipid), and the mixtures were incubated 10 min further at 0°C before addition of CHCl\(_3\)/MeOH 1:2, and extraction as described under "Experimental Procedures." Calpactin I, when used, was 7.5 \(\mu\)g/assay, and phospholipase A\(_2\), when used, was 174 ng/assay. The average of duplicates is reported. Data are expressed as percent of total radioactivity.
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The solution was overlaid with a solution of vesicles of [14C]PS and PE in a buffer containing either 1 µM free Ca2+ (a, c) or 10 µM free Ca2+ (b, d, e, f) in 60% sucrose. The solution was centrifuged at 120,000 x g for 2 h. Fractions were analyzed for radioactivity (E) by scintillation counting and for protein (F) by SDS-PAGE, Coomassie staining, and quantitation with a densitometer. The phospholipid codistributes with the calpactins and 73-kDa protein only at 10 µM Ca2+.

Combination with calpactin I (at a concentration which previously resulted in 65% phospholipase inhibition) the inhibitory effects of both agents are abrogated. This suggests that calpactin and PS form a complex which is not an inhibitor of phospholipase A2.

Inhibition by Calpactin Is Dependent on Phospholipid Substrate Concentration—The entire E. coli membrane is not needed in order to observe inhibition. E. coli were extracted with chloroform/methanol to obtain their phospholipid components free of proteins and polysaccharides. When the phospholipid mixture was reconstituted into vesicles and used to assay phospholipase A2, calpactin I still showed an ability to inhibit the enzyme in a sigmoidal dose-dependent manner if the substrate concentration was very low, as is shown in Fig. 6. The ratio of calpactin I to phospholipase A2 at half-maximal inhibition is 1000:1 when the phospholipid is 5 µM. Time courses of phospholipase A2 action on this substrate are linear up to about 10% hydrolysis with the exception of a small rapid burst immediately after addition of the enzyme, lasting less than 15 s and accounting for less than 1% hydrolysis (data not shown). Thus, by taking 6–8 time points/assay in the linear region, it is possible to determine rates of hydrolysis with which to carry out a kinetic analysis of the inhibition. When this was done, it was found that inhibition by calpactin I is totally abolished at sufficiently high concentrations of substrate (Fig. 7A). Furthermore, the shape of the inhibition curve is sigmoidal and, significantly, not hyperbolic. In this experiment, calpactin and phospholipase A2 concentrations are held constant, and only the substrate concentration is varied. If the inhibition were due to a simple noncompetitive mechanism, as has been suggested (26), then under these conditions the inhibited sample should show a hyperbolic substrate dependence, and the rates of hydrolysis in inhibited versus control samples should be a constant ratio regardless of substrate concentration. But, as can be seen in Fig. 7B, the percent inhibition by calpactin I goes from 100 to 0% as the
Lipase described under "Experimental Procedures." Note that half-maximal concentration and the velocity determined from the time course as phospholipid vesicles in the presence of potent inhibitors of phospholipase A2, and regulated by phospholipid which had been extracted from E. coli sonicated vesicles was kept constant at 5 µM. The reaction was started by the addition of phospholipase A2 (5.1 x 10^{-10} M). Standard assay conditions were used. Six time points were taken for each calpactin concentration and the velocity determined from the time course as described under "Experimental Procedures." Note that half-maximal inhibition was achieved with approximately 3 µg of calpactin I or 5.3 x 10^{-9} M.

Fig. 6. Dose response of calpactin I on the rate of phospholipase A2 hydrolysis of phospholipid vesicles. Substrate phospholipid which had been extracted from E. coli and reconstituted into sonicated vesicles was kept constant at 5 µM. The reaction was started by the addition of phospholipase A2 (5.1 x 10^{-10} M). Standard assay conditions were used. Six time points were taken for each calpactin concentration and the velocity determined from the time course as described under "Experimental Procedures." Note that half-maximal inhibition was achieved with approximately 3 µg of calpactin I or 5.3 x 10^{-9} M.

Fig. 7. Dependence of calpactin I inhibition on the concentration of extracted E. coli phospholipid. A, velocity versus substrate concentration for phospholipase A2 acting on extracted phospholipid vesicles in the presence (C) or absence (○) of calpactin I. The concentration of phospholipase A2 (5.1 x 10^{-10} M) and calpactin I (1.1 x 10^{-1} M) was kept constant as the concentration of E. coli-derived phospholipid vesicles was increased. Each point (including error) was calculated from a time course as described under "Experimental Procedures." B, percent inhibition is shown as a function of the substrate concentration for the same system.

substrate concentration is raised.

The result so obtained for inhibition as a function of E. coli-derived phospholipid concentration is consistent with the preliminary observation that micromolar amounts of calpactin I did not inhibit phospholipase A2 at millimolar amounts of pure phospholipid substrate. To confirm that this substrate concentration effect is also operative in the E. coli whole cell assay, the corollary experiment was done (Fig. 8). As can be seen, at high concentrations of E. coli, the inhibition is reduced to zero, although a much broader substrate concentration range must be covered in order to achieve the effect.

DISCUSSION

Lipocortins and Calpactins—The "lipocortins" comprise a class of proteins which are thought to be induced by steroids, potent inhibitors of phospholipase A2, and regulated by phosphorylation (1, 2). These observations have been incorporated into a general model for cellular responses such as the activation of thymocytes and platelets (26, 36). According to this scheme, cellular activation leads to the phosphorylation of lipocortin which then loses its inhibitory activity toward phospholipase A2. This would cause an increase in phospholipase A2 activity, thereby increasing formation of one of its products, arachidonic acid, which is a precursor of prostaglandins and leukotrienes. Although this is an attractive model, key features have not been rigorously established. Most importantly, it has not yet been demonstrated that lipocortin is a specific inhibitor of phospholipase A2 in vitro or in vivo. In fact, earlier work (26) suggested that it may inhibit phospholipases C and D equally as well as phospholipase A2.

In our recent investigations on Ca^{2+} binding by calpactins, proteins which have been shown to be equivalent to lipocortins, it was noticed that phospholipids increase the affinity of calpactin for Ca^{2+} (12). This was due to the direct interaction of calpactin with lipid and suggested that this interaction could also be responsible for the phospholipase A2 inhibitory effect others have found. Three inhibitory proteins were tested herein: (i) calpactin I which is equivalent to the 36-kDa tyrosine kinase substrate first identified in chick embryo fibroblasts transformed by Rous sarcoma virus; (ii) calpactin II, a substrate of the epidermal growth factor receptor tyrosine protein kinase; and (iii) a related 73-kDa protein which has been shown to co-purify with calpactin and partially colocalize with spectrin in fibroblasts (37). This protein is probably one of the calcineurins (38) or calselectins (39), has also been isolated from lymphocyte membranes (40), and has the property of binding to hydrophobic resins in a Ca^{2+}-dependent manner. It may also be the 70-kDa inhibitor of phospholipase A2 that has been noted in cell extracts in studies of lipocortin. When assayed for inhibition of phospholipase A2 using E. coli as substrate, all three proteins were found to be inhibitory, although at ratios far from stoichiometric with the enzyme.

In order to analyze the mechanism of this inhibition, we tested calpactins for (i) their ability to interact with enzyme and/or substrate, (ii) their ability to bind to pure phospholipid, (iii) inhibition of phospholipase A2 after preincubation
of inhibitor with phosphatidylserine, (iv) dose-dependent inhibition of phospholipase A₂ using lipids extracted from E. coli, and (v) inhibition of phospholipase A₃ as a function of substrate concentration, using both E. coli cells and extracted lipid. All three proteins were found to bind E. coli substrate and synthetic PS/PE vesicles directly. PS was capable of abolishing the inhibitory effect of calpactin I, perhaps by removing the protein from solution, and no evidence was seen for a phospholipase A₂-calpactin complex.

**Phospholipase A₂ Inhibition toward E. coli Membranes**—In initial experiments using standard assays for phospholipase A₂, calpactins were found to have no significant effect on synthetic phospholipid hydrolysis. However, a commonly used method to test for lipocortin-type activity involves the use of [³H]oleic acid-labeled E. coli, and when we tested the calpactins against this substrate, we found a marked inhibition of phospholipase A₂ in the assay. In vitro assays for phospholipase A₂ inhibition by lipocortin, which have relied primarily on the activity of an extracellular phospholipase A₂ toward phospholipid in these radiolabeled autoclaved E. coli or else toward synthetic PC, have one striking feature in common. They employ very low concentrations of a substrate on which the enzyme has unusually low activity. Phosphatidylcholine is generally 0.2 mM or less (26, 41, 42), while published reports of lipocortin inhibition on E. coli have not always provided exact growth conditions or specific radioactivities of the labeled phospholipid with which to reproduce substrate concentrations exactly (for example, see Ref. 29). Following the well-established methods of Elsbach and co-workers (28, 34), however, we obtained [³H]oleic acid-labeled cells that are probably labeled to an extent similar to that for cells used in previous lipocortin studies. The amounts used in our assay were chosen on the basis of total cpm to correspond with them, and the resulting concentration of lipid was always less than 10 μM.

Whole E. coli cells did not, however, make a satisfactory substrate with which to explore the kinetics of inhibition further. For this reason, the bacterial phospholipids were quantitatively extracted and reconstituted into sonicated vesicles. On this substrate, inhibition of phospholipase A₂ was still observed by calpactin I so long as the substrate concentration was very low. It is noteworthy that although the calpactin inhibition was dose-dependent, the midpoint of inhibition occurred at a molar ratio of 1000:1 calpactin to phospholipase A₂ when the substrate was 5 μM, and we have observed that this ratio varies with the concentration of substrate used.

**Kinetics of Phospholipase A₂ Inhibition**—Previous reports suggested that the inhibition of phospholipase A₂ by lipocortin is noncompetitive (26, 41). At least in the case of one form, calpactin I, our results are in substantial disagreement. This is seen in the sigmoidal shape of the velocity versus substrate concentration curve for the calpactin-containing sample in Fig. 7A. This sigmoid behavior could not be obtained for simple noncompetitive inhibition in which the inhibitor alters the $V_{\text{max}}$ but not the $K_{\text{m}}$ of the enzyme for substrate. In addition, competitive inhibition and inhibition requiring substrate plus calpactin to be the true inhibitor should both produce hyperbolically shaped curves in an experiment of this type, in contrast to the observed results. On the other hand, the sigmoidal shape observed here is that which would be expected for inhibition due to substrate depletion. Such a kinetic scheme is shown in Equation 1.

$$
K_{\text{m}} \frac{E + S}{K_{\text{m}} S + I} \rightarrow E + P
$$

where $E$ is phospholipase A₂, $S$ is substrate, $P$ is product, and $I$ is calpactin. $E$ is the Michaelis complex, and $SI$ the substrate-calpactin complex which is inactive. As in standard Michaelis-Menten kinetics, the velocity ($v$) is still given by Equation 2,

$$
v = \frac{V_{\text{max}} [S]}{K_{\text{m}} + [S]}
$$

but $[S]$, the concentration of free substrate is now given by Equation 3,

$$
[S] = \frac{([I]_o - [I]_t) + K_{I}^4 + 4K_{I}[S])^{1/2}}{2}
$$

where $[S]_o$ is the total substrate concentration and $[I]_o$ is the total calpactin concentration (43). The degree of sigmoidicity of the resulting velocity curve is dependent on the relative values of $K_I$ and $K_{I}^4$, as well as the concentrations of enzyme and inhibitor used in the assay. No attempt was made to determine $K_I$ and $K_{I}^4$ in this complex lipid system since these constants would in fact be complex functions of the lipid composition. However, it should now be possible to investigate this distinctive kinetic behavior on pure lipid substrates, for which these constants can be determined. Although the sigmoidal velocity curve obtained argues against simple competitive or noncompetitive inhibition mechanisms, it will be fully diagnostic for substrate depletion only if the exact velocity curve can be predicted on the basis of independently determined constants, $K_{m}, K_{I}$, and $V_{\text{max}}$.

The substrate depletion model appears the most attractive at this stage, especially in light of the binding and competition studies with PS, but it is important to know why the inhibition was previously attributed to noncompetitive inhibition. This might be explained by the very low affinities of phospholipase A₂ and possible differences in affinities of the calpactins for the different substrates used. For example, in the substrate dependence curve for E. coli whole cells, the same type of concentration dependence is seen as with vesicles, but an unusually large concentration range has to be covered, on the order of 200-fold. If only the lower substrate concentrations (1–100 μM) had been investigated, the percent inhibition could have been construed to be the same, within error, regardless of substrate concentration. This would have been the expected result for noncompetitive inhibition. The reason that such a large concentration range must be covered, relative to the vesicles, in order to see depletion of inhibition could be due to different $K_m$ and/or $K_I$ values for the two substrate mixtures. A different composition and smaller quantity of phospholipids may be exposed to the surface in cells than is the case with vesicles at a given total lipid concentration (44). In fact, it has been our observation that the enzyme appears to have a 10-fold lower apparent $K_m$ for the sonicated vesicles than for the cells, although the highest overall rate achieved is approximately the same on both substrates. In a similar vein, long-chain phosphatidylcholine, the other substrate used in lipocortin studies, usually displays a $K_m$ for porcine pancreatic phospholipase A₂ that is in the millimolar range, in contrast to the micromolar apparent $K_m$ observed for phospholipase A₂ on these E. coli lipid vesicles, although differences in lipid preparation can drastically affect these constants (21, 45).

It is well known that extracellular phospholipase A₂ activity is highly dependent on the physical state of the substrate (21, 45). The hydrolysis products, lysophospholipid and fatty acid, when present together, cause concentration-dependent changes in the phase transition properties of aggregated phos-
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In summary, the most likely explanation for calpastatin inhibition of pancreatic phospholipase A₂ in vitro is substrate depletion. The physiological significance of this mechanism of inhibition is not yet clear. It does appear that inhibition by calpastatin I, and perhaps also calpastatin II, is not specific for phospholipase A₂ in terms of enzyme-inhibitor interactions. Rather, the inhibitor appears to be specific for the phospholipid substrate. If in fact calpastatin does inhibit phospholipase A₂ in vivo, it may be due to interactions of calpastatin with phospholipids (and possibly the cytoskeleton) that affect local membrane organization or else block access to the enzyme. But if this were the case, other lipolytic enzymes and other membrane components besides phospholipase A₂ would be expected also to be affected. This type of activity could explain the early observation by Hirata (26) of phospholipase C and phospholipase D inhibition by lipomodulin in vitro (26). It is still possible that crude cellular extracts contain a mixture of the calpactins and one or more other similarly sized proteins that specifically bind phospholipase A₂. As the assays used until now to monitor lipocortin activity do not distinguish between lipid-binding and phospholipase A₂-binding activities, investigators may have been misled, particularly in view of the relative abundance of the calpactins (which account for up to 1% of cellular protein). It is thus evident that the role of lipocortin in phospholipase A₂ inhibition requires further definition. Of primary importance will be the evaluation of lipocortins from different sources to see whether they too show the behavior demonstrated here for bovine lung calpastatin I.

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