A Calcium-dependent Mechanism for Associating a Soluble Arachidonoyl-hydrolyzing Phospholipase A2 with Membrane in the Macrophage Cell Line RAW 264.7*

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Arachidonoyl-hydrolyzing phospholipase A2 plays a central role in providing substrate for the synthesis of the potent lipid mediators of inflammation, the eicosanoids, and platelet-activating factor. Although Ca\(^{2+}\) is required for arachidonic acid release in vivo and most phospholipase A2 enzymes require Ca\(^{2+}\) for activity in vitro, the role of Ca\(^{2+}\) in phospholipase A2 activation is not understood. We have found that an arachidonoyl-hydrolyzing phospholipase A2 from the macrophage-like cell line, RAW 264.7, exhibits Ca\(^{2+}\)-dependent association with membrane. The intracellular distribution of the enzyme was studied as a function of the Ca\(^{2+}\) concentration present in homogenization buffer. The enzyme was found almost completely in the 100,000 x g soluble fraction when cells were homogenized in the presence of Ca\(^{2+}\) chelators and there was a slight decrease in soluble fraction activity when cells were homogenized at the level of Ca\(^{2+}\) in an unstimulated cell (80 nM). When cells were homogenized at Ca\(^{2+}\) concentrations expected in stimulated cells (230-450 nM), 60-70% of the phospholipase A2 activity was lost from the soluble fraction and became associated with the particulate fraction in a manner that was partly reversible with EGTA. Membrane-associated phospholipase A2 activity was demonstrated by [\(^{3}H\)]arachidonic acid release both from exogenous liposomes and from radiolabeled membranes. With radiolabeled particulate fraction as substrate, this enzyme hydrolyzed arachidonic acid but not oleic acid from membrane phospholipid, and [\(^{3}H\)]arachidonic acid was derived from phosphatidylycholine, phosphatidylethanolamine, and phosphatidylinositol/phosphatidylserine. We suggest a mechanism in which the activity of phospholipase A2 is regulated by Ca\(^{2+}\): in an unstimulated cell phospholipase A2 is found in the cytosol; upon receptor ligation the cytosolic Ca\(^{2+}\) concentration increases, and the enzyme becomes membrane-associated which facilitates arachidonic acid hydrolysis.

The enzyme, phospholipase A2, is thought to play a central role in providing arachidonic acid for subsequent metabolism to prostaglandins and leukotrienes, potent lipid mediators of inflammation. Hydrolysis of the ether linked phospholipid, 1-O-alkyl-2-arachidonoyl-glycerophosphocholine, by phospholipase A2 also releases the precursor of another potent lipid mediator, platelet-activating factor. Hence, an understanding of the regulation of arachidonoyl-hydrolyzing phospholipase A2 may ultimately allow us to control for some of the deleterious aspects of inflammation. The mechanisms involved in the regulation of intracellular phospholipase A2 enzymes in general are incompletely understood. A role for Ca\(^{2+}\) in arachidonoyl-hydrolyzing phospholipase A2 activation is strongly suggested by the following findings: incubation of permeabilized rat mesangial cells with increasing concentrations of Ca\(^{2+}\) resulted in increased arachidonic acid release (1); in inflammatory cells Ca\(^{2+}\) ionophore could induce arachidonic acid release (2, 3) and immune complex- and calcium ionophore-induced arachidonic acid release was absolutely dependent on extracellular Ca\(^{2+}\) (3). Furthermore, many arachidonoyl-hydrolyzing enzymes showed an absolute requirement for Ca\(^{2+}\) in vitro (4-8). However, it is not known whether the role of Ca\(^{2+}\) is to regulate the activity of the enzyme itself or to regulate some other transduction mechanism(s) leading to phospholipase A2 activation. In platelets (6) and mesangial cells (8), the intracellular distribution of arachidonoyl-hydrolyzing phospholipase A2 activity has been shown to be dependent on the Ca\(^{2+}\) content of the buffer used for cell disruption. When cells were homogenized in the presence of Ca\(^{2+}\) chelators, phospholipase A2 activity was almost entirely cytosolic (5-8); in the presence of a high concentration of Ca\(^{2+}\) (>1 mM), there was a loss of cytosolic activity and a concomitant increase in membrane-associated activity (6, 8). These observations may reflect a regulatory role for Ca\(^{2+}\) in the activation of phospholipase A2 by promoting its association with membrane, thus facilitating arachidonic acid hydrolysis. However, the level of Ca\(^{2+}\) that is required for this phenomenon has not been investigated previously. We have described the purification of an arachidonoyl-specific phospholipase A2 from the macrophage-like cell line RAW 264.7 (7). We now report that the intracellular distribution of this phospholipase A2 changes from cytosolic, when cells are homogenized in Ca\(^{2+}\) concentrations expected in unstimulated cells, to membrane-associated, when cells are homogenized at Ca\(^{2+}\) concentrations expected in stimulated cells. When membrane-associated, the phospholipase A2 was found to hydrolyze arachidonic acid, but not oleic acid, from membrane phospholipid.

EXPERIMENTAL PROCEDURES

Isolation of Soluble and Particulate Fractions from Cells in Suspension Culture—The murine macrophage cell line, RAW 264.7, was...
Intracellular Phospholipase A2 Distribution and Ca**

To determine the distribution of intracellular phospholipase A2 following cell disruption in buffer containing varying amounts Ca**+, cells were washed twice in phospho-buffered saline, pH 7.2, and once in homogenization buffer (140 mM KCl, 10 mM NaCl, 2.5 mM MgCl2, 0.5 mM EDTA, and 10 mM HEPES/KOH, pH 7.2, with or without calcium) by centrifugation at 250 × g for 10 min. The washed cell pellet was resuspended to a concentration of 125 × 10^6 cells/ml in homogenization buffer containing the protease inhibitors, phenylmethylsulfonyl fluoride (PMSF) (1 mM) and leupeptin (1 μg/ml). Homogenization buffer containing free Ca**+ concentrations less than 1 μM was prepared using the fluorescent calcium indicator, indo-1 (Molecular Probes, Eugene, OR), as described (10). The solution was first depleted of Ca**+ by the addition of a final concentration of 100 mM EGTA and then Ca**+ was added back until the required concentration was reached. Cells in homogenization buffer were sonicated (Bransonic 2000, B. Braun Instruments, Bridgewater, NJ), which resulted in 100% disruption. The homogenate was ultracentrifuged at 100,000 × g for 60 min to produce a soluble fraction containing cytosol and resuspended in 50 mM Tris buffer, pH 7.3, containing 10 mM EGTA, 1 mM NaCl, 1 mM PMSF, and 1 μg/ml leupeptin. After 30 min incubation on ice, the particulate fraction was ultracentrifuged (100,000 × g for 60 min), the supernatant was removed, and the pellet was resuspended in incubation buffer. This incubation cycle was repeated and the final pellet was resuspended in 50 mM Tris buffer, pH 8, containing 1 mM PMSF and 1 μg/ml leupeptin. Particulate fraction phospholipase A2 activity was measured by assaying the pellet and both supernatants. Protein was determined by the method of Lowry et al. (11) using bovine serum albumin as a standard.

Isolation of Soluble and Particulate Fractions from Radiolabeled Cells—Membranes labeled with [H]arachidonic acid were used as substrate in an alternate approach to determine the distribution of intracellular phospholipase A2 activity and also in experiments to determine the phospholipid species that were hydrolyzed by both partially purified soluble fraction phospholipase A2 and membrane-associated phospholipase A2. Membranes labeled with [H]arachidonic acid or with [3H]oleic acid were used in experiments to determine the specificity of membrane-associated phospholipase A2 for membrane phospholipid fatty acid. Cells are suspension culture, and homogenized as described above, and containing 140 mM NaCl and the protease inhibitors, PMSF and 1 μg/ml leupeptin. Approximately 100,000 x g centrifugation were obtained as described above. The particulate fraction was washed twice in homogenization buffer to remove contaminating cytosol and resuspended in 50 mM Tris buffer, pH 8, containing 1 mM NaCl and 1 μg/ml leupeptin. Approximately 80-85% of the [H]arachidonic acid was taken up by the cells, and phospholipid species from particulate fractions contained [H]arachidonic acid in the following proportions: PC = 18 ± 1%; PI/PS = 20 ± 1%; PE = 48 ± 2%; where values are expressed as the mean ± S.E. for eight experiments. The values for [H]oleic acid incorporation into phospholipid were: PC = 50%; PI/PS = 11%; PE = 23%, where values are representative of two experiments.

Liposomal Substrate—The phospholipase A2 assay measured the hydrolysis of [H]arachidonic acid from 1-O-hexadecyl-2-[H]arachidonoyl-glycerophosphocholine as described previously (9). The reaction mixture consisted of a sonicated dispersion of 30 μM 1-O-hexadecyl-2-[H]arachidonoyl-glycerophosphocholine (75,000 dpm) containing 30 mol% 1-stearoyl-2-arachidonoyl-sn-glycerol (Sigma), 500 μg/ml delipidated human serum albumin, and 10 mM CaCl2 in 50 mM Tris buffer, pH 8 (100 μl). The diacylglycerol and delipidated human serum albumin (7) have been shown to enhance the activity of arachidonoyl-hydrolyzing phospholipase A2 isolated from RAW 264.7 cells. The reaction was started by the addition of macrophase protein (50 μg) and incubated at 37°C in a shaking water bath for 1 min.

Membrane Substrate—In experiments using radiolabeled cells, phospholipase A2 activity associated with the particulate fraction was determined by measuring the hydrolysis of [H]-fatty acid from endogenously-labeled membrane phospholipid substrate. The assay mixture contained [3H]-fatty acid-labeled particulate fraction (100,000 dpm) and 10 mM CaCl2 in 50 mM Tris buffer, pH 8 (100 μl). Immediately after the addition of particulate fraction (0 min) and at the indicated times, lipids were extracted (12) and PC/PS were separated by thin layer chromatography. The solvent system was chlorofluoromethanol/acetate/water (50:25:8:4) where PI and PS were scavenged together. Lipid standards were visualized with iodine, lipid species were scraped, and radioactivity was determined by liquid scintillation spectrometry. Membranes were tested as a substrate for soluble fraction phospholipase A2, the enzyme was partially purified as described previously (9).

RESULTS AND DISCUSSION

The Effect of the Concentration of Ca**+ in Homogenization Buffer on the Intracellular Distribution of Arachidonoyl-Hydrolyzing Phospholipase A2 Determined Using Liposomal Substrate. To examine the effect of Ca**+ on the intracellular distribution of arachidonoyl-hydrolyzing phospholipase A2, RAW 264.7 cells were disrupted in the presence of 100 μM EGTA or increasing amounts of Ca**+, soluble and particulate fractions were isolated, and the phospholipase A2 activity of each fraction was determined in the presence of 10 mM Ca**+ using a liposomal substrate. As shown in Fig. 1, soluble fraction phospholipase A2 activity was highest when cells were homogenized in the presence of 100 μM EGTA and increasing amounts of Ca**+, soluble and particulate fractions were isolated, and the phospholipase A2 activity of each fraction was determined in the presence of the range of Ca**+ concentrations (10-100 μM). The concentration of Ca**+ found in an unstimulated cell (15). A decrease in soluble fraction phospholipase A2 activity of 60 and 70% was seen when cells were homogenized in the presence of 235 and 450 mM Ca**+, respectively, the range of Ca**+ in a stimulated cell (13). Only a small additional decrease in soluble fraction phospholipase A2 activity was seen when cells were homogenized in the presence of higher concentrations of Ca**+, demonstrating that the process was almost complete at physiological Ca**+ levels. For these experiments an homogenization buffer containing 140 mM NaCl and 1 mM KCl was used to simulate the cytosolic environment of the enzyme. The possibility that these results could in part be due to depolarization caused by the high [K+] of this buffer was excluded as similar results were seen when cells were homogenized in 5 mM HEPES or in buffer containing 140 mM NaCl and 10 mM KCl (data not shown). Neither could this effect be explained by major differences in protein distribution following exposure to Ca**+ as approximately 50% of the total protein was found in the soluble fraction for each of the homogenates. Similar results were obtained whether cells were homogenized by sonication or by nitrogen cavitat

1 The abbreviations used are: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine.

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Intracellular Phospholipase A<sub>2</sub> Distribution and Ca<sup>2+</sup>

The decrease in soluble fraction activity. Fig. 1 shows a maximum of 33% of the decrease in soluble fraction phospholipase A<sub>2</sub> activity recoverable from the particulate fraction when cells were homogenized at Ca<sup>2+</sup> concentrations greater than 230 nM.

When cells were homogenized in the presence of 100 μM EGTA and then the homogenate was adjusted to 100 μM Ca<sup>2+</sup> before ultracentrifugation, the same loss of soluble fraction phospholipase A<sub>2</sub> activity was observed, suggesting an intact cell was not required for this phenomenon to be observed.

We then tested the hypothesis that the decrease in soluble fraction phospholipase A<sub>2</sub> activity, seen when increasing amounts of Ca<sup>2+</sup> were present during homogenization, was concomitant with an increase in membrane-associated phospholipase A<sub>2</sub> activity. A direct relationship between the decrease in soluble fraction phospholipase A<sub>2</sub> activity and the increase in membrane-associated activity was found (Fig. 1).

Very little activity was detected in the particulate fraction when cells were homogenized in the presence of 100 μM EGTA, and highest activity was detected when cells were homogenized in the presence of 450 nM Ca<sup>2+</sup>. When cells were homogenized at Ca<sup>2+</sup> concentrations greater than 230 nM, the activity recovered from the particulate fraction was less than the decrease in soluble fraction activity. Fig. 1 shows a maximum of 33% of the decrease in soluble fraction phospholipase A<sub>2</sub> activity recoverable from the particulate fraction when cells were homogenized in the presence of 450 nM Ca<sup>2+</sup>. Recovery was found to be variable and at best up to 75% of the decrease in soluble fraction phospholipase A<sub>2</sub> activity was recoverable on the particulate fraction under the optimal conditions described below. The inability to quantitatively recover the enzyme from the particulate fraction may be due to losses of enzyme activity sustained by experimental procedures, to the presence of Ca<sup>2+</sup>-dependent proteases inactivating phospholipase A<sub>2</sub> even in the presence of PMSF and leupeptin, or to a fraction of the phospholipase A<sub>2</sub> becoming integral membrane protein as has been suggested for protein kinase C (14).

To detect phospholipase A<sub>2</sub> activity from the particulate fraction that was isolated from cells homogenized in the presence of 100 μM Ca<sup>2+</sup>, it was necessary to dissociate the enzyme from the membrane with the Ca<sup>2+</sup> chelator, EGTA (Fig. 2). When particulate fraction was incubated in the presence of 100 μM Ca<sup>2+</sup> and then ultracentrifuged, very little phospholipase A<sub>2</sub> activity was recoverable in the supernatant. When Ca<sup>2+</sup> was chelated with EGTA, the total measurable phospholipase A<sub>2</sub> activity from the particulate fraction increased 3- to 4-fold compared to keeping membranes in the presence of Ca<sup>2+</sup>, suggesting a Ca<sup>2+</sup>-dependent association of the enzyme with the membrane. Increasing the ionic strength of the incubation buffer to 1 M NaCl only slightly increased the amount of phospholipase A<sub>2</sub> activity recovered. If the particulate fraction was incubated with 10 mM EGTA and 1 M NaCl but was not ultracentrifuged, the total phospholipase A<sub>2</sub> activity was always less than that measurable following an ultracentrifugation step. When the pellet was resuspended in incubation buffer for an additional 30 min on ice and ultracentrifuged, approximately 50% of its phospholipase A<sub>2</sub> activity was now recoverable in the resulting supernatant. A third incubation and ultracentrifugation did not release any further phospholipase A<sub>2</sub> activity into the supernatant.

In experiments not shown, particulate fraction was also tested with a variety of detergents in an attempt to improve recovery of the enzyme. However, the phospholipase was completely inhibited by detergents at their critical micellar concentration and it was necessary to dilute the detergent 10- to 100-fold in the phospholipase A<sub>2</sub> assay to minimize inhibition. Using this approach, no further enzyme was recoverable. The properties of the soluble enzyme recovered from the particulate fraction were similar to those described previously for soluble fraction arachidonoyl-specific phospholipase A<sub>2</sub> (7): activity was dependent on the protein concentration and increased linearly up to 26 μg; when assayed as a function of incubation time, a nonlinear relationship was observed after 30 s to 1 min, corresponding to 2% hydrolysis of substrate.

The Activity of Arachidonoyl-hydrolyzing Phospholipase A<sub>2</sub> with Membrane Phospholipid as Substrate—An alternate approach of demonstrating membrane-associated arachidonoyl-hydrolyzing phospholipase A<sub>2</sub> activity was to measure arachidonic acid release from the phospholipids in the membrane that the enzyme became bound to. Initially, to ensure that this enzyme could hydrolyze phospholipids presented as membrane, we measured the activity of the partially-purified phospholipase A<sub>2</sub> from the soluble fraction using [3H]arachidonic acid-labeled membranes as substrate. As shown in Fig. 3, the phospholipase did hydrolyze [3H]arachidonic acid from this substrate, exhibiting nonlinear kinetics after 1-min incubation when more than 5% of substrate was hydrolyzed, which is similar to the kinetics described with liposomal substrate.
It was interesting to determine the membrane phospholipid substrates that were hydrolyzed by membrane-associated phospholipase A2 and to compare them to those hydrolyzed by partially purified soluble fraction phospholipase A2. Table I shows that arachidonic acid was hydrolyzed from PC, PE, and PI/PS by enzyme from both soluble and particulate fractions, consistent with earlier observations in which arachidonic acid hydrolysis from liposomes containing PC, PE, and PI/PS by enzyme from both soluble and particulate fractions, consistent with earlier observations in which arachidonic acid hydrolysis from liposomes containing PC, PE, and PI/PS was 5-fold that of the particulate fraction isolated from cells homogenized in 100 μM EGTA, resulting in 10% compared to 2% release of 1H]arachidonic acid from the membrane, respectively (Fig. 4). In contrast, there was no difference in 1H]oleic acid release from radiolabeled membranes isolated from cells homogenized in either 100 μM CaCl2 or 100 μM EGTA. From both membrane fractions there was 2% release of the total membrane 1H]oleic acid by 40 min. These results suggest that the phospholipase A2, which redistributes from the soluble to the particulate fraction in the presence of >80 nM Ca2+, shows specificity for arachidonic but not oleic acid, in accord with the properties of the arachidonoyl-specific phospholipase A2.

![Graph](http://www.jbc.org/)

**Fig. 3.** Kinetics of partially purified soluble fraction arachidonoyl-hydrolyzing phospholipase A2 and membrane-associated phospholipase A2, using radiolabeled membranes as substrate. To determine the kinetics of the partially purified soluble fraction phospholipase A2, radiolabeled cells were homogenized in the presence of 100 μM EGTA and particulate fraction was isolated as described under "Experimental Procedures." Particulate fraction (100,000 dpm) was added to a reaction mixture containing 10 mM CaCl2 and partially purified arachidonoyl-hydrolyzing phospholipase A2 (0.6 μg of protein) in a final volume of 100 μl of 50 mM Tris, pH 8.5. To determine the kinetics of membrane-associated phospholipase A2, particulate fraction (100,000 dpm) isolated from radiolabeled cells homogenized in the presence of 100 μM CaCl2 (100,000 dpm) isolated from radiolabeled cells was separated by thin layer chromatography as described under "Experimental Procedures." Phospholipase A2 activity is expressed as the difference in 1H]arachidonic acid hydrolysis at the indicated time minus 1H]arachidonic acid hydrolysis at 0 min. The protein content of particulate fractions resulting in radioactivity of 100,000 dpm ranged from 10 to 15 μg. Data are representative of n = 4 experiments.

![Graph](http://www.jbc.org/)

**Fig. 4.** Specificity of phospholipase A2 for membrane phospholipid fatty acid. Particulate fractions (100,000 dpm) isolated from 1H]arachidonic acid-labeled membranes by arachidonoyl-hydrolyzing phospholipase A2 were assayed for phospholipase A2 activity in the presence of 100 μM CaCl2 (100,000 dpm) isolated from cells homogenized in the presence of 100 μM CaCl2, or 100 μM EGTA, and the kinetics of 1H]arachidonic acid release from the membrane, respectively (Fig. 3). Having established that 1H]arachidonic acid-labeled membranes are a substrate for the phospholipase A2 enzyme, radiolabeled cells were homogenized in the presence of 100 μM CaCl2, or 100 μM EGTA, and the kinetics of 1H]arachidonic acid release, and hence phospholipase A2 activity, were determined for each particulate fraction. At all time points there was much greater arachidonic acid release from the particulate fraction from cells homogenized with Ca2+ than with EGTA (Fig. 3), demonstrating that an increase in membrane-associated phospholipase A2 activity induced by Ca2+ can be detected by this approach. As we have reported previously (7), both the crude and partially purified soluble fraction phospholipase A2 exhibit a biphasic calcium dose response in which activity is measurable at physiological levels of calcium followed by a sharp rise in activity using millimolar concentrations of calcium. The calcium dose response of membrane-associated phospholipase A2 also showed measurable activity at physiological calcium levels, but 5- to 4-fold higher activity in the presence of 10 mM calcium (data not shown), the level used in these assays.

**Table I**

| Phospholipid species hydrolyzed from 1H]arachidonic acid-labeled membranes by arachidonoyl-hydrolyzing phospholipase A2 |
|-------------------------------------------------|
| Phospholipid | % decrease in 1H]arachidonic acid content of phospholipid |
| PC | 43 ± 6 | 26 ± 7 | 36 ± 4 |
| PI/PS | 24 ± 5 | 20 ± 4 | 21 ± 5 |

* Values are expressed as mean ± S.E. for three experiments.

1 In a single experiment, a similar trend but with a smaller percent change in the 1H]arachidonic acid content of the phospholipid species was seen after 30-s incubation.
phospholipase A₂ we have described previously (7). The low level of membrane-associated phospholipase activity that is evident on membranes from EGTA-homogenized cells may represent the membrane-bound, Ca²⁺-dependent phospholipase A₂, with an alkaline pH optimum, which has been described in the macrophage-like cell line, P388D₁ (15). Its substrate specificity has not been studied in detail, but it is known to hydrolyze both sn-2 palmitic and arachidonic acids.

**The Effect of the Ca²⁺ Concentration of Homogenization Buffer on Membrane Association of Arachidonoyl-hydrolyzing Phospholipase A₂.** Detected Using [³H]Arachidonic Acid-labeled Membrane as Substrate—The effect of Ca²⁺ concentration on the membrane association of phospholipase A₂ was then measured using [³H]arachidonic acid-labeled membranes as substrate. Fig. 5 shows that particulate fraction phospholipase A₂ activity was lowest when cells were homogenized in the presence of Ca²⁺ chelators, increased only slightly when cells were homogenized at levels of Ca²⁺ expected in an unstimulated cell (80 nM), and showed the greatest increase when cells were homogenized at levels of Ca²⁺ expected in stimulated cells (200-650 nM). Soluble fraction phospholipase A₂ activity showed an identical trend to that depicted in Fig. 1 (data not shown). These results provide further evidence that the decrease in soluble fraction phospholipase A₂ activity seen when increasing amounts of Ca²⁺ are present in homogenization buffer is exactly paralleled by an increase in particulate fraction phospholipase A₂ activity. Moreover, a functional association of the phospholipase A₂ with membrane phospholipid is induced by Ca²⁺, leading to the hydrolysis of arachidonic acid, but not oleic acid. Hence we suggest a mechanism whereby the activity of phospholipase A₂ is regulated: in an unstimulated cell phospholipase A₂ is found in the cytosol where, in the absence of substrate, there is no arachidonic acid release; upon receptor ligation the cytosolic Ca²⁺ concentration increases, and the enzyme becomes membrane-associated predominantly in a Ca²⁺-dependent manner which facilitates arachidonic acid release.

Although a Ca²⁺-induced decrease in soluble fraction arachidonoyl-hydrolyzing phospholipase A₂ activity and a concomitant increase in membrane-associated activity has been reported (6, 8), this is the first report that this phenomenon occurs at physiological levels of Ca²⁺, supporting a biological role for this process. The partial translocation of phospholipase A₂ from soluble to particulate fraction has been reported in mouse bone marrow-derived macrophages stimulated with 1-oleoyl-2-acetyl-glycerol (16) and in rat mesangial cells stimulated with phorbol myristate acetate (8). However, whether the Ca²⁺ concentration of the homogenization buffer was not specified (16) or cells were homogenized under conditions which would themselves be expected to alter the intracellular distribution of the enzyme (8), making interpretation of reported results difficult in the light of our present findings. It is possible that an increase in intracellular Ca²⁺ is not the only mechanism that may promote phospholipase A₂ association with membrane. Since phorbol myristate acetate induces arachidonic acid release (3) but no increase in intracellular Ca²⁺ (1), a protein kinase C-mediated phosphorylation mechanism, either of phospholipase A₂ or of a phospholipase A₂-modulating protein, may be involved. It is also possible that both mechanisms may act synergistically, as suggested for the maximal release of arachidonic acid from rat mesangial cells (1).

We have demonstrated recently that arachidonoyl-hydrolyzing phospholipase A₂ shows enhanced activity in the presence of anionic phospholipids. Furthermore, phosphatidylinositol-4,5-bisphosphate was shown to decrease the Ca²⁺ concentration required for full enzyme activity to the nanomolar range. Consequently, anionic phospholipids, together with Ca²⁺, may play a role in binding the enzyme to membrane. Hence, phospholipase A₂ exhibits properties similar to the amphiprotic Ca²⁺/phospholipid binding proteins (17), which include the enzymes, protein kinase C (18-20) and calpain (21), and a variety of cytoskeletal proteins (22). The characteristics of these proteins include a change in intracellular distribution from cytosol to membrane in the presence of increasing Ca²⁺ concentration, where membrane association is mediated through interactions with Ca²⁺ and phospholipids.

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