A Calcium-dependent Mechanism for Associating a Soluble Arachidonoyl-hydrolyzing Phospholipase A\textsubscript{2} with Membrane in the Macrophage Cell Line RAW 264.7*

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Arachidonoyl-hydrolyzing phospholipase A\textsubscript{2} 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\textsuperscript{2+} is required for arachidonic acid release in vitro and most phospholipase A\textsubscript{2} enzymes require Ca\textsuperscript{2+} for activity in vitro, the role of Ca\textsuperscript{2+} in phospholipase A\textsubscript{2} activation is not understood. We have found that an arachidonoyl-hydrolyzing phospholipase A\textsubscript{2} from the macrophage-like cell line, RAW 264.7, exhibits Ca\textsuperscript{2+}-dependent association with membrane. The intracellular distribution of the enzyme was studied as a function of the Ca\textsuperscript{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\textsuperscript{2+} chelators and there was a slight decrease in soluble enzyme activity when cells were homogenized at the level of Ca\textsuperscript{2+} in an unstimulated cell (80 nM). When cells were homogenized at Ca\textsuperscript{2+} concentrations expected in stimulated cells (230-450 nM), 60-70% of the phospholipase A\textsubscript{2} 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 A\textsubscript{2} activity was demonstrated by \([\text{H}]\)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 \([\text{H}]\)arachidonic acid was derived from phosphatidylycholine, phosphatidylethanolamine, and phosphatidylserine and phosphatidylinositol/phosphatidylcholine. We suggest a mechanism in which the activity of phospholipase A\textsubscript{2} is regulated by Ca\textsuperscript{2+}: in an unstimulated cell phospholipase A\textsubscript{2} is found in the cytosol; upon receptor ligation the cytosolic Ca\textsuperscript{2+} concentration increases, and the enzyme becomes membrane-associated which facilitates arachidonic acid hydrolysis.

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EXPERIMENTAL PROCEDURES

Isolation of Soluble and Particulate Fractions from Cells in Suspension Culture—The murine macrophage cell line, RAW 264.7, was...
cultured as described previously (9). To determine the distribution of intracellular phospholipase A₂, following cell disruption in buffer containing varying amounts Ca²⁺, 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 MgCl₂-6H₂O 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 × 10⁶ cells/ml in homogenization buffer containing the protease inhibitors, phenylmethylsulfonyl fluoride (PMSF) (1 mM) and leupeptin (1 μg/ml). Homogenization buffer containing free Ca²⁺ concentrations less than 1 mM was prepared using the fluorescent calcium indicator, indo-1 (Molecular Probes, Eugene, OR), as described (10). The solution was first deproteinized by addition of a final concentration of 100 mM EGTA, and then Ca²⁺ was added back until the required concentration was reached. Cells in homogenization buffer were sonicated at 4 °C for 90 s using a microprobe (Braunsonic 2000, B. Braun Instruments, Burglingame, CA), which resulted in 100% disruption. The homogenate was ultracentrifuged at 100,000 × g for 60 min to produce a soluble and a particulate fraction (pellet). The particulate fraction was washed twice in homogenization buffer to remove contaminating cytosol and resuspended in incubation buffer (10 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 [³H]arachidonic acid were used as substrates 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 [³H]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 from suspension culture were plated into 100-mm diameter tissue culture dishes (Costar, Cambridge, MA) at a density of 10 × 10⁶/dish in Dulbecco's modified Eagle's medium/RPMI 1640 (1:1, v/v) (Hazeltone, Lenexa, KS) containing 10% iron-supplemented bovine calf serum (HyClone Laboratories, Inc., Logan, UT). Approximately 6 h later the medium was removed, and 5 μCi of either [³H]arachidonic acid (100 Ci/mmol; Du Pont-New England Nuclear) or [³H]oleic acid (10 Ci/mmol; Du Pont-New England Nuclear), which had been dried in the presence of nitrogen and brought up to a final concentration of 1 μCi/ml in homogenization buffer containing the protease inhibitors, PMSF, leupeptin, and 140 mM NaCl, was added per dish. After 16 h, cells were washed three times with Dulbecco's modified Eagle's medium containing 0.25% delipidated lipopolysaccharide-free human serum albumin to remove unbound fatty acid. Cells were then washed twice with 20 ml of Hanks' balanced salt solution containing 80 mM Ca²⁺ (Hanks' balanced salt solution/80 mM Ca²⁺), scissored into 2 ml of Hanks' balanced salt solution/80 mM Ca²⁺, centrifuged at 250 × g for 10 min, and then resuspended in 2 ml of homogenization buffer. It was necessary to wash the scraped cells twice with the Ca²⁺ concentration of the cell suspending medium, which was verified using the indo-1 assay. Cells were homogenized, and 100,000 × g soluble and particulate fractions 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 PMSF and 1 μg/ml leupeptin. Approximately 80–85% of the [³H]arachidonic acid was taken up by the cells, and phospholipid species from particulate fractions contained [³H]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 [³H]oleic acid incorporation into phospholipid

Results and Discussion

The Effect of the Concentration of Ca²⁺ in Homogenization Buffer on the Intracellular Distribution of Arachidonoyl-hydrolyzing Phospholipase A₂, Detected Using Liposomal Substrates—To examine the effect of Ca²⁺ concentration in 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 Ca²⁺, soluble and particulate fractions were isolated, and the phospholipase A₂ activity of each fraction was determined in the presence of 10 μM Ca²⁺ 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 1.0 mM CaCl₂, and 75% of the homogenates. Similar results were obtained whether cells were homogenized in the presence of 250 and 450 mM Ca²⁺, respectively, the range of Ca²⁺ in a stimulated cell (13). Only a small additional decrease in soluble fraction phospholipase A₂ activity of 60 and 75% was seen when cells were homogenized in the presence of 250 and 450 mM Ca²⁺, respectively, the range of Ca²⁺ 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 Ca²⁺, demonstrating that the process was almost complete at physiological Ca²⁺ levels. For these experiments an homogenization 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 Ca²⁺ 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 cavitation

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

The decrease in soluble fraction activity. Fig. 1 shows a maximum of 33% of the decrease in soluble fraction phospholipase A₂ activity was recoverable in the supernatant. A third approach, no further enzyme was recoverable. The properties of the soluble enzyme were similar to those described previously for soluble fraction arachidonoyl-specific phospholipase A₂ (7): activity was now recoverable in the resulting supernatant. A third incubation and ultracentrifugation did not release any further phospholipase A₂ 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 A₂ 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 A₂ (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 A₂ with Membrane Phospholipid as Substrate—An alternate approach of demonstrating membrane-associated arachidonoyl-hydrolyzing phospholipase A₂ 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 A₂ from the soluble fraction using [1H]arachidonic acid-labeled membranes as substrate. As shown in Fig. 5, the phospholipase did hydrolyze [1H]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.
Purification of soluble and particulate fractions of phospholipase A2 enzyme, radiolabeled cells were homogenized in the presence of 100 μM CaCl2 and partially purified arachidonoyl-hydrolyzing phospholipase A2 (0.6 pg of protein) in a final volume of 100 μl of 50 mM Tris, pH 8 (T). 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, was assayed for phospholipase A2 activity in the presence of 10 mM CaCl2 for the indicated times, as described under "Experimental Procedures." Immediately after the addition of particulate fraction (0 min) and at the times indicated, lipids were extracted and arachidonic acid was separated by thin layer chromatography as described under "Experimental Procedures." Phospholipase A2 activity is expressed as the difference in [3H]arachidonic acid hydrolysis at the indicated time minus [3H]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.

Having established that [3H]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 [3H]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 2- to 4-fold higher activity in the presence of 10 mM calcium (data not shown), the level used in these assays.

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, PI/PS, and PI by enzyme from both soluble and particulate fractions, consistent with earlier observations in which arachidonic acid hydrolysis from liposomes containing PC, PE, or PI was observed using soluble fraction phospholipase A2 (7). We then examined the specificity of membrane-associated phospholipase A2 for the fatty acid of membrane phospholipid using cells radiolabeled with either [3H]arachidonic acid or [3H]oleic acid. Cells were homogenized in the presence of either 100 μM EGTA or 100 μM CaCl2, and the kinetics of [3H]fatty acid release were determined for each particulate fraction. After 40-min incubation, phospholipase A2 activity from the particulate fraction isolated from [3H]arachidonic acid-labeled cells homogenized in 100 μM CaCl2 was 5-fold that of the particulate fraction isolated from cells homogenized in 100 μM EGTA, resulting in 10% compared to 2% release of [3H]arachidonic acid from the membrane, respectively (Fig. 4). In contrast, there was no difference in [3H]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 [3H]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 phos-
pholipase 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 A₁, 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 A₁ 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 A₁ is regulated: in an unstimulated cell phospholipase A₁ 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 A₁ 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 A₁ from soluble to particulate fraction has been reported in mouse brain macrophage-derived microglia stimulated with 1-oleoyl-2-acethyl-glycerol (16) and in rat mesangial cells stimulated with phorbol myristate acetate (8). However, either 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 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 A₁ 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 A₁ or of a phospholipase A₁-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 A₁ shows enhanced activity in the presence of anionic phospholipids. Furthermore, phosphatidyl-inositol-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 A₁ exhibits properties similar to the amphitropic 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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