Phospholipase A₂ Activity Specific for Phosphatidic Acid
A POSSIBLE MECHANISM FOR THE PRODUCTION OF ARACHIDONIC ACID IN PLATELETS*

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Activation of platelets induces the formation of phosphatidic acid, which results from the combined actions of a phosphatidylinositol-specific phospholipase C and a 1,2-diacylglycerol kinase. It has been proposed that this phosphatidic acid leads in some way to the subsequent production of arachidonic acid. The present studies reveal the existence of a particulate phospholipase A₂ enzyme that appears to act selectively on phosphatidic acid to produce arachidonic acid. This enzyme has been assayed by degradation of exogenously added [32P]lysophosphatidic acid. The rate of this enzyme activity is optimal at pH 7.0. Activity is maximal at 10 μM Ca²⁺ and is completely inhibited by ethyleneglycol bis(amoineether) N,N,N,N-tetraacetic acid. The present studies reveal the existence of a particulate phospholipase A₂ enzyme that appears to act selectively on phosphatidic acid to produce arachidonic acid. This enzyme has been assayed by degradation of exogenously added [32P]lysophosphatidic acid or by the conversion of exogenously added 2-[14C]arachidonylphosphatidic acid to [14C]arachidonic acid. The degradation of phosphatidic acid to lysophosphatidic acid is optimal at pH 7.0. Activity is maximal at 10 μM Ca²⁺ and is completely inhibited by ethyleneglycol bis(amoineether) N,N,N,N-tetraacetic acid. The rate of [32P]lysophosphatidic acid formation is 2-4 nmol/min/mg of protein and is linear up to 12 μM of exogenously added [32P]lysophosphatidic acid. This phospholipase A₂ activity is relatively specific for phosphatidic acid as compared to other major phospholipids. Detergents such as sodium deoxycholate and Triton X-100 inhibit the formation of lysophosphatidic acid. This activity is thus distinctly different from that of other platelet phospholipase A₂ activities which preferentially degrade phosphatidyethanolamine and phosphatidylcholine and which require alkaline pH values, millimolar concentrations of Ca²⁺, and deoxycholate for maximal activation. Quinacrine, an inhibitor of the production of arachidonic acid in stimulated platelets, inhibits this phosphatidic acid-specific activity in a calcium-dependent manner. We propose that this phosphatidic acid-specific phospholipase A₂ might play an important role in the formation of arachidonic acid in stimulated platelets.

Arachidonic acid is esterified in position 2 of phospholipids (1–3), and it has generally been accepted that its production is induced by a receptor-mediated activation of phospholipase A₂ activity (4–9). We have recently shown (10) that platelet phospholipases of the A₂ type degrade phosphatidylethanolamine and phosphatidylcholine. Phosphatidylinositol is, however, instead degraded rapidly and specifically by a phospholipase C activity (10–13). The product of the phospholipase C action is 1,2-diacylglycerol, which can either be degraded by 1,2-diacylglycerol lipase (14) or be phosphorylated to phosphatidic acid by 1,2-diacylglycerol kinase (13, 15). The latter reaction appears to be favored since a net accumulation of phosphatidic acid is observed after stimulation of intact platelets by thrombin or collagen (16, 17). This occurs prior to the release of arachidonic acid and is independent of the generation of arachidonic acid metabolites (16). Furthermore, phosphatidic acid production closely parallels the degradation of phosphatidylinositol (17). It is thus likely that the arachidonic acid derived from phosphatidylinositol may be released via phosphatidic acid (18). In support of such a view, we have found in platelets a phosphatidic acid-specific phospholipase A₂ activity. This activity differs substantially from that of phospholipases of the A₂ type that degrade phosphatidylethanolamine and phosphatidylcholine.

EXPERIMENTAL PROCEDURES

Materials—[1-14C]Arachidonic acid (56.2 mCi/mmol) was from Amersham Corp., and [32P]orthophosphoric acid (carrier-free in 0.02 N HCl) was obtained from New England Nuclear. Phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylinositol, sphingomyelin, phospholipase A₂ from Crotaulus adamanteus, and quinacrine were obtained from Sigma. Phosphatidic acid was from Supelco. Lysophosphatidic acid was obtained from Serdery, London, Ontario, Canada. Arachidonic acid and Silica Gel H plates (Prekote, 0.25 mm thick) were purchased from Applied Science Laboratories, Inc., State College, PA. Silica Gel G (SiI  G-25) plates were from Brinkmann Instruments.

Isolation of Platelets and Preparation of Total Particulate Fraction—Platelets were isolated from 2 units of horse blood in acid-citrate-dextrose medium. The blood was centrifuged at 300 x g for 15 min at room temperature. The platelet-rich plasma was centrifuged once more to remove erythrocytes. The platelets were then pelleted from plasma by centrifugation at 3,000 x g for 20 min at 4 °C. The pellet was resuspended gently in 50 ml of NaCl/Tris-HCl (120 mM/30 mM) buffer, pH 7.4 (NaCl/Tris buffer), and centrifuged once more at 3,000 x g for 20 min. The pellet was finally resuspended in 20 ml of NaCl/Tris buffer and frozen at -20 °C. The following morning it was thawed and sonicated (3 times, 30 s) using a Branson Sonifier at maximum output. The suspension of broken cells was then centrifuged at 100,000 x g for 60 min. The supernatant was removed and the particulate fraction was washed once with NaCl/Tris buffer. The particulate material was then suspended in 10 ml of the same buffer and again sonicated as before in order to obtain a homogenous suspension. Protein was assayed (19) and the suspension was then diluted to a protein concentration of 4 mg/ml. Approximately 20% of the total platelet protein was recovered in the particulate fractions.

Assay of Phosphatidic Acid-specific Phospholipase A₂—The standard incubation mixture contained NaCl/Tris buffer, pH 7.0, 10 mM NaF, 10 μM phosphatidic acid (labeled with either [32P]orthophosphate or [14C]arachidonic acid), 10 μM Ca²⁺. Appropriate Ca²⁺ concentrations were achieved by using 5 mM Ca/EGTA¹ buffer, pH 7.0 (20). Samples of phosphatidic acid were dried under N₂ and resuspended in NaCl/Tris buffer by sonication. Tubes were placed in a shaking water bath (37 °C) and the reaction was then started by adding appropriate amounts of the membrane suspension. Control incubations with and without boiled membranes (3 min, 100 °C) were

¹ The abbreviation used is EGTA, ethyleneglycol bis(amoineether) N,N,N,N-tetraacetic acid.

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Phosphatidic Acid-specific Phospholipase A2

Carried out simultaneously. The reaction was stopped by adding a solution of chloroform/methanol/concentrated HCl (10:3:1, v/v). The phases were split with 0.6 ml of chloroform and 0.5 ml of 2 M KCl. This procedure provided almost complete extraction of phosphatidic acid and 85-90% extraction of lysophosphatidic acid (22). The lower chloroform phase was removed using a Pasteur pipette and dried under nitrogen gas at 25°C.

Chromatographic Separation of Lipids—The dried lipids were redissolved in chloroform and applied onto Silica Gel G plates that were previously impregnated with 0.5 M oxalic acid (22). The plates were run in a solvent mixture of chloroform/methanol/10 N HCl (87:10:3, v/v) (System 1). In this system, lysophosphatidic acid (Rf 0.20) is completely separated from phosphatidic acid (Rf 0.61). Other major phospholipids remain close to the origin and neutral lipids move close to the solvent front. Other phospholipids were analyzed on Silica Gel H plates in a solvent system of chloroform/methanol/acetic acid/water (75:45:12, v/v) (System 2) (23). The separation of labeled phosphatidic acid to be used as substrate for the enzymatic assay described above was performed on Silica Gel G plates using the upper phase from a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (9:5:2:10, v/v). In this system, phosphatidic acid is clearly separated from all the other phospholipids and prostaglandin products (System 3) (16). Similarly, labeled phosphatidic acid was separated on formaldehyde-impregnated papers by descending chromatography with the upper phase of 1-butanol/acetic acid/water/diethyl ether (8:2:10:1, v/v) (24). Lipids were localized by autoradiography or exposure to iodine vapors and specific areas were counted in a liquid scintillation counter with Aquasol II. Phospholipid phosphates were quantitated according to Bartlett (26).

Preparation of [32P]Phosphatidylglycerol and [32P]Phosphatidic Acid—Washed platelets from 2 units of blood were resuspended in 20 ml of NaCl/Tris buffer containing glucose (10 mM) and EGTA (2 mM) and incubated with 10 mCi of [32P]orthophosphate at 37°C for 2 h. Platelets were then washed once with 50 ml of NaCl/Tris buffer and resuspended in 20 ml of the same buffer. Lipids were extracted as described above and phosphatidylglycerol was separated on formaldehyde-treated papers (24), localized by a brief autoradiography, and eluted with 30 ml of methanol. [32P]Phosphatidic acid was obtained from platelets prelabelled with [32P]orthophosphate as described before and stimulated with thrombin (1 unit/ml) for 3-5 min. [32P]Phosphatidic acid was separated from all the other phospholipids on Silica Gel G plates (System 3) and was localized by brief exposure to iodine vapor. [32P]Phosphatidic acid from the specific silica gel area was eluted with 20 ml of methanol.

Preparation of [3H]Arachidonylphosphatidic Acid—Platelets were labeled with [3H]arachidonic acid as described previously (10, 13). [3H]labeled platelets were then treated with 25 mM deoxycholate in the presence of 5 mM ATP and 10 mM MgCl2 for 30 min at 37°C (13). Under these conditions, the 1,2-diacylglycerol produced from phosphatidylglycerol by phosphatidylglycerol-specific phospholipase C was phosphorylated to phosphatidic acid. Phosphatidic acid was then separated as described before (System 3). The [3H]Arachidonic acid is esterified in position 2 of the phosphatidic acid, since treatment with phospholipase A1 (C. adamanteus) releases [3H]arachidonic acid (systems 1 and 3).

Purity of the radiolabeled phosphatidic acid was tested by thin layer chromatography using Systems 1 and 2. In either system, 90% of the radioactivity was found to be associated with the material that moved with authentic phosphatidic acid (Supelco). The remainder of the radioactivity migrated with various other phospholipids. Further purification of the lipid by silica gel thin layer chromatography (Cay) had no appreciable effects on the release of [3H]Arachidonic acid or on the formation of [3H]Lysophosphatidic acid, following treatment with C. adamanteus phospholipase A2 or with total particulate fractions from platelets.

Characterization of Lysophosphatidic Acid by Alkaline Hydrolysis—[3H]Lysophosphatidic acid obtained in the enzymatic assay described above was separated on oxalate-impregnated thin layer plates (System 1) and the specific silica gel area was resuspended in 0.8 ml of methanol plus 0.2 ml of 1 N NaOH and incubated at 45°C overnight. Then, the pH was brought to 4 by addition of formic acid and the plates were split by addition of 0.8 ml of water, 0.4 ml of methanol, and 1.2 ml of chloroform. The upper aqueous phase was evaporated under N2 and the residue was redissolved in water and chromatographed on thin layer plates (0.1 mm thickness) of MN 300 cellulose using two different solvent systems (27), acetate/1-propanol/phenol/water (10:20:10:1, v/v, System 4) and methanol/pyridine/0.5 N ammonia (40:10:10, v/v) (System 5).

More than 98% of the radioactivity was recovered in the aqueous extracts and was found to move with the same Rf values as the authentic glycerol 3-phosphate in both solvent systems.

RESULTS

Phosphatidic Acid Degraded by a Phospholipase A2 Activity—Table I shows that prolonged incubation of [3H]arachidonate-labeled phosphatidic acid with phospholipase A2 (C. adamanteus) or with total particulate fractions of platelets produces extensive degradation of phosphatidic acid liberating [3H]arachidonate acid and nonradioactive lysophosphatidic acid. Similar results were obtained using a lower concentration of phospholipase A2 (1-5 units) from C. adamanteus and at shorter incubations (10-30 min). These changes were not observed with boiled particulate fractions. These results suggest that [3H]arachidonate acid is esterified in position 2 of phosphatidic acid and that the total particulate fractions of platelets contain a heat-labile, enzymatic activity which releases [3H]arachidonate acid from position 2 of phosphatidic acid. This activity thus resembles a phospholipase of the A2 type.

Further characterization of the products of this enzymatic activity has been achieved by using [3P]phosphatidic acid as substrate. Particulate preparations of platelets degrade [3P]phosphatidic acid to 3P]lysophosphatidic acid, as does the phospholipase A2 from C. adamanteus. Alkaline hydrolysis of

Table I

<table>
<thead>
<tr>
<th>Additions</th>
<th>Phosphatidic Acid</th>
<th>Lysophosphatidic Acid</th>
<th>Arachidonate Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>33,440</td>
<td>3,090</td>
<td>518</td>
</tr>
<tr>
<td>Phospholipase A2</td>
<td>10,636</td>
<td>2,372</td>
<td>23,888</td>
</tr>
<tr>
<td>Boiled membranes</td>
<td>35,260</td>
<td>2,736</td>
<td>1,474</td>
</tr>
<tr>
<td>Membranes</td>
<td>22,642</td>
<td>2,168</td>
<td>12,302</td>
</tr>
</tbody>
</table>

Fig. 1. Properties of phosphatidic acid-specific phospholipase A2. a, time course of the enzyme reaction (100 μg of protein); b, effect of protein concentration (5-min incubations); c, effect of pH (10-min incubations, 80 μg of protein). Buffers used were 50 mM imidazole-NaOH in the pH range of 6.9 to 7.0 and 30 mM Tris-HCl in the pH range of 7.0 to 9.0. Incubations (0.5 ml) were carried out at 37°C and contained 120 mM NaCl, appropriate buffer, and 1 mM CaCl2. For a and b, Tris-HCl buffer, pH 7.0, was used. Reactions were stopped and lipids were separated on oxalate-impregnated thin layers (Silica Gel G), using chloroform/methanol/10 N HCl (87:30:0.8, v/v), localized by autoradiography, and counted. For further details, see "Experimental Procedures."
Phosphatidic Acid-specific Phospholipase A2

The formation of lysophosphatidic acid is linear up to 10 min and 125 µg of particulate protein (Fig. 1, a and b). Maximal activity is observed at pH 7.0 (Fig. 1c). This phospholipase A2 activity is dependent on calcium ions, and maximal activity is obtained between 7 and 10 µM Ca²⁺ (Fig. 2).

The effect of varying the concentration of phosphatidic acid on the formation of lysophosphatidic acid is shown on Fig. 3. Under present assay conditions, the rate of [³²P]lysophosphatidic acid formation reaches a maximum of 1.8 nmol/min/mg of protein at 12 µM of exogenously added [³²P]phosphatidic acid. However, the Lineweaver-Burk plot of the linear portion of the data gives an apparent Michaelis constant of 20 µM and a Vₘₚ₁ of 4 nmol/min/mg of protein. This discrepancy may be due to different factors, such as low solubility of phosphatidic acid.

Detergents such as deoxycholate and Triton X-100 are widely used for activation of platelet phospholipases A₁ and C activities (10, 13, 28). In contrast, deoxycholate inhibits the platelet phospholipase A₂ activity that degrades phosphatidic acid (Fig. 4). This activity is also inhibited by Triton X-100 (Fig. 4).

![Fig. 2. Effect of different concentrations of calcium on lysophosphatidic acid formation by platelet particulate fractions. Assays (0.5 ml) contained 150 µg of particulate protein, 10 nmol of [³²P]phosphatidic acid (5000 cpm/nmol), NaCl/Tris-HCl buffer, pH 7.0, and varying amounts of calcium. Incubation was carried out at 37 °C for 10 min. Free calcium concentrations at or below 10 µM were achieved by using 5 mM Ca/EGTA buffer, pH 7.0. Lipids were analyzed as in Fig. 1.](image)

![Fig. 3. Effect of phosphatidic acid concentration on lysophosphatidic acid formation. Assays (0.5 ml) contained various amounts of [³²P]phosphatidic acid obtained from thrombin-stimulated platelets (specific activity, 65,000 cpm/nmol), 80 µg of particulate protein, and 0.1 mM Ca²⁺. All other conditions are the same as in Fig. 2.](image)

![Fig. 4. Effect of deoxycholate and Triton X-100 on lysophosphatidic acid formation. Assays (0.5 ml) containing 150 µg of particulate protein, 5 nmol of [³²P]phosphatidic acid (6000 cpm/nmol), and 10 µM Ca²⁺ were incubated for 10 min with varying amounts of sodium deoxycholate (●) or Triton X-100 (△). All other conditions are as in the legend to Fig. 2.](image)

### Table II

Effects of different phospholipids on the formation of [³²P]-lysophosphatidic acid

| Phospholipid                  | Concentration of nonradioactive phospholipid
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>10 µM</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>97</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>100</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>105</td>
</tr>
<tr>
<td>Phosphatidyserine</td>
<td>114</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>96</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>77</td>
</tr>
</tbody>
</table>

**Effect of Unlabeled Phospholipids on the Degradation of Radioactive Phosphatidic Acid**—This was determined by measuring the formation of lysophosphatidic acid from a fixed amount of [³²P]phosphatidic acid (10 µM) in the presence of 2 concentrations (10 and 60 µM) of different phospholipids. Table II shows that, at 10 µM, none of the major phospholipids except phosphatidic acid had any appreciable effect on lysophosphatidic acid formation. At 60 µM, phosphatidylethanolamine and anionic phospholipids such as phosphatidyserine and phosphatidylinositol show substantial inhibitory effects. Under these conditions, phosphatidic acid itself exerts the most pronounced inhibition on [³²P]lysophosphatidic acid formation (Table II and Fig. 5). These data suggest that [³²P]-lysophosphatidic acid formation is reduced because of the dilution of [³²P]phosphatidic acid with unlabeled phosphatidic acid.

The lack of effects of phosphatidylcholine and sphingomyelin (Table II) suggests that these two phospholipids might not be substrates for the activity that degrades phosphatidic acid. As to the inhibition of [³²P]lysophosphatidate formation, by phosphatidyserine and phosphatidylinositol, it is possible that these anionic phospholipids are actually inhibiting the enzyme rather than acting as substrates. These possibilities have been directly tested by comparing the formation of a radioactive product that chromatographs as authentic glycerol-3-phosphate.

Properties of the Phospholipase A₂ That Degrades Phosphatidic Acid in Platelets—The formation of lysophosphatidic acid produces a radioactive product
Phosphatidic Acid-specific Phospholipase A₂

Effect of Quinacrine on the Phosphatidic Acid-specific Phospholipase A₂—The antimalarial drug quinacrine is known to block the release of arachidonic acid from platelet phospholipids (6). Quinacrine also inhibits the phosphatic acid-phospholipase A₂ from platelet total particulate fractions (Fig. 7). This inhibition depends on the concentration of quinacrine (Fig. 7). The efficiency of that inhibition is, however, dependent on the Ca²⁺ concentration in the medium. At 1 mM Ca²⁺, 60% inhibition is observed by 1 mM quinacrine. At 10 μM Ca²⁺, the activity is completely blocked by 1 mM quinacrine, and the quinacrine concentration for half-maximal inhibition is 170 μM. In the absence of added Ca²⁺ (Ca²⁺ in the medium is sufficient for enzymatic activity), 50% inhibition of the formation of lyso phosphatic acid is observed at 60 μM quinacrine. Under the latter conditions, 150 μM quinacrine inhibits arachidonate release from thrombin-stimulated platelets by 50% (Fig. 7). These results suggest that quinacrine might exert its effects by chelating calcium or otherwise by competing for calcium-binding sites.

DISCUSSION

The data presented here clearly demonstrate the existence in platelets of a phospholipase A₂ activity (Table I) which seems to favor the specific degradation of phosphatic acid (Table II; Figs. 5 and 6). The activity is optimally active at neutral pH (Fig. 1c), is highly sensitive to Ca²⁺ (Fig. 2), and is inhibited by deoxycholate. This activity thus contrasts sharply with platelet phospholipases A₂ that degrade phosphatidylethanolamine and phosphatidylcholine which require alkaline pH, millimolar concentrations of Ca²⁺, and deoxycholate for optimal activity (10, 28–30).

Phosphatic acid is a product of platelet stimulation (16, 17) and arises as a consequence of the degradation of phosphatidylinositol (13, 16, 17). More than 50% of the platelet phosphatidylinositol is degraded by a phosphatidylinositol-specific phospholipase C soon after platelet stimulation by thrombin (17, 31). Most of the 1,2-diacylglycerol produced is phosphorylated to phosphatic acid (17). When thrombin is the stimulus, up to 70% of the degraded phosphatidylinositol is recovered as phosphatic acid (17), whereas in collagen-stimulated platelets, the production of phosphatic acid is stoichiometrically and reciprocally related to the degradation of phosphatidylinositol (17). These results strongly suggest an efficient phosphorylation of 1,2-diacylglycerol to phosphatic acid rather than decylation of the 1,2-diacylglycerol by 1,2-diacylglycerol lipase (4). Furthermore, we have been unable to detect any appreciable diacylglycerol lipase activity in our membrane preparations.

The presence of a phosphatic acid-specific phospholipase

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**Fig. 5.** Effect of nonradioactive phosphatic acid on the formation of [³²P]lyso phosphatic acid from [³²P]phosphatic acid. Assays (0.5 ml) contained 80 μg of particulate protein, 2.0 nmol of [³²P]phosphatic acid (150,000 cpm/nmol), 1 mM Ca²⁺, and varying amounts of nonradioactive phosphatic acid. Other conditions are as in Fig. 2.

**Fig. 6.** Relative degradation of radioactive phosphatic acid, phosphatidylinositol, and phosphatidylcholine by platelet particulate fractions. Assays (0.5 ml) contained 40 μg of particulate protein and varying amounts of [³²P]phosphatic acid (●), [³²P]phosphatidylinositol (△), and [³²P]phosphatidylcholine (■). All the other conditions are the same as in the legend to Fig. 2.

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A<sub>2</sub> in platelets suggest that the formation of arachidonic acid from phosphatidic acid might be of functional significance. Such a physiological role is favored by certain properties such as calcium sensitivity (Fig. 2) and substrate affinity (Fig. 3). In this relation, we have observed<sup>3</sup> that thrombin stimulation of platelets also produces lysophosphatidic acid (also see Ref. 22), although the lysophosphatidic acid which is present in stimulated platelets represents only about 10% of the accumulated phosphatidic acid. It is possible that a rapid reacylation of the lysophosphatidic acid occurs by a specific transfer of arachidonic acid from phosphatidylethanolamine and phosphatidylcholine. Such a transfer could be achieved by a unique acyltransferase of the type described recently (32).

Here, one can speculate that the phospholipase A<sub>2</sub> activities that degrade phosphatidylethanolamine and phosphatidylcholine (10, 28-30) might constitute an integral part of this acyltransferase activity and might be dissociated under conditions such as alkaline pH values and the presence of detergents (10, 28).

The deacylation of phosphatidic acid might then be the initial step in a cascade of reactions that leads to a net production of arachidonic acid from various phospholipids (4-9) with the concomitant accumulation of various lysophospholipids (17). The postulated cycle of reactions involving deacylation of phosphatidic acid and subsequent specific reacylation of lysophosphatidic acid may explain why platelet stimulation releases only arachidonic acid and not other fatty acids (4, 5, 14). Quinacrine, interestingly, inhibits both the in vitro phosphatidic acid-specific phospholipase A<sub>2</sub> (Fig. 7) and the net production of arachidonic acid in intact, stimulated platelets (6).

The platelet phosphatidic acid-specific phospholipase A<sub>2</sub> generates 2-4 nmol of lysophosphatidic acid/min/mg of membrane protein. This rate is at least an order of magnitude higher than the phospholipase A<sub>2</sub> activities that degrade phosphatidylethanolamine and phosphatidylcholine (29). The phosphatidic acid-specific phospholipase A<sub>2</sub> activity, however, does not account for the 5-8 nmol of arachidonic acid released/10<sup>8</sup> platelets (±0.5 mg of total particulate protein) upon thrombin stimulation. It might well be that the rates of various phospholipase A<sub>2</sub> activities as obtained under in vitro assay conditions do not reflect the rapidity with which the enzymes appear to function during stimulation.

REFERENCES


