Deoxycholate treatment of horse platelets previously labeled in their phospholipids with \([^{14}C]\)arachidonate produces selective conversion of \([^{14}C]\)phosphatidylcholine to \([^{14}C]\)2-diacylglycerol. This phospholipase C activity, which has a pH optimum of 7.5, is specific for phosphatidylcholine since other phospholipids or neutral lipids are not affected. Although exogenous Ca\(^{2+}\) is not required for activity, ethylene glycol bis(\(\beta\)-aminoethyl ether)N,N,N',N'-tetraacetic acid or EDTA abolishes phosphatidylcholine degradation. However, in the presence of added Ca\(^{2+}\), other phospholipases such as phosphatidylethanolamine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) are also degraded but by a phospholipase A\(_2\) activity. This activity generates the respective lyso-derivatives as well as various \([^{14}C]\)arachidonate metabolites. The phospholipase A\(_2\) activity is further enhanced by increasing the pH (7.5 to 9.5), a condition which greatly suppresses the phospholipase C activity. Most of the platelet phospholipase A\(_2\) activity is associated with the particulate fractions of the cell, while the phospholipase C activity appears to be almost completely restricted to the soluble fraction. Deoxycholate treatment of the particulate fractions results in cleavage by phospholipase A\(_2\) of phosphatidylethanolamine and not of phosphatidylcholine. The preferred substrates for platelet phospholipase A\(_2\) appear to be phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine, while phosphatidylcholine seems to be degraded nearly exclusively by phospholipase C.

One important consequence of platelet activation by such physiological stimuli as thrombin is the release of arachidonate from membrane phospholipids (1-8). This fatty acid plays a key role as a precursor of cyclooxygenase and lipoxygenase products (9). Arachidonate is uniquely located in position 2 of a variety of biological processes such as secretion (19), chemotaxis (20), Ca\(^{2+}\) mobilization (21-23), and prostaglandin synthesis (1-6, 24, 25). Our data indicate that the activities of these two phospholipases can discriminate among various endogenous phospholipids. Phospholipase C and phospholipase A\(_2\) also differ with regard to other properties such as pH optima, Ca\(^{2+}\) requirements, and cellular localization.

**EXPERIMENTAL PROCEDURES**

Materials—\([^{1,4}C]\)Arachidonic acid (56.2 mCi/mmol) was obtained from Amersham Corp., Arlington Heights, IL. Carrier-free (\(^{32}P\)) orthophosphoric acid in 0.02 N HCl was purchased from New England Nuclear, Boston, MA. Phosphatidylethanolamine, phosphatidycholine, phosphatidylserine, and phosphatidylinositol were obtained from Sigma. Lyso- and lysophosphatidylethanolamine, lyso- and lysophosphatidylcholine and 1,2-diacylglycerol were from Scpeclo, Bellfonte, PA. Arachidonic acid and Silica Gel H plates (Preciote, 0.25-mm thick) were purchased from Applied Science Laboratories, Inc., State College, PA. Silica Gel G (Sil G-25) plates were from Brinkmann Instruments, Westbury, NY.

Preparation of Labeled Platelets—Washed horse platelets were obtained from 500 ml of acid citrate-dextrose blood (9). The blood was centrifuged at 300 \(\times\) g at room temperature. The supernatant, platelet-rich plasma, was removed and centrifuged twice more to remove red cells. Samples of 50 ml of platelet-rich plasma were then incubated at 37°C for 2 h with 2 \(\mu\)l of \([^{14}C]\)arachidonate. At the end of the incubation period, EDTA was added to a final concentration of 2 mM and the platelets were pelleted by centrifugation at 3000 \(\times\) g for 20 min at 0-4°C. The pellet was resuspended gently in 50 ml of NaCl-Tris-HCl buffer (290 mM/tris buffer, pH 7.4 (Tris/NaCl buffer). The platelets from 500 ml of blood were finally suspended in 20 to 25 ml of buffer and used within 1 h.

Incubation Procedures and Lipid Extraction—Incubations were performed in duplicate or triplicate in a shaking water bath at 37°C for 30 min. The reaction volume was usually 1 ml unless otherwise mentioned and contained platelets equivalent to 4 mg of protein (26) (approximately 2 \(\times\) 10\(^{10}\) cells). The incubation mixture contained buffer (pH 7.4) plus appropriate additions as described in the legends to the figures and tables. In order to lyse the platelets, the suspension was frozen once and thawed and then sonicated (three times, 30 s) using a Branson Sonifier at maximum output. The sonicated lysate was then centrifuged at 30,000 \(\times\) g for 30 min to remove unbroken cells. One portion of this cell-free lysate was kept at 0-4°C and the other portion was centrifuged at 100,000 \(\times\) g for 30 min.
Platelet Phospholipases A₂ and C

RESULTS

Activation of Phospholipase A₂ by Deoxycholate—Previous studies have indicated that the activity of phospholipase A₂ is dependent on Ca²⁺ and optimal at alkaline pH values (10, 11). In those reports, however, there is no characterization of the lysophospholipids induced by phospholipase A₂ activity. Fig. 1 shows the iodine staining of phospholipids from platelets treated with increasing concentrations of deoxycholate. There are no detectable amounts of lysophospholipids in the absence of deoxycholate. Lyso-PE and lyso-PC are formed in the presence of 0.3 mM deoxycholate and increased up to 2.5 mM (Fig. 1).

Differential Ca²⁺ Requirements for Phospholipase C and Phospholipase A₂—We have previously shown (12) that the expression of platelet phospholipase C requires the presence of deoxycholate. Such enhancement of phospholipase C activity occurs at pH 7.4 and needs no exogenous, additional supply of Ca²⁺. Under these conditions, phosphatidylinositol (PI) is the only phospholipid which is degraded; diacylglycerol accumulates; and arachidonate or arachidonate metabolites are not released (Fig. 2) (12). However, after addition of Ca²⁺, substantial amounts of arachidonate metabolites are produced in a concentration-dependent manner (Fig. 2). Addition of Ca²⁺ further increases diacylglycerol production with a concomitant increase in the breakdown of PI. The breakdown of PI accounts for the diacylglycerol produced but not for all of the arachidonate which is released. However, the loss of arachidonate from phosphatidylethanolamine (PE) and phosphatidylcholine (PC) is nearly equivalent to the arachidonate metabolites which accumulate (Fig. 3). At the same time, substantial quantities of the lyso-derivatives of PE and PC are formed (Table I) which can be visualized by iodine staining (Fig. 1). These experiments clearly show that platelet phospholipase A₂ activity results in the release of arachidonate from PE and PC but not from PI. Phospholipase C hydrolyzes only PI, and this activity requires less Ca²⁺ than does phospholipase A₂. Furthermore, the use of deoxycholate not only activates the phospholipase activities but also greatly enhances their sensitivity to the presence of Ca²⁺ (compare with Refs. 10 and 11).

Fig. 1. Profile of iodine-stained phospholipids from platelets treated with different concentrations of deoxycholate (DOC). Assays (1 ml) containing 4 mg of protein were incubated at 37°C for 30 min in the presence of 1 mM Ca²⁺, pH 9.0. Lipids were separated on TLC (Silica Gel H) using chlorormethanol/acetate/water (75:45:12.5, v/v) as described under "Experimental Procedures." SM, sphingomyelin.
Activities—Phospholipase C and Phospholipase A2 activities fell substantially, as indicated by the reduced production of arachidonate metabolites at pH 7.4 as a function of Ca²⁺ concentration. Ca²⁺ was added to sonicated platelets (4 mg of protein, approximately 2 × 10⁸ cells) before addition of deoxycholate at a final concentration of 2.5 mM. Incubations (1-ml assays) were carried out at 37°C for 30 min. For details, see “Experimental Procedures.” Values for arachidonate metabolites represent free arachidonate plus arachidonate metabolites. Production of [¹⁴C]diacylglycerol (Δ, ▲) and of [¹⁴C]arachidonate metabolites (○, ●) was measured with no additions (Δ, ○) or in the presence of 2.5 mM deoxycholate (▲, ●).

Effect of pH on Phospholipase C and Phospholipase A₂ Activities—Phospholipase C and Phospholipase A₂ activities could be further differentiated by varying the pH of the medium. There is a considerable increase in the phospholipase C activity when the pH is raised from 7.5 to 9.5, as measured by the accumulation of arachidonate plus arachidonate metabolites (Fig. 4) and also by the loss of arachidonate from PE and PC (Fig. 5). At the same time, the phospholipase C activity falls substantially, as indicated by the reduced production of diacylglycerol (Fig. 4) and the diminished loss of PI (Fig. 5). At pH 9.0 to 9.5, the activity of phospholipase A₂ is nearly maximal, while the activity of phospholipase C is profoundly decreased. Arachidonate is not released from triglycerides under these conditions.

Formation of Lysophospholipids—Fig. 1 shows the initial observation that indicated the formation of lysophospholipids. Further measurements of these lysophospholipids were performed after determination of the optimal conditions for phospholipase C and phospholipase A₂ on endogenous phospholipids. Table I summarizes an analysis of platelet phospholipids in the presence and absence of deoxycholate at two different pH values (i.e. 7.4 and 9.0). The loss of individual phospholipid...
Platelet Phospholipases A₂ and C

Table II

Degradation of [³²P]Phosphatidylinositol by platelets in the presence of deoxycholate

Various amounts of [³²P]PI (approximately 500 cpm/nmol) were transferred into assay tubes and the solvent (chloroform) was evaporated. After sonication in 0.25 ml of Tris/NaCl buffer, pH 9.0, 0.25 ml of platelet suspension (2 mg of protein), also at pH 9.0, was added. Incubations at 37°C for 30 min were carried out without additions or in the presence of 2.5 mM deoxycholate. [³²P]PI and [³²P]lyso-PI were separated, localized, and quantitated as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>PI concentration (nmol)</th>
<th>Lyso-PI formed (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>1.6</td>
</tr>
<tr>
<td>45</td>
<td>4.9</td>
</tr>
<tr>
<td>120</td>
<td>15.7</td>
</tr>
<tr>
<td>330</td>
<td>42.3</td>
</tr>
</tbody>
</table>

Fig. 6. Production of [¹⁴C]diacylglycerol (●, △) and [¹⁴C]arachidonate metabolites (○, □) in whole homogenates and in the particulate fractions as a function of the Ca²⁺ concentration. Experiments were performed at pH 7.5. For experimental conditions, see the legend to Fig. 2. △, ○, particulate fractions; ●, □, homogenates.

Fig. 7. Degradation of [¹⁴C]-labeled phospholipids by total homogenate and particulate fractions from platelets. Samples are from the same experiment as described in the legend to Fig. 6. a, degradation of [¹⁴C]PI by particulate fraction (●, ○) and total homogenate (△, △) in the absence (○, ○) or in the presence (●, △) of 2.5 mM deoxycholate. b, degradation of [¹⁴C]PE by particulate fraction (●, ○) and total homogenate (△, △) in the absence (○, ○) or in the presence (●, △) of 2.5 mM deoxycholate.

Discussion

The present data indicate that PI is a poor substrate for phospholipase A₂ activity and confirm that PI is instead cleaved primarily (if not exclusively) by phospholipase C (12). Phospholipase A₂ activity degrades other phospholipids, PE, PC, and PS. These two phospholipases thus clearly act on different substrates.

In addition, these two phospholipases differ in other important properties. Phospholipase C is present mainly in the cytosolic fraction, while phospholipase A₂ is associated with the particulate fraction of platelets. Although both activities require Ca²⁺, phospholipase A₂ requires much higher concentrations than does phospholipase C. The maximal activity of phospholipase C is observed at a physiological pH, while phospholipase A₂ is more active at higher pH values.

Moreover, upon stimulation of platelets with thrombin, phospholipase C is activated before phospholipase A₂ (8). This implies that, following a physiological stimulus, the metabolic route to arachidonate production involves the sequential activation of phospholipase C followed by phospholipase A₂. It is possible that phosphatidic acid is a product of the combined activities of phospholipase C and diacylglycerol kinase (8, 12), which in turn could stimulate phospholipase A₂ activity (11, 25). The present data indicate that phospholipase C is fully expressed at pH 7.4 and that additional Ca²⁺ at this pH activates phospholipase A₂. Inde-
ndependent studies indicate that addition of phosphatidic acid to intact platelets results in the release of arachidonate from phospholipids as well as the production of arachidonate metabo-

lites. In stimulated platelets, arachidonic acid is not only released from PE and PC but also from PI (1-8, 11, 14), pointing to the possible involvement of other enzymatic activities. Recently, it has been proposed that diacylglycerol lipase releases arachidonate from the diacylglycerol released from PI by phospholipase A2. Diacylglycerol is also an effective substrate for diacylglycerol kinase, which appears to be much more active than diacylglycerol lipase in intact platelets (36). Diacylglycerol kinase phosphorylates very rapidly the diacylglycerol produced from PI, leading to the formation of phosphatidic acid (12). Eventually, phosphatidic acid may also release its arachidonic acid. This could be achieved directly by the action of phospholipase A2 on phosphatidic acid, or indirectly through the involvement of an acyltransferase activity which might catalyze an exchange of arachidonate from phosphatidic acid to PE and PC (25). PE and PC would then be degraded specifically by phospholipase A2 to release arachidonate, as shown in the present studies. We have been unable, by a variety of procedures, to detect degradation of phosphatidic acid by phospholipase A2. It will now be crucial to study the putative acyltransferase activity which could establish the relationship of phosphatidic acid with PE and PC in the mihilization of arachidonate from PI. These considerations could also explain why there is no accumulation of diacylglycerol or lysophospholipids during platelet activation. The diacylglycerol produced by the action of phospholipase C must be immediately decaylated (14) or phosphorylated (8, 12), and the lysophospholipid produced by phospholipase A2 would have to be reacylated very rapidly. Diacylglycerol or lysodervatives can only be detected in the presence of deoxycholate, which almost certainly is perturbing and uncoupling reactions that in the intact cell are probably very closely linked. The fact that during platelet activation PI is the only phospholipid which is decreased in mass, while PC and PE also liberate arachidonate (without a change in mass) (8, 16), suggests a rapid and efficient process of fatty acid mobilization and transfer among different lipids.

The results of the present studies, together with previously reported information (8, 25), indicate that stimuli acting on cell surface receptors might first be stimulating a cytosolic enzyme (phospholipase C) followed by a membrane-bound enzyme (phospholipase A2). Although this could be interpreted through the action of a second messenger, an alternative possibility is that thrombin produces conformational changes in the platelet membrane that allow the exposure of PI to a cytosolic enzyme. This could lead to a rapid production of phosphatidic acid in the plasma membrane and induction of phospholipase A2 activity.

REFERENCES


