Are Lysophosphatidic Acids or Phosphatidic Acids Involved in Stimulus Activation Coupling in Platelets?

By A. M. Benton, J. M. Gerrard, T. Michiel, and S. E. Kindom

Phosphatidic acids and/or lysophosphatidic acids are potentially pivotal compounds produced during metabolism of phosphatidylinositol, a very early event in the response of platelets to thrombin and other stimuli. In the present study, we have reevaluated the platelet aggregation produced by these agents to assess whether one or both could possibly function as intermediates in the transmission of the activating signal. Liposomes could be used to deliver lysophosphatidic acids but were not more effective than using 25% ethanol, dimethylsulfoxide, or even good suspensions in Hank’s balanced salt solution. Aggregation induced by lysophosphatidic acid was considerably influenced by the concentration of albumin in the medium, with the highest percentage of aggregation in the absence of albumin and decreasing degrees of aggregation as more albumin was present. Aggregation in 1 mg/ml albumin was optimum at 0.5–1.0 mM Mg, when calcium was held constant at 0.5 mM. When magnesium was 0.5 mM, then the response to lysophosphatidic acid increased with calcium concentration from 0 to 2 mM. The optimum pH for lysophosphatidic-acid-induced aggregation was 7.50–7.75. The presence of 1 mg/ml fibrinogen lowered the mean concentration of lysophosphatidic acid required for 50% aggregation from 10 μM to 1.2 μM. Submicromolar concentrations of lysophosphatidic acid were sufficient to convert a weak ADP response to full aggregation. In contrast, purified phosphatidic acid added alone did not cause platelet aggregation or stimulate calcium flux. Only when added in liposomes did any phosphatidic acid species enhance ADP-induced aggregation, and this was seen only at concentrations approaching 100 μM. The results show that lysophosphatidic acids are much more potent than phosphatidic acids and support the concept that it is lysophosphatidic acid rather than phosphatidic acid that is a significant stimulus produced during phosphatidylinositol metabolism and involved in stimulus activation coupling.

STIMULUS ACTIVATION COUPLING is a critical process in many cells, including platelets. It is widely believed that calcium forms an essential common pathway for platelet activation and that there must therefore exist mechanisms for coupling the interactions of stimulating agents with their receptors to internal fluxes of calcium. One of the earliest events to occur following stimulus of platelets by thrombin is the metabolism of phosphatidylinositol with the formation of phosphatidic acid. Substantial evidence now links this early process to release of arachidonic acid from cell phospholipids. The arachidonic acid can be converted to thromboxane-A2, which may function as one pathway for stimulation of a flux of calcium. However, it is also abundantly clear that many of the responses to thrombin are not dependent on the production of thromboxane and that there must be at least one other mechanism for stimulating a flux of calcium.

Recent evidence implicates phosphatidic and lysophosphatidic acids as critical intermediates. Both of these compounds are produced in significant quantities, with current evidence suggesting lysophosphatidic acid production may be about 10% of that of phosphatidic acid. Both compounds have also been reported to cause platelet aggregation, though the only published report showing activity with phosphatidic acid suggests it is about tenfold less active than lysophosphatidic acid. Based on the lesser relative production but greater effect of lysophosphatidic acid, one could conclude that both these compounds may be equally important. However, we have found that all commercial preparations of phosphatidic acids are contaminated by lysophosphatidic acids, and therefore, it was critical in the present study to reevaluate this question with purified phosphatidic acid.

A consideration that has tended to make lysophosphatidic acids unlikely endogenous mediators is that the effects of these agents on human platelets have been found primarily at higher concentrations than would be expected to be produced within the cell. We have therefore carefully evaluated the conditions required for optimum aggregation to see if the higher concentrations found in earlier studies actually resulted from suboptimal conditions for assessment of platelet aggregation in response to this agent. The results of the present study strongly support the concept that lysophosphatidic acid, not phosphatidic acid, is a critical intermediate in stimulus activation coupling.

MATERIALS AND METHODS
Preparation of Washed Platelets

Blood was drawn directly from the antecubital vein of normal donors, following informed consent, into plastic syringes containing...
1 ml of citrate, citric acid, dextrose anticoagulant (93 mM sodium citrate, 7 mM citric acid, 140 mM dextrose, pH 6.5) per 9 ml of whole blood. Platelet-rich plasma (PRP) was obtained by centrifugation of anticoagulated whole blood at 100 g for 20 min at room temperature. The PRP was then removed and an equal volume of the above anticoagulant solution was added. The citrated PRP was cooled on ice for 5 min, then the platelets were pelleted by centrifugation at 900 g for 10 min at 4°C. The supernatant was removed and the platelet pellet was routinely resuspended in Hank's balanced salt solution, pH 7.4, containing 10 mM glucose, 0.5 mM MgCl₂ and 1 mg/ml bovine serum albumin (BSA) at a platelet concentration of 2-4 x 10⁸/ml. In selected experiments where the cation concentration was varied, the magnesium was omitted from this resuspending buffer. In experiments where the albumin concentration was varied, the albumin was added in the appropriate concentration and aggregation at 900 g/min was performed. In experiments where the pH was varied, the albumin concentration was varied, the pH of the washed platelet suspension was adjusted with either 7% NaHCO₃ or 0.1 N HCl just before study on the aggregometer. Fibrinogen (1 mg/ml) was added to the resuspending buffer in experiments designed to assess the influence of this protein.

Platelet Aggregation Studies

Washed platelets were kept at 4°C until just before use. They were then incubated for 3 min at 37°C in a water bath to allow the temperature of the platelet suspension to reach 37°C before the response of the platelets was studied on a Payton Dual Channel aggregometer (Payton Corp., Scarborough, Ont.) Calcium (0.5 mM except where indicated) was added 1 min before addition of lysophosphatidic acid or phosphatidic acid. Percent aggregation was the maximum extent of platelet aggregation achieved within the first 4 min. All aggregation studies reported here are based on the results of at least three separate experiments with similar results. Analysis of lactate dehydrogenase levels and serotonin secretion were performed using previously published methods.

Formation of Liposomes

Phosphatidylinositol liposomes (yeast PI, Serdary Research Co., London, Ont.) containing lysophosphatidic acid or phosphatidic acid were prepared at 20 mM phosphatidylinositol and 4 mM lysophosphatidic acid or phosphatidic acid by sonication in distilled water using a bath type sonicator (15 min at 37°C). Control liposomes containing phosphatidylinositol only were prepared in a similar fashion and were always used to check that such liposomes had no effect by themselves. On occasions, other ratios of phosphatidylinositol to lysophosphatidic acid and phosphatidic acid were evaluated, as was the use of phosphatidycholine liposomes from beef heart. These were found to be less effective, and all the studies presented here were done using the ratio 20 mM phosphatidylinositol to 4 mM lysophosphatidic acid or phosphatidic acid.

Calcium Flux Studies

Calcium flux was studied using membrane vesicles prepared according to Gerrard et al., which is a further modification of the method of Robblee et al., following the first modification by Kaser-Glanzmann et al. These vesicles were equilibrated with calcium 45 in the presence of adenosine triphosphate and magnesium for 10 min, at which time a sample was taken to assess uptake of calcium by the vesicles. To prevent further uptake of calcium, 600 μM EDTA was added, followed immediately by lysophosphatidic acid, phosphatidic acid, or buffer control. Samples were then taken at 15 and 20 min to assess calcium release stimulated by each condition.

Materials

Palmitoyl-lysophosphatidic acid, oleoyl-lysophosphatidic acid, dipalmitoyl-phosphatidic acid and dioleoyl-phosphatidic acid were purchased from the Serdary Research Laboratories (London, Ontario, Canada). Egg-lecithin-derived phosphatidic acid was purchased from the Sigma Chemical Co. (St. Louis, Mo.). Phosphatidic acids were found to contain contaminating lysophosphatidic acids and so were prepared free of these contaminants using either of two procedures. Studies with each procedure gave similar results. In the first method, phosphatidic acid was first converted to its sodium salt, then partially purified by separation on silicic acid as described by Renkonen with elution of phosphatidic acid using 5 column volumes of chloroform:methanol:acetic acid (95:4:1). The phosphatidic acid residue was then taken to dryness under nitrogen, and as it still contained a trace of lysophosphatidic acid, this was removed using a second silicic acid column in which the phosphatidic acid was eluted with 100% chloroform (45 ml/0.5 g silicic acid).

The second preparation of phosphatidic acids involved using thin-layer chromatography on silica gel G plates (Analtech Inc., Newark, Del.). Phosphatidic acid and lysophosphatidic acid were separated using the solvent system diethyl-ether:ethanol:formic acid:HCl 150:1:2:0.5 (v/v). The phosphatidic acids ran with an RF of 0.5, while the lysophosphatidic acids remained at the origin. The region containing the phosphatidic acid was scraped immediately into chloroform:methanol 2:1 and extracted by vortexing for 3 min. After centrifugation at 200 g for 10 min the chloroform:methanol was removed and the silicic acid reextracted with further chloroform:methanol 2:1. The two chloroform:methanol fractions were pooled and evaporated to 1 ml under nitrogen. After filtration using fluorosphere filters (0.45 μ) (Millipore Corp.) to remove traces of silicic acid, the phosphatidic acid was taken to dryness under nitrogen and immediately resuspended in 1 ml chloroform. Purity of the phosphatidic acid was evaluated immediately before use to insure that none had broken down to lysophosphatidic acid. The chloroform was reagent grade (Fisher), and was distilled twice before use. Methanol was HPLC grade. Lysophosphatidic acids and phosphatidic acids were routinely dissolved in 25% ethanol for study unless stated otherwise. For aggregation studies, the final amount of 25% ethanol added was such that the concentration of ethanol did not exceed 0.5% in the final solution. The concentration of purified phosphatidic acid was determined by analysis of phosphorous content according to the method of Gerlach and Deuticke.

RESULTS

The Influence of Vehicle on Aggregation by Lysophosphatidic and Phosphatidic Acids

Since lysophosphatidic acid does not easily go into aqueous solution, it was important to compare its effectiveness when delivered in various vehicles. Figure 1 shows that there was not a great deal of difference in the effectiveness of lysophosphatidic acids when delivered in 25% ethanol, in dimethylsulfoxide, in liposomes, or in Hank's balanced salt solution containing bovine serum albumin (HBSS/BSA). However, on occasion, difficulty was obtained in achieving a good suspension of lysophosphatidic acid in HBSS/BSA, and 25% ethanol was used as the solvent for the rest of the studies. Measurement of lactate dehydrogenase levels in the supernatant of lysophosphatidic-acid-aggregated platelets showed less than 1% lysis of
platelets at concentrations of up to 25 \( \mu M \) lysophosphatidic acid.

Phosphatidic acids (dipalmitoyl, dioleoyl, and egg lecithin derived) were similarly evaluated in 25% ethanol, in dimethylsulfoxide, in HBSS/BSA and in a variety of liposome preparations. At no concentration of phosphatidic acid in any of these vehicles did we see any platelet aggregation (see Fig. 2 for a comparison of phosphatidic acid and lysophosphatidic acid). Some shape change was occasionally seen at concentrations greater than 50 \( \mu M \). Phosphatidic acids were not further evaluated except in experiments designed to study the enhancement of ADP-induced aggregation.

**The Influence of Albumin on Lysophosphatidic-Acid-Induced Aggregation**

When the platelets were washed and resuspended in HBSS containing 0–50 mg/ml albumin, the concentration of lysophosphatidic acid needed to achieve full aggregation increased as the concentration of albumin was increased (Fig. 3). The results shown in Fig. 3 were repeated in three separate experiments. However, in one other experiment, no aggregation was seen when lysophosphatidic acids were added to platelets suspended without albumin, while aggregation occurred normally in HBSS containing 1 mg/ml albumin. We have no explanation for this result, except that a small amount of albumin may help preserve platelets, but for all other experiments, HBSS containing 1 mg/ml albumin was used.

**The Influence of Ca\(^{2+}\) and Mg\(^{2+}\) Ion Concentration**

When the calcium concentration was kept constant at 0.5 mM and the magnesium ion concentration varied from 0 to 2 mM, optimum platelet aggregation

---

**Fig. 1.** The influence of the vehicle used on platelet aggregation stimulated by palmitoyl-lysophosphatic acid (LPA). LPA dissolved in chloroform:methanol 2:1 was dried under nitrogen and then dissolved in dimethylsulfoxide (DMSO), or 25% ethanol, or dispersed in Hank's balanced salt solution (HBSS) containing 1 mg/ml albumin, or in phosphatidylinositol liposomes. As shown, there was relatively little difference between the various vehicles. LPA concentration (on the abscissa) in this and the other figures is plotted on a logarithmic scale.

**Fig. 2.** A comparison of the response of platelets to palmitoyl-lysophosphatic acid (LPA) and dioleoyl-phosphatic acid (PA). Platelets were washed once and resuspended in HBSS containing 1 mg/ml BSA. LPA and PA in 25% ethanol were added at the time point shown by the arrow at the indicated concentrations. Dipalmitoyl-phosphatic acid and egg-lecithin-derived phosphatic acid (not shown) were no more effective at initiating platelet aggregation than dioleoyl-PA. There was no difference in the results if the PAs were resuspended in DMSO, HBSS/BSA, or in various liposome preparations.
PHOSPHOLIPIDS AND PLATELET AGGREGATION

was seen at 0.5–1 mM Mg (Fig. 4). When the magnesium ion concentration was kept constant at 0.5 mM, and the calcium ion concentration varied from 0 to 2.0 mM, there was a progressive increase in lysophosphatidic-acid-induced platelet aggregation as the calcium ion concentration was increased.

The Influence of pH on Lysophosphatidic-Acid-Induced Platelet Aggregation

When the pH of the platelets suspended in HBSS/BSA was varied from 6.50 to 8.50, the pH optimum for lysophosphatidic-acid-induced aggregation was 7.50.

Fig. 3. The influence of albumin concentration on aggregation induced by lysophosphatidic acid (LPA). Platelets were washed as described in the text and resuspended in HBSS containing various albumin concentrations. Palmitoyl-LPA in 25% ethanol was added to platelets stirred on the platelet aggregometer and aggregation evaluated. On some occasions, addition of LPA in excess of that required to produce maximal aggregation in HBSS and in HBSS containing 1 mg/ml BSA was associated with a subsequent fall off in the total extent of aggregation. The reasons for this decrease in total extent of aggregations with higher concentrations of LPA was not explored and the explanation is not known. LPA was tested at concentrations lower than those shown, but no aggregation was seen.

Fig. 4. The influence of altering the calcium and magnesium ion concentrations on aggregation induced by 2.5 μM palmitoyl-lysophosphatidic acid (LPA). When the calcium (Ca++) concentration was varied, the magnesium ion concentration was held constant at 0.5 mM. When the magnesium (Mg++) ion concentration was varied, the calcium ion concentration was held constant at 0.5 mM. Results are shown as the mean ± SE of three experiments.
With increased or decreased pH, a higher concentration of lysophosphatidic acid was needed to see full aggregation (Fig. 5).

The Influence of Fibrinogen

Without fibrinogen, 50% aggregation required an average of 10.2 μM palmitoyl-lysophosphatidic acid (SD 9.7, n = 11 different donors). With 1 mg/ml fibrinogen present in the resuspending buffer, aggregation required an average of 1.2 μM palmitoyl-lysophosphatidic acid (SD = 1.4, n = 5 different donors).

Enhancement of ADP-Induced Aggregation

Both palmitoyl and oleoyl-lysophosphatidic acid were effective in enhancing ADP-induced aggregation. Palmitoyl-lysophosphatidic acid was more effective than oleoyl-lysophosphatidic acid, for the former could convert a small degree of ADP-induced aggregation to full aggregation at 0.25 μM, while the latter required 2.5 μM. In contrast, dioleoyl-phosphatidic acid produced only partial enhancement of aggregation at a concentration of 85 μM, and even then it was effective.
Fig. 7. The influence of palmitoyl-lysophosphatidic acid (P-LPA), oleoyl-lysophosphatidic acid (O-LPA), and dioleoyl-phosphatidic acid (0-PA) on enhancement of ADP-induced aggregation. All three compounds were tested over a range of concentrations from 10⁻⁷ to 10⁻⁵ M with the results shown. The LPAs were dissolved in 25% ethanol, while 0-PA was prepared in P1 liposomes. 0-PA in 25% ethanol or in DMSO had no effect to enhance ADP-induced aggregation at concentrations up to 10⁻⁵ M. It was only when 0-PA was prepared in liposomes that it had any effect. The P1 liposomes alone had no effect (not shown).

Only when added in liposomes, dipalmitoyl-phosphatidic acid and egg-lecithin-derived phosphatidic acid were completely ineffective in enhancing ADP-induced aggregation when used alone or in liposomes at concentrations up to 10⁻⁵ M.

The minimum difference between the phosphatidic acid and lysophosphatidic acid is therefore 80-350-fold (Fig. 6 and 7).

The Role of Thromboxane Synthesis and Secretion in Lysophosphatidic Acid Effects on Platelets

Preincubation of platelets for 15 min with 100 μM aspirin to inhibit thromboxane synthesis blocked serotonin secretion in response to lysophosphatidic acid regardless of whether or not lysophosphatidic acid was used alone or together with a small dose of ADP. (Secretion in the absence of aspirin was 10%-40%; secretion in the presence of aspirin was less than 2%. The results were consistent through 5 separate experiments.) However, a full dose-response curve for lysophosphatidic-acid-induced aggregation on five different donors showed a significant effect to partially suppress platelet aggregation in only one of these donors. The effect of lysophosphatidic acid to enhance ADP-induced aggregation was more susceptible to inhibition by aspirin (significant inhibition in 2 of 3 donors).

Calcium Release From a Platelet Membrane Fraction

Since earlier studies suggested that phosphatidic acid might act intracellularly to release calcium from a platelet calcium-sequestering membrane these studies were repeated using purified phosphatidic acid. The data shown in Table 1 demonstrate that phosphatidic acid had no effect when compared to lysophosphatidic acid added in similar concentrations. The results confirm that lysophosphatidic acid is, at the very least, much more effective than phosphatidic acid.

DISCUSSION

The results of the present study show that lysophosphatidic acids are considerably more effective as agonists of human platelets than has been previously demonstrated. Previous studies have shown effects on human platelets at concentrations of 5-100 μM while the present study shows significant effects at concentrations as low as 0.25 μM. The effectiveness of the lysophosphatidic acids depends critically on the conditions used to study aggregation, with albumin content, fibrinogen content, calcium content, and pH of the resuspending buffer all being important. Consistent results also require good solubilization of this agent. In addition, our studies demonstrate that lysophosphatidic acids are at a minimum 80-350-fold more effective than phosphatidic acid in enhancing ADP aggregation.

Recent results of Lapetina show that lysophosphatidic acid production is at least 5%-10% of the platelet phosphatidic acid production in response to thrombin. It is possible that this estimate may considerably understate the relative production of lysophosphatidic acid if there is rapid deacylation and then reacylation of phosphatidic acid. Thus, from studies of both production and effectiveness, it would be expected,
based on present data, that lysophosphatidic acid should contribute more to the effect of the phosphatidylinositol response than phosphatidic acid.

There are certain limitations to the above analysis. First, it is possible that lysophosphatidic acid was able to reach the site of action better than phosphatidic acid. For this reason, we tried several vehicles and several different types of liposomes to deliver both the phosphatidic and lysophosphatidic acids. It was only with liposomes that an effect of phosphatidic acids could be seen perhaps reflecting the fact that phosphatidic acid alone may cross membranes much less well than lysophosphatidic acid. However, even when delivered to an isolated membrane fraction, only the lysophosphatidic acid was effective in initiating calcium release. Earlier studies suggesting that phosphatidic acids were effective probably results from contamination with lyso-derivatives. Indeed, we can not completely exclude the possibility that the weak effect seen in the present study with dioleoyl phosphatidic acid was due to the presence of a tiny and undetectable (by thin-layer chromatography) amount of lysophosphatidic acid. Thus, though we cannot unequivocally rule out the possibility that we were unable to deliver phosphatidic acid to a specific intracellular site of action, these studies performed to the best of our current ability to test this question show that phosphatidic acid is far less effective than lysophosphatidic acid.

Another factor that must be considered is that phosphatidylinositol breakdown in platelets results in production primarily of arachidonyl-stearoyl phosphatidic acid. To achieve the closest approximation of this phosphatidic acid species we used egg-lecithin-derived phosphatidic acid, which contains some of this species. The egg-lecithin-derived phosphatidic acid was ineffective. We therefore conclude that it is highly unlikely that arachidonyl-stearoyl phosphatidic acid is more effective than dioleoyl-phosphatidic acid.

Another question to be addressed is the concentration of lysophosphatidic acid produced in the cell relative to the concentration of lysophosphatidic acid required to stimulate platelets when added externally. As shown in the present study, lysophosphatidic acid added externally has significant effects on ADP-induced aggregation when added at concentrations as low as 0.25 μM. How does this compare with the best current estimate of lysophosphatidic acid production in stimulated platelets? Broekman et al. have shown that on thrombin stimulation, 1.97–5.65 nmole phosphatidic acid is produced per 10^7 platelets (data from Tables I and II of reference 8). In a suspension of 3 x 10^7 platelets/ml, it would be expected that there would be 0.6–1.7 μM phosphatidic acid or 0.03–0.17 μM lysophosphatidic acid achieved (using Lapetina’s estimate of lysophosphatidic acid as 5%–10% of phosphatidic acid). However, considerable evidence suggests that these compounds produced during the phosphatidylinositol response do not leave the cell but stay inside. Thus, production of phosphatidic acid and lysophosphatidic acid distributed solely within the cell would achieve concentrations within the cell of 270–770 μM phosphatidic acid and 14–77 μM lysophosphatidic acid based on a mean cell volume of 7.3 cu. μm. There would appear to be little doubt that the amount of lysophosphatidic acid produced in response to thrombin is enough to have a significant effect on platelet function. We therefore conclude that some of the effects of thrombin on platelets are almost certainly mediated through production of lysophosphatidic acid. To refine this analysis, it will be necessary to have better estimates of lysophosphatidic acid production in the cell and to know the concentrations of lysophosphatidic acid reached within the cell when lysophosphatidic acid is added under the conditions used in these experiments. It should also be noted that the comparative role of lysophosphatidic acid and other factors in the thrombin response have yet to be analyzed.

The mechanism of action of lysophosphatidic acid is not known with certainty, though it can act as a calcium ionophore, and evidence suggests a role for it to initiate a flux of calcium to raise the cytoplasmic levels of this ion. Lysophosphatidic acids can also promote thromboxane-A2 production, and such production is associated with enhanced granule secretion. This thromboxane production and granule secretion may under some circumstances, and in some donors be associated with enhancement of platelet aggregation. The role of this thromboxane synthesis in aggregation appears most similar to studies with the calcium ionophore A23187, which can promote thromboxane production, which is in some circumstances associated with a small but significant enhancement of A23187-induced aggregation. However, lysophosphatidic acid-induced secretion appears more susceptible to aspirin inhibition than A23187-induced granule secretion for reasons not presently understood. While studies reported here and previously cannot completely rule out a requirement for a very small amount of extracellular ADP for lysophosphatidic-acid-induced aggregation, the primary effect of lysophosphatidic acid on platelets appears to be a direct one that requires neither thromboxane production nor granule secretion.

ACKNOWLEDGMENT

We thank Dr. L. Seargent for performing the LDH assays and J. M. McCrea and S. Glover for helpful discussion and assistance during the course of these studies.
PHOSPHOLIPIDS AND PLATELET AGGREGATION

REFERENCES

Are lysophosphatidic acids or phosphatidic acids involved in stimulus activation coupling in platelets?

AM Benton, JM Gerrard, T Michiel and SE Kindom