The Initial Action of Thrombin on Platelets

CONVERSION OF PHOSPHATIDYLINOSITOL TO PHOSPHATIDIC ACID PRECEDING THE PRODUCTION OF ARACHIDONIC ACID*

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Measurements of phosphatidylinositol (PI) and phosphatidic acid (PA) by phosphorus assays and by radioactivity ([14C]arachidonate) indicate that thrombin induces the degradation of a given fraction of the total PI to PA. The maximal conversion of PI to PA represents approximately one-third of the total PI which can be degraded by thrombin. This same amount of PI is converted to PA even in the presence of 1 mM quinacrine, which completely inhibits the release of arachidonic acid from phospholipids and which reduces by two-thirds the loss of labeled PI. In this case the fall in PI is equal to the amount of PA formed. If thrombin is added to platelets previously maximally stimulated by ionophore A23187, PA is produced from PI in amounts equal to those produced by thrombin in the absence of other stimuli. Furthermore, the resynthesis of PI from PA is also unaffected by quinacrine, and thus the entire thrombin-stimulated PI-cycle is maintained. The data thus indicate the existence of a quinacrine-insensitive phospholipase C which can initially convert a given amount of PI to PA and which is closely associated to the thrombin receptor. The further breakdown of PI and production of arachidonic acid might result from the action of quinacrine-sensitive activities (i.e. phospholipase A2). The simplest scheme is one in which thrombin specifically produces an active fraction of PA which in some way results in the subsequent production of arachidonic acid from various phospholipids (including PI), perhaps by activation of quinacrine-sensitive phospholipase A2.

The formation of phosphatidic acid in stimulated platelets (1-10) results from the combined activities of phospholipase C (which breaks down phosphatidylinositol to diacylglcerol) and diacylglcerol kinase (which phosphorylates the diacylglcerol to PA) (5, 6). The production of PA is observed prior to the formation of arachidonic acid (4), is independent of cyclooxygenase and lipoygenase products (4, 9), is blocked by cyclic AMP and serine esterase inhibitors (2, 4, 7, 8), and parallels the release of serotonin (4). The early generation of PA in response to thrombin might be related to several physiological processes, such as Ca2+ fluxes (11) and reactions leading to the formation of arachidonic acid (4, 6), which may participate in the mediation of shape changes, release reaction, and aggregation. The present studies indicate that PA in platelets is formed from a fraction of PI that is associated with thrombin receptors and which can occur unaltered even when the release of arachidonic acid from phospholipids is completely inhibited.

MATERIALS AND METHODS

Radioactive compounds and other chemicals were obtained as described before (2-5, 9, 10). Quinacrine was from Sigma. Labeling of horse platelets with [14C]arachidonic acid was carried out as previously reported (2-5, 9). Labeling of horse platelets with [3P]orthophosphate was done after separating the platelets from 1 unit of blood as described previously (2-5, 9). Platelets were then resuspended in 10 ml of buffer (134 mM NaCl, 15 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM glucose), 5 mCi of [3P]orthophosphate were added, and the platelets were incubated at 37° C for 2 h. The platelets were then sedimented at 3000 x g for 20 min, resuspended again in 100 ml of the same buffer and centrifuged. In all cases, appropriate dilutions of the platelet pellet were performed to achieve a final concentration of 1 x 107 platelets/ml, which was the volume used for the assays. Preincubations with quinacrine were usually for 5 min and additions of thrombin or ionophore A23187 were usually for 5 min. Two different batches of ionophore A23187 were bought from Eli Lilly and Co. and were used in our experiments. These batches had different potencies. One produced maximal release of arachidonic acid from platelets at 1 mM while the other was maximally active at 5-10 μM. Lipid extraction and chromatographic separation of lipids have been detailed before (4, 5, 9, 10). PA and arachidonic acid metabolites were separated on thin layer chromatography (4, PI was best separated on formaldehyde-impregnated papers (4, 9), and phosphatidylycholine and phosphatidylenolamine were separated on a thin layer chromatographic system used for separation of phospholipids (4). Radioactive spots were localized by radioautography and nanoradioactive lipids were detected by iodine vapors or Nile blue (paper chromatography). Phosphate determinations were performed as described earlier (4, 9).

Enzyme Assays—PI-specific phospholipase C was assayed as previously described (5). Diacylglycerol lipase was assayed as recently reported (12). The assay was carried out in a 0.2 ml volume containing 50 mM Hepes-NaOH (pH 7.0), 100 mM NaCl, 5 mM CaCl2, 12.5 mM reduced glutathione, and 200 μg of particulate protein (total particulate fraction from human or horse platelets was obtained as described before (9)). The reaction was started by adding 100 nmol of ([3H]arachidonoyl)diacylglycerol (1000 cpm/nmol) which had previously been resuspended by sonication in the buffer described above. Incubations were for 30 min at 37°C and reactions were stopped by the addition of 3.0 ml of chloroform/methanol/heptane (125:140:100, v/v) and 1 ml of buffer containing 50 mM K2CO3 and 50 mM H2BO3, pH 10.0. Phases were then separated and the upper aqueous phases (containing free fatty acids) were carefully transferred into scintillation vials containing 15 ml of Aquasol II fluid.

Preparation of ([3H]Arachidonoyl)diacylglycerol—Platelets from 2 units of horse blood were labeled with [3H]arachidonate (5,6,8,9,11,12,14,15-[3H]N)arachidonic acid, 78.2 Ci/nmol from New England Nuclear) as described elsewhere (2-5, 9). Lipids were extracted and dried under N2 and the residue was resuspended in chloroform (1 ml) and applied to a silicic acid column (0.6 x 4.0 cm; Unisil, Clarkson Chemical, PA). The neutral lipid fraction was eluted with 20 ml of methanol/chloroform (10:90, v/v). All phospholipids with the exception of sphingomyelin, were then eluted with 50 ml of...
polipid fraction was dried under nitrogen and resuspended in 10 ml of buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM CaCl₂, and 1 mM ZnSO₄. The suspension was then incubated with 50 units of phospholipase C from Bacillus cereus (Sigma) for 1 h at 37°C. Diacylglycerol was then extracted twice with 20 ml of diethyl ether and the upper phases were combined and dried under N₂. The residue was redissolved in 0.5 ml of diethyl ether/hexane (10:90, v/v) and applied onto another silicic acid column as described above which was first washed with 15 ml of diethyl ether/hexane (10:90, v/v). The diacylglycerol fraction was then eluted with 30 ml of diethyl ether/hexane (20:80, v/v). Thin layer chromatography of this fraction (petroleum ether/diethyl ether/acetone, 40:60:1, v/v) shows the presence of both 1,2-diacylglycerol (80% of the total radioactivity) and 1,3-diacylglycerol (20% of the total radioactivity) which co-chromatographed with authentic standards. Further characterization and purification of the 1,2-diacylglycerol was achieved by high pressure liquid chromatography on silicic acid (Whatman, PXS 10/25) with hexane/isopropanol/acetone (99:1:8, v/v). Again 80% of the total radioactivity was associated with the 1,2-diacylglycerol fraction. The 1,2-diacylglycerol was stored at −20°C in diethyl ether to prevent isomerization.

RESULTS

Thrombin Still Induces Degradation of PI and Formation of PA When the Production of Arachidonic Acid from Platelets Is Completely Inhibited—Quinacrine is known to inhibit the release of arachidonic acid from platelet phospholipids (13). This inhibitory action can be assessed by measuring the formation of arachidonic acid metabolites in thrombin-stimulated platelets (14, 15). Quinacrine at a concentration of 1 mM totally inhibits the appearance of arachidonic acid and its metabolites (Fig. 1). In the presence of quinacrine, arachidonic acid is not released from phosphatidylcholine and phosphatidylethanolamine (Fig. 1). However, quinacrine does not alter the thrombin-induced formation of PA and the disappearance of a corresponding quantity of PI (Figs. 1 and 2). Nearly two-thirds of the thrombin-induced loss of PI is prevented by quinacrine, despite the fact that the total amount converted to PA is not affected (Figs. 1 and 2). These results therefore indicate that quinacrine does not alter the thrombin-activated, PI-specific phospholipase C or the diacylglycerol kinase. Quinacrine does, however, inhibit the release of arachidonic acid from phosphatidylcholine and phosphatidylethanolamine as well as from PI (Figs. 1 and 2); thus, considerably less [14C]PI is lost in the presence of quinacrine (Figs. 1 and 2). PI therefore

![Fig. 1. Quinacrine inhibits the release of arachidonic acid from phospholipids in platelets. Samples (0.5 ml) that contained 1 × 10⁷ platelets prelabeled with [14C]arachidonic acid were preincubated with or without 1 mM quinacrine for 5 min at 37°C. Thrombin (1 unit/ml) was then added for 5 min. PC, phosphatidylcholine; PE, phosphatidylethanolamine; HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; HHT, 12-hydroxy-5,8,10-hepatecatrienoic acid. Open bars, control platelets; cross-hatched bars, thrombin-stimulated platelets; stippled bars, thrombin-stimulation of platelet pre-treated with quinacrine.](http://www.jbc.org/)

![Fig. 2. Quinacrine allows the degradation of PI to PA while inhibiting the release of arachidonic acid. Samples (0.5 ml) that contained 1 × 10⁷ platelets prelabeled with [14C]arachidonic acid were preincubated with different concentrations of quinacrine, as indicated, for 5 min at 37°C. Thrombin (1 unit/ml) was then added for 5 min (△, ○, ■, □, ◇, ○), controls in which thrombin was not added; □, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; ■, 12-hydroxy-5,8,10-hepatecatrienoic acid.](http://www.jbc.org/)

![](http://www.jbc.org/)
contributes not only through its conversion to PA but also through release of its arachidonic acid. The latter effect may occur by the action of a phospholipase A₂, perhaps utilizing the same mechanisms involved in the release of arachidonic acid from other phospholipids (9).

Specific Formation of PA by Thrombin—When platelets are stimulated with concentrations of thrombin or ionophore A23187 that maximally produce arachidonic acid from platelet phospholipids, a further challenge with any of these stimuli does not produce more arachidonic acid (4). However, these experiments indicate a major difference with regard to PI and PA. In platelets that have already been maximally stimulated to form arachidonic acid with ionophore A23187, thrombin still induces a quantitatively similar degradation of PI and the concomitant formation of equivalent amounts of PA (Fig. 3).

Quinacrine Does Not Inhibit the Resynthesis of PI Induced by Thrombin—Thrombin increases the incorporation of [³²P]Pi into PI in a time-dependent manner while the labeling of [³²P]PA is initially increased but decreases at later times (Fig. 4). Activation of the PI-specific phospholipase C by thrombin induces formation of diacylglycerol which is phosphorylated to PA (Fig. 4) (5, 6). PA is further transformed to CD[P-diacylglycerol and to PI, completing the so-called PI cycle (16–18), which is clearly not inhibited by quinacrine (Figs. 1, 2, and 4). Experiments with [³²P]labeled also show that thrombin is much more effective than ionophore A23187 in inducing formation of PA (Fig. 5) (4). Again, in these experiments thrombin exerts a specific effect on PA after treatment of the platelets with ionophore A23187 (Fig. 5). By prolonging the exposure of platelets to thrombin a fall in PA is detected which most probably represents its conversion into PI (Figs. 4 and 5). This action of thrombin in channeling PA to PI is further enhanced by ionophore A23187 (Fig. 5).

Content of PA and PI in Stimulated Platelets—Thrombin (5-min incubation) increases the total quantity of PA in platelets by more than 10-fold (Table I). The increase with ionophore A23187 is only about 3-fold. These increases correlate well with those observed under the same incubation conditions measuring [⁴⁺C]PA (Fig. 5) and [³²P]PA (Figs. 4 and 5) (4). In addition, thrombin further increases the amount of PA in platelets previously treated with ionophore A23187 or quinacrine (Table I). The amount of PA formed upon thrombin treatment is about one-third of the amount of PI that is degraded under the same conditions (Table I). The fact that the amount of PA after incubation (ionophore A23187 with or without thrombin) for 10 min is lower than that observed at 5 min is consistent with the conversion of PA to PI during longer periods of incubation (8, 19).

Effect of Quinacrine on Diacylglycerol Lipase activity of platelet membranes

Assays containing 200 μg of particulate protein from horse or human platelets were performed as described under "Materials and Methods." Quinacrine was used at a final concentration of 1 mM. Results are representative of three similar experiments.

| Additions | [³²P] Radioactivity
|-----------|-------------------|
| None      | 1015              |
| Membranes | 1055              |
| Membranes plus quinacrine | 965 |

TABLE II

Effect of quinacrine on diacylglycerol lipase activity of platelet membranes

Additions: [³²P] Radioactivity (counts per minute/mg protein)

| Additions | Horse | Human |
|-----------|-------|-------|
| None      | 1015  | 985   |
| Membranes | 1055  | 1955  |
| Membranes plus quinacrine | 965 | 2155 |

FIG. 4. Thrombin stimulation of [³²P]-labeling of PI and PA in the presence and absence of quinacrine. Samples (0.5 ml) of [³²P]-platelets (1 × 10⁹) were incubated with (Δ, ○) and without (Δ, □) 1 mM quinacrine for 5 min at 37 °C and then further incubated with thrombin (1 unit/ml) for the times as indicated.

FIG. 5. Effect of thrombin and ionophore A23187 on [³²P]-labeling of PA and PI. Samples (0.5 ml) of [³²P]-platelets (1 × 10⁹) were incubated with thrombin (1 unit/ml) or ionophore A23187 (1 μM) as indicated. (This batch of ionophore A23187 produced maximal release of [¹⁴C]arachidonic acid from platelets at 1 μM.)
is not affected by 1 mM quinacrine (Table II). Quinacrine (1 mM) also has no effect on the PI-specific phospholipase C (Fig. 6) as measured by the formation of 1,2-[14C]diacylglycerol after addition of deoxycholate and Ca2+ to platelets prelabelled with [14C]arachidonic acid (5). Fig. 6 also shows that prolonging the quinacrine treatment of platelets up to 30 min does not affect the phospholipase C activity or the thrombin-induction of phosphatidic acid.

**DISCUSSION**

The interaction of thrombin with platelet receptors appears to selectively activate the transient formation of PA from PI in a cycle of reactions that ultimately leads back to the resynthesis of PI (16–18). The conversion of PI to PA occurs prior to the release of arachidonic acid (4), and it is unaffected by quinacrine, which effectively inhibits the release of arachidonic acid from all phospholipids without affecting diacylglycerol lipase (Table II) or PI-specific phospholipase C (Fig. 6). Furthermore, this PA formation is also observed when thrombin is added to platelets that have been activated to release arachidonic acid by a different stimulus, ionophore A23187.

The fact that thrombin induces the formation of the same amount of PA under different conditions of stimulation (i.e. thrombin alone or after treatment with quinacrine or ionophore A23187) suggests that the principal initial effect of the activation of phospholipase C is the production of PA. This implies that the diacylglycerol initially formed by phospholipase C activity is effectively and completely phosphorylated rather than degraded by diacylglycerol-lipase (12, 19, 20). Significantly, the putative diacylglycerol-lipase is undetectable in horse platelets (Table II). This is further supported by recent results (8) that demonstrate that in platelets collagen induces stoichiometric, reciprocal changes in the amounts of PI degraded and PA formed.

It is known that arachidonic acid is derived from other phospholipids as well as PI on stimulation by thrombin (2, 8, 14). Thrombin degrades substantially more PI than can be accounted for by the initial formation of PA. The additional degradation of PI results in the formation of arachidonic acid metabolites. The contribution of PI to arachidonic acid metabolites might be explained by the action of a phospholipase A2 activity which could act on several different phospholipids (8, 9), including PI and PA itself.

Previously, we have proposed that the first step in the overall sequence that leads to the formation of arachidonic acid in thrombin-stimulated platelets is the activation of a phospholipase C which degrades PI to produce diacylglycerol and PA (4, 6, 9). This is then somehow followed by the action of phospholipase A2 to produce arachidonic acid from various phospholipids. It is pertinent that inhibition of phospholipase C by cyclic AMP (9) or serine esterase inhibitors (7) prevents the formation of arachidonic acid. However, phospholipase C is unaffected even when the production of arachidonic acid is completely inhibited by quinacrine. The production of PA by thrombin might result in cellular calcium gating (11, 18) which subsequently, among other effects, might also activate this putative phospholipase A2 and result in the production of arachidonic acid. Such a mechanism might also explain why ionophore A23187 bypasses the formation of PA during the release of arachidonic acid (i.e. possible direct stimulation of phospholipase A2) and why thrombin would still degrade PI and form PA after treatment of platelets with ionophore A23187.

**REFERENCES**

1. Lloyd, J. V., and Mustard, J. F. (1974) Br. J. Haematol. 26, 243–253
2. Lapetina, E. G., Chandraborse, K., and Cuatrecasas, P. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 818–822
3. Lapetina, E. G., and Cuatrecasas, P. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 121–125
4. Lapetina, E. G., and Cuatrecasas, P. (1979) Biochim. Biophys. Acta. 573, 394–402
5. Billah, M. M., Lapetina, E. G., and Cuatrecasas, P. (1979) Bioch. Biophys. Res. Commun. 90, 92–98
6. Lapetina, E. G., Billah, M. M., and Cuatrecasas, P. (1980) in The Regulation of Coagulation (Mann, K. G., and Taylor, F. B., eds) pp. 491–497, Elsevier North-Holland, Amsterdam
7. Walenga, R., Vanderhoek, J. Y., and Feinstein, M. B. (1980) J. Biol. Chem. 255, 6024–6027
8. Broekman, M. J., Ward, J. W., and Marcus, A. J. (1980) J. Clin. Invest. 66, 275–283
9. Billah, M. M., Lapetina, E. G., and Cuatrecasas, P. (1980) J. Biol. Chem. 255, 10227–10231
10. Lapetina, E. G., Billah, M. M., and Cuatrecasas, P. (1980) J. Biol. Chem. 255, 10966–10970
11. Gerrard, J. M., Butler, A. M., Peterson, S. A., and White, J. G. (1978) Prostaglandins Med. 1, 387–396
12. Bell, R. L., Kennerly, D. A., Standord, N., and Majerus, P. W. (1978) Proc. Natl. Acad. Sci. U. S. A. 76, 3238–3241
13. Blackwell, G. L., Duncombe, W. G., Flower, R. J., Parsons, M. F., and Vane, J. R. (1977) Br. J. Pharmacol. 59, 353–366
14. Lapetina, E. G., Schmitges, C. J., Chandraborse, K., and Cuatrecasas, P. (1977) Biochem. Biophys. Res. Commun. 76, 828–835
15. Lapetina, E. G., Schmitges, C. J., Chandraborse, K., and Cuatrecasas, P. (1978) in Advances in Prostaglandin and Thromboxane Research (Galli, C., Galli, G. and Porcellati, G., eds) Vol. 3, pp. 127–135, Raven Press, New York
16. Lapetina, E. G., and Hawthorne, J. N. (1971) Biochem. J. 122, 171–179
17. Lapetina, E. G., and Michell, R. H. (1973) FEBS Lett. 31, 1–10
18. Michell, R. H. (1975) Biochim. Biophys. Acta 415, 81–147
19. Bell, R. L., and Majerus, P. W. (1980) J. Biol. Chem. 255, 1790–1792
20. Rittenhouse-Simmons, S. (1980) J. Biol. Chem. 255, 2259–2262
21. Billah, M. M., Lapetina, E. G., and Cuatrecasas, P. (1981) J. Biol. Chem., 256, in press.
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