Phospholipid Metabolism in Stimulated Human Platelets: *CHANGES IN PHOSPHATIDYLINOSITOL, PHOSPHATIDIC ACID, AND LYSOPHOSPHOLIPIDS*

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ABSTRACT Endogenous phospholipid metabolism in stimulated human platelets was studied by phosphorus assay of major and minor components following separation by two-dimensional thin-layer chromatography. This procedure obviated the use of radioactive labels. Extensive changes were found in quantities of phosphatidylinositol (PI) and phosphatidic acid (PA) as a consequence of thrombin or collagen stimulation. Thrombin addition was followed by rapid alterations in the amount of endogenous PI and PA. The decrease in PI was not precisely reciprocated by an increase in PA when thrombin was the stimulus. This apparent discrepancy could be explained by removal of a transient intermediate in PI metabolism, such as diglyceride, formed by PI-specific phospholipase C (Rittenhouse-Simmons, S., *J. Clin. Invest.* **63:** 580–587, 1979). Diglyceride would be unavailable for PA formation by diglyceride kinase, if hydrolyzed by diglyceride lipase (Bell, R. L., D. A. Kennerly, N. Stanford, and P. W. Majerus. *Proc. Natl. Acad. Sci. U. S. A.* **76:** 3238–3241, 1979) to yield arachidonate for prostaglandin endoperoxide formation. Thrombin-treated platelets also accumulated lysophosphoglycerides. Specifically, lysophosphatidyl ethanolamines accumulated within 15 s following thrombin addition. Fatty acid and aldehyde analysis indicated phospholipase A_2 activity, with an apparent preference for diacyl ethanolamine phosphoglycerides. In the case of collagen, these changes occurred concomitantly with aggregation and consumption of oxygen for prostaglandin endoperoxide formation.

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These studies of endogenous phospholipid metabolism provide information supporting the existence of two previously postulated pathways for liberation of arachidonic acid from platelet phospholipids: (*a*) the combined action of PI-specific phospholipase C plus diglyceride lipase yielding arachidonate derived from PI; and (*b*) a phospholipase A_2 acting primarily on diacyl ethanolamine phosphoglyceride.

INTRODUCTION

Interest in the effects of stimulation upon platelet lipid metabolism focused initially on inositol-containing phospholipids. As in many secretory tissues (1), platelet stimulation increases the turnover of phosphatidylinositol (PI)[1] and related phospholipids, such as phosphatidic acid (PA) and di- and triphosphoinositide (2–4). The metabolism of these phospholipids is also relatively active in unstimulated platelets (5, 6). The turnover of ethanolaminephosphoglycerides (PE), phosphatidylcholine (PC), and sphingomyelin was reported to be very low in both stimulated and unstimulated platelets (5, 6). However, platelet stimulation increases *de novo* synthesis of phosphatidyl serine (7) and PI (8).

Recently the important role of arachidonic acid and its oxygenation products in platelet function has initiated new interest in platelet lipid metabolism (for review, 9). In platelets, arachidonic acid is esterified to the 2-position of phospholipids (10–12). Investigations on the mechanism by which arachidonate is made available to cyclooxygenase and lipoxygenase have initially centered on a putative phospholipase A_2 (13–17).

[1] *Abbreviations used in this paper*: DPG, diphosphatidylglycerol, cardiolipin; LPC, lysophosphatidylcholine; LPE, lysophosphatidyl ethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine, lecithin; PE, ethanolamine phosphoglycerides; PI, phosphatidylinositol; TLC, thin-layer chromatography.

A relationship between two aspects of lipid metabolism in stimulated platelets, i.e., PI turnover and arachidonate liberation, was recently postulated by Bell et al. (18), and is based upon their demonstration of a diglyceride lipase in platelets. This enzyme would release arachidonate from diglyceride produced by the PI-specific phospholipase C identified by Rittenhouse-Simmons (19).

In the present study we provide evidence for two pathways for hydrolysis of the 2-position fatty acid from platelet phospholipids: one involves a phospholipase A_2 of considerable activity, with diacyl ethanolamine phosphoglycerides as primary substrates; the other involves operation of the aforementioned pathway of PI-specific phospholipase C plus diglyceride lipase. In addition, we have shown that upon collagen stimulation PI-PA metabolism, arachidonate oxygenation, and aggregation follow similar time-courses.

METHODS

Platelet collection and processing. For each experiment 2 U of whole blood were drawn into a plastic pack system (Fenwal 4R1718, Fenwal Laboratories, Div. Travenol Laboratories, Inc., Deerfield, Ill.), and washed platelets were prepared as previously described (20). The blood donors denied having taken medications during the preceding 2 wk. Nevertheless, platelet cyclooxygenase activity was checked before platelet processing by stimulating samples of platelet-rich plasma with collagen and measuring O_2 consumption as previously described for washed platelets (20). After removal of platelet-poor plasma, the 2 U were combined, washed twice in Tris-citrate (20), resuspended in 0.15 M KCl, and adjusted to a concentration of 10^{10} platelets/ml (21).

Experimental design. Incubations (37°C) for lipid studies were carried out in a 2-ml total volume in open tubes, shaken at 120 excursions/min in a Braun shaker equipped with Thermomix 1460 BKU circulator (B. Braun Instruments, San Francisco, Calif.). O_2 consumption and aggregation studies were performed using smaller volumes (20) containing platelets and buffer components in the same proportions as in the lipid studies. Time-course experiments, including controls, were also done in open tubes in a constant temperature bath with magnetic stirring at ~1,000 rpm. Platelets, 0.5 ml (5×10^9), were added to 1.5 ml of a solution containing 75 mM Tris, 100 mM KCl, 5 mM glucose, and 0.35% defatted bovine serum albumin at pH 7.4. Where indicated, EGTA (5 mM, neutralized to pH 7.4 with KOH) was also included. Inhibitors (20) (Table III) were added during a 5-min preincubation, after which the appropriate stimulus was introduced. Incubations were continued as required and then stopped by addition of 7 ml chloroform/methanol 2:5, vol/vol (12).

Lipid extraction and thin-layer chromatography (TLC). Lipids were extracted as previously described (5, 11, 12). Aggregation did not interfere with this procedure. KCl-EDTA (5) was not used, to avoid interference during spotting of the lipid extract. TLC plates were prepared by slurrying 35 g silica gel H in 85 ml 2.5% magnesium acetate, and spreading 0.50-mm layers onto acid-cleaned 20 × 20-cm glass plates using a Quickfit apparatus (Corning Glass Works, Science Products Div., Corning, N. Y.). (12, 22). After air drying for at least 24 h, the plates were stored in a desiccant-containing cabinet. Plates were "activated" for at least 1 h in a flow of nitrogen kept at 55% relative humidity (22). Lipid extracts were spotted in this same atmosphere. Between first (chloroform/methanol/ammonia, 65:35:5.5, vol/vol) and second (chloroform/acetone/methanol/acetic acid/water, 3:4:1:1:0.5, vol/vol) dimensional developments (12, 22), plates were dried for 1 h in a stream of dry nitrogen. With this TLC system, separation of major phospholipids, including PI and phosphatidyl serine, as well as such minor components as PA, diphosphatidylglycerol (DPG), lysophosphatidyl ethanolamine (LPE), and lysophosphatidylcholine (LPC), was readily and reproducibly accomplished (Fig. 1) (12, 22).

Phosphorus analysis. After two-dimensional TLC, plates were air dried and stained in iodine vapor. Areas containing phosphorus (detected in separate experiments with a modified Dittmer-Lester spray reagent [23]) were scraped and phosphorus content was determined (24). PI, LPE, PA, and DPG were quantitated with high precision, as evidenced by small standard deviations (Table II). Measurements of the phosphorus content of other phospholipids showed larger standard deviations, contributing to the lack of significance in changes observed after stimulation (Fig. 2) (Table II).

Gas-liquid chromatography. TLC plates with samples for fatty acid and aldehyde analysis were kept in an atmosphere of dry nitrogen after 2-dimensional TLC until dried. Areas to be analyzed were identified, scraped, methylated (BF_3/methanol), and analyzed as previously described (11, 12, 25). Results were expressed as weight percent of methylated components.

Chemicals. Reagents used were obtained as previously described (20). In addition, albumin was from Sigma Chemical Co., St. Louis, Mo. (A6003); silica gel H, type 60 (Merck & Co., Rahway, N. J.), from Brinkmann Instruments, Inc., Westbury, N. Y.; solvents were pesticide grade (Nanograde) from Mallinckrodt, Inc., St. Louis, Mo. TLC and gas chromatography supplies were from Supelco, Inc., Bellefonte, Pa. Human α-thrombin (2,300 U/mg protein) was a gift from Dr. John Fenton II. Collagen was from Hormon-Chemie, Munich, West Germany.

Statistical analysis was performed on a Hewlett-Packard 97 calculator (Hewlett-Packard, Palo Alto, Calif.) using a program to determine the t statistic for two means.

RESULTS

Effects of thrombin stimulation on the phospholipid composition of washed human platelets. After the addition of thrombin to a washed platelet suspension, two major differences between phospholipids in the control and stimulated samples were discernible (Fig. 1): First, the quantity of platelet PI decreased, whereas the amount of PA increased dramatically. Second, an additional phospholipid was evident (designated LPE in Fig. 1, located between PI and sphingomyelin). The latter was identified as lysophosphatidyl ethanolamine since it was ninhydrin-positive, contained phosphorus, and co-chromatographed with authentic LPE. In addition, gas chromatographic analysis of methylated samples indicated the presence of a large proportion of dimethylacetal derivatives (Table I). In human platelets measurable amounts of plasmalogens (as dimethylacetals) are found only in the ethanolamine phosphoglyceride class (10–12). The absence of polyunsaturated fatty acids in these samples

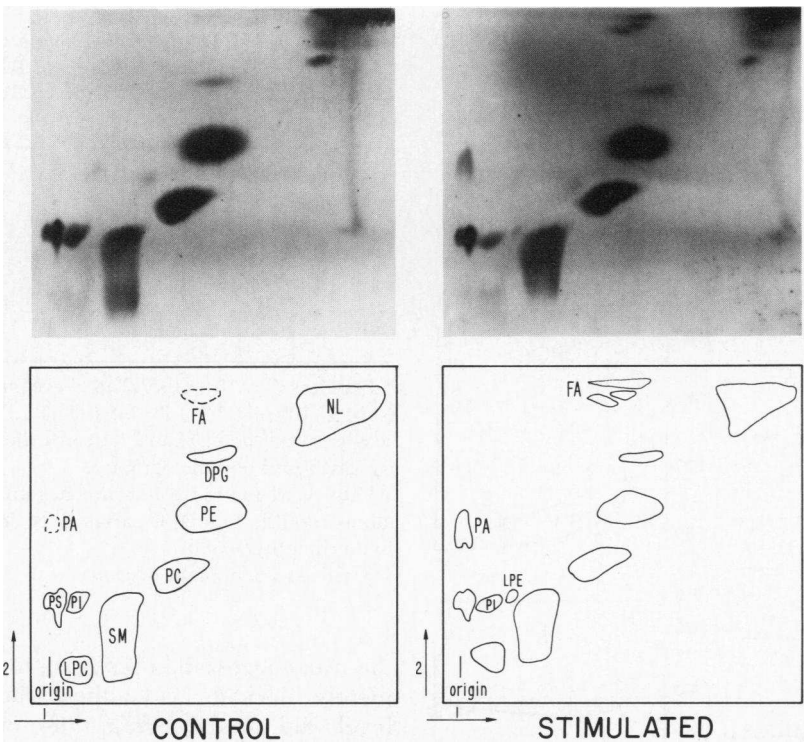

FIGURE 1 Comparison of lipid distribution by two-dimensional TLC of control and thrombin-stimulated platelets. Photograph (top) and tracing (bottom) of a heavily iodine-stained TLC plate of a control incubation (5×10^9 platelets) depict the base-line lipid profile (left). Only trace amounts of PA and fatty acids (FA) are discernible (broken lines); no LPE is visible. Stimulation with thrombin (0.6 U/10^8 platelets) for 3 min (right) altered the lipid pattern: The quantity of PI decreased, PA increased, and a new spot, LPE (absent in the control), appeared. Three distinct spots are seen in the fatty acid area (FA) of the stimulated sample. These were tentatively identified as the hydroxy acids 12-hydroxyeicosatetraenoic acid (HETE) and 12-hydroxyheptadecatrienoic acid (HHT), and free fatty acids. (Thromboxanes are not extracted by the procedure used.) All three zones were radioactive when the platelets were prelabeled with [^{14}C]arachidonic acid (data not shown). The line above the word "origin" indicates the position of application of the lipid extract. NL, neutral lipids; for other abbreviations, see text.

(Table I) suggested that the LPE was formed by a phospholipase A_2-like activity.

The quantitative changes in platelet phospholipids due to thrombin stimulation are listed in Table II, while their time-courses are depicted by Fig. 2. The changes in PI and PA are rapid (50% complete in 20 s), large, and statistically significant ($P < 0.001$, Table II). However, it should be noted that the decreases in PI (17.8 nmol at 60 s) were greater than the increases in PA (10 nmol at 60 s). The phosphorus content of the LPE area (Fig. 1) rose rapidly from barely detectable levels to a plateau value of 5 nmol, a statistically significant change (Fig. 2 and Table II). The level of LPC rose more slowly after thrombin treatment, and the increase was of less statistical significance (Fig. 2 and Table II). Downward changes of variable magnitude were observed in the quantities of PE and PC, but were not statistically significant. The time-course of changes in PE and LPE differed from those in PC and LPC. The ethanolamine phospholipids rapidly attained plateau levels, but PC and LPC changed at a later time and more slowly (Fig. 2). These trends were observed in every experiment, despite variability in magnitude of the alterations in PE and PC. There were no changes in level of DPG (Fig. 2). Alterations in phosphatidylserine and sphingomyelin content did not follow an identifiable pattern.

Effect of inhibitors of platelet function on PI-PA metabolism. Treatment of 3.7×10^9 platelets with thrombin (0.14 U/10^8 platelets) for 10 min induced a loss of >40% of platelet PI and a 17-fold gain in the quantity of PA (Table III).

5 min before thrombin addition in an experiment as described above, inhibitors of platelet function were preincubated with the platelet suspension. Arachidonic acid oxygenation was blocked with 5,8,11,14-eicosatetraynoic acid (30 μM)—an inhibitor of both

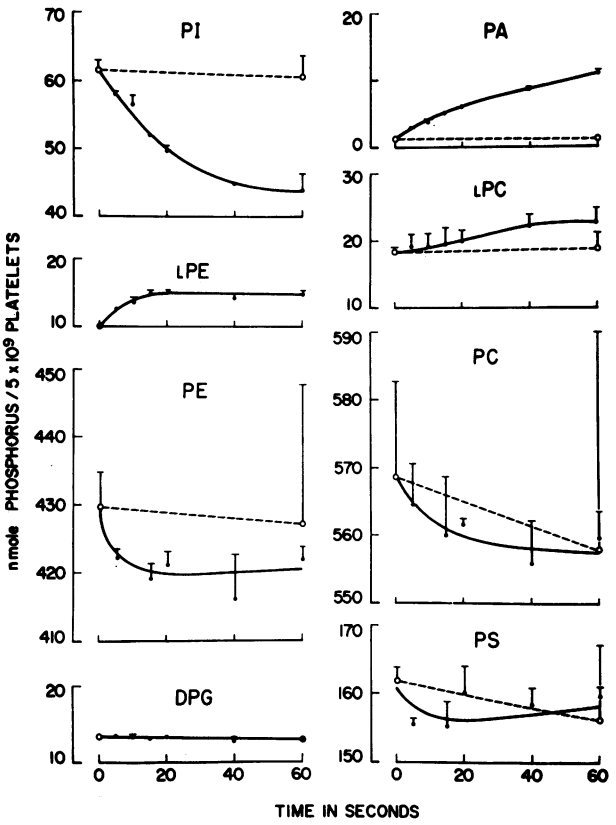

FIGURE 2 Composite representation of quantitative changes in platelet phospholipids following stimulation with thrombin. Washed platelets (5×10^9) were preincubated for 4 min, then stimulated with thrombin (0.2 U/10^8 platelets). Incubations were stopped at the indicated time points; lipids were extracted, separated (see Fig. 1), and phosphorus was quantitated as described in Methods. Of note are the increases in PA, LPE, and LPC, as well as the pronounced decrease in PI, and the slight decrease in PE. Equal distances on the ordinate represent identical changes in phosphorus content. Vertical lines indicate SD, which at some points (especially with DPG and PA) were too small to be shown in this figure. O ----- O control samples; ● —— ● stimulated samples. For abbreviations, see text.

TABLE I
*Fatty Acid and Aldehyde Composition of LPE from Thrombin-stimulated Platelets**

Component‡	Percent of Total§
16:0 DMA	5
16:0	8
18:0 DMA	18
18:1 DMA	4
18:0	56
18:1	10
20:4	Trace

* Platelets (7×10^9 cells/2.2 ml) were incubated with thrombin (0.14 U/10^8 platelets) for 60 s. The LPE of four incubations was isolated, pooled, and transesterified before quantitation by gas-liquid chromatography.
‡ Fatty acid methyl esters are designated by number of C atoms: double bonds of parent fatty acids. DMA, fatty aldehyde dimethylacetals.
§ Expressed as weight percent.

the lipoxygenase and cyclooxygenase enzymes (26). Absence of arachidonic acid oxygenation was verified in an oxygen consumption apparatus (20). Indomethacin (Sigma Chemical Co.) (10, 50, or 100 μM), a cyclooxygenase inhibitor, was also used. As shown in Table III, preincubation with either inhibitor had no influence on PI-PA metabolism. Imidazole (Sigma Chemical Co.) (5 mM), a thromboxane synthetase inhibitor, was also without effect on this pathway. In additional experiments, aspirin, whether added in vitro in concentrations up to 200 μM or ingested by the blood donor before collection, had no effect on PI-PA metabolism after thrombin stimulation.

When platelet cyclic AMP levels were elevated by preincubation with dibutyryl cyclic AMP (3 mM), the usual aggregation response to thrombin was completely blocked. Under these conditions PI and PA levels did not differ from those of unstimulated controls (Table III).

The influence of ionized calcium was studied by addition or removal of extracellular calcium as well as by inhibition of intracellular calcium translocation with 8-(*N,N*-diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride (TMB-8). The latter compound slightly inhibited metabolism of PI and PA (Table III). This inhibition could be reversed by addition of extracellular calcium. Addition of extracellular calcium per se did not affect metabolism of PI and PA but removal of extracellular calcium by EGTA (5 mM) resulted in strong inhibition (Table III). This effect of EGTA was not observed when incubations were terminated within 3 min after thrombin addition. This phenomenon was explored in greater detail with a time-course study (Fig. 3): metabolism of PI and PA was unaffected by the presence of EGTA up to 2 min following thrombin addition. In the absence of EGTA, maximal changes in PI and PA content were attained at 10 min (Fig. 3). However, in the presence of EGTA values close to those seen in unstimulated controls were obtained at the 30-min time point (Fig. 3). Thus EGTA did not influence the initial phase of PI-PA metabolism following thrombin stimulation, but did exert an effect later in the time-course.

In separate experiments, ionophore A23187 (1 μM, 3 min) induced only a 19% decrease in the level of PI, whereas thrombin (0.6 U/10^8 platelets, 3 min) reduced the level of PI by 40%. The increase in PA after ionophore addition (to 300% of unstimulated control) was minor when compared with that induced by throm-

TABLE II

*Phospholipid Content of Control and Thrombin-stimulated Platelets**

Phospholipid class	Control		Stimulated			
			15 s		*60 s*	
PA	1.25‡	(0.06)§	5.05‖	(0.20)	11.11‖	(0.56)
PI	61.55	(1.51)	51.96‖	(0.19)	43.73‖	(2.46)
LPC	18.25	(0.68)	19.45	(2.41)	22.64¶	(2.08)
PC	568.65	(14.10)	559.96	(8.65)	559.74	(3.88)
LPE	0.23	(0.13)	5.01**	(0.61)	4.81**	(0.39)
PE	429.49	(5.25)	418.98¶	(2.23)	421.77	(1.93)
PS	161.92	(1.95)	155.28	(3.89)	160.37	(1.27)
DPG	12.57	(0.14)	12.21	(0.07)	11.89	(0.31)
SM	220.61	(12.66)	203.94	(19.45)	219.93	(12.77)

* Platelets (5×10^9 cells/2 ml) were preincubated for 4 min. Incubations were stopped at 15 and 60 s after stimulation (0.2 U thrombin/10^8 platelets). Controls were stopped after preincubation. Phospholipid phosphorus in each phospholipid class was determined as described in Methods. PS, phosphatidyl serine; SM, sphingomyelin.

‡ Mean of three incubations is expressed as nanomoles phosphorus per 5×10^9 platelets.

§ SD of three incubations.

‖ Compared with control, $P < 0.001$.

¶ Compared with control, $P < 0.05$.

** Compared with control, $P < 0.01$.

bin (1,300% of control). This increase in PA following ionophore addition was less than the decrease in PI.

Collagen-induced PI-PA metabolism. Platelet content of PI and PA was measured following 3-min incubations with varying amounts of collagen. As in the case of thrombin stimulation, levels of PI and PA changed markedly (Fig. 4). These changes were readily observed, even when the amount of collagen added was <10 μg/5×10^9 platelets (Fig. 4), an amount comparable to 0.3 μg collagen added to 0.5 ml platelet-

TABLE III

*Effects of Inhibitors on Changes in PA and PI Content of Platelets Following Thrombin Stimulation**

Inhibitor	PA		PI	
Unstimulated control	1.30‡	(100)§	44.91	(100)
Stimulated control	22.21	(1,700)	25.71	(57)
ETYA (30 μM)	20.67	(1,600)	22.97	(51)
Indomethacin (100 μM)	20.23	(1,550)	26.16	(58)
Imidazole (5 mM)	24.70	(1,900)	23.99	(53)
Dibutyryl cyclic AMP (3 mM)	1.50	(115)	42.33	(94)
$CaCl_2$ (2 mM)	20.83	(1,600)	22.59	(50)
EGTA (5 mM)	5.02	(385)	38.77	(86)
TMB-8 (600 μM)	16.07	(1,200)	28.42	(63)
TMB-8 (600 μM) + $CaCl_2$ (2 mM)	23.00	(1,750)	20.55	(46)

* Platelets (3.7×10^9 cells/2 ml) were preincubated (5 min) with buffer or inhibitor(s) at the final concentrations indicated, prior to stimulation with thrombin (0.14 U/10^8 platelets) for 10 min. Incubations were stopped, and amounts of PA and PI quantitated as described in Methods.

‡ Mean of duplicate experiments, expressed in nanomoles of phosphorus per 3.7×10^9 platelets.

§ Percent of unstimulated control.

ETYA, 5,8,11,14-eicosatetraynoic acid; TMB-8, 8-(*N*, *N*-diethylamino)-octyl-3, 4,5-trimethoxybenzoate hydrochloride.

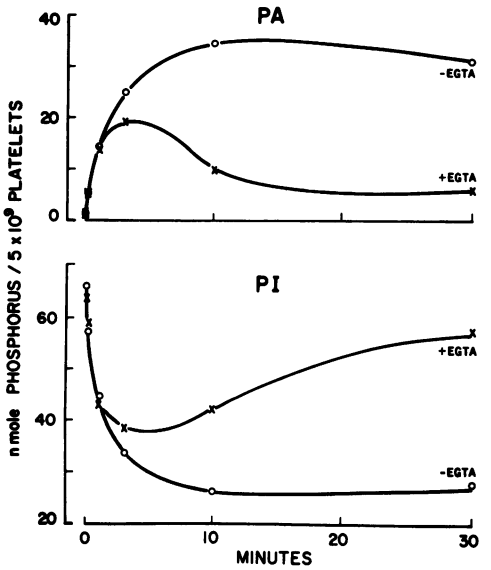

FIGURE 3 Effect of EGTA on the time-course of changes in quantity of PA (top) and PI (bottom). In the absence of EGTA (○, −EGTA) the changes were initially very rapid, then slowly leveled off. In the presence of EGTA (×, +EGTA) the changes were initially equally rapid, but leveled off sooner, and then reversed to approach base-line values between 10 and 30 min after addition of stimulus (5×10^9 platelets, 0.2 U thrombin/10^8 cells).

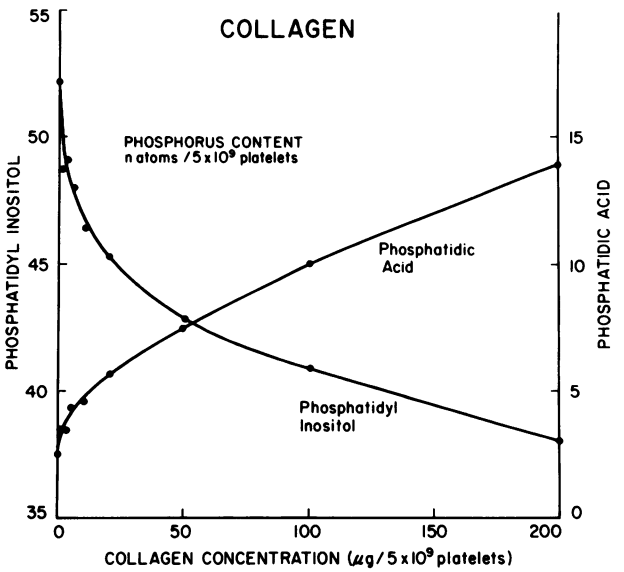

FIGURE 4 Effect of collagen concentration on the phosphatidylinositol and phosphatidic acid content of platelets. Incubations containing 5×10^9 platelets each were stopped 3 min after stimulation with the indicated amounts of collagen. Phospholipid phosphorus was quantitated as described in Methods. Small amounts of collagen generate large changes in phosphorus content of phosphatidylinositol (left ordinate) and phosphatidic acid (right ordinate).

rich plasma containing 300,000 platelets/μl. Although accumulation of LPE was observed following collagen stimulation, the primary emphasis of experiments with this stimulus was on the time-course of changes in PI and PA content.

A representative time-course of PI-PA metabolism following collagen stimulation is shown in Fig. 5, together with an aggregation curve and an oxygen consumption trace. Following a lag phase of about 20 s the rate of oxygen consumption increased in a burst (prostaglandin endoperoxide formation), returning to base-line values ~ 1 min later (Fig. 5, [20]). Aggregation, as well as changes in PI and PA content, followed a time-course very similar to that of the burst (Fig. 5). However, whereas PI levels remained at base line before decreasing, PA levels consistently decreased slightly before increasing. Thereafter the gain in endogenous PA equalled the loss in PI (Fig. 5). This was in contrast to the data obtained after thrombin stimulation, where the loss in PI was greater than the gain in PA.

DISCUSSION

Thrombin-induced changes in endogenous platelet phospholipids. Thrombin stimulation of platelets induced major changes in endogenous PI and PA content (Fig. 1). A discrepancy was observed between loss of PI and gain in PA: the loss of PI was greater than the gain in PA (Figs. 2 and 3; Table II). A possible explanation for this may be a series of reactions originating with accumulation of diglyceride within seconds after thrombin addition, as reported by Rittenhouse-Simmons (19) and subsequently by Bell et al. (18). Rittenhouse-Simmons demonstrated that this diglyceride was generated from PI by a PI-specific phospholipase C (19). A similar activity in platelet lysates has been reported by Mauco et al. (27). It was suggested that the diglyceride produced may participate in platelet secretion (19). On the other hand, Bell and associates proposed that the diglyceride is substrate for a diglyceride lipase, the presence of which they demonstrated in platelets (18). Since diglyceride derived from PI is very rich in arachidonic acid (10–12), combined action of PI-specific phospholipase C and diglyceride lipase would render arachidonate available for cyclooxygenase and lipoxygenase (18). Thus, the difference observed between loss of PI and gain in PA may be accounted for by hydrolysis of arachidonic acid from the intermediate diglyceride and its transformation into oxygenated products. This would render the diglyceride unavailable for formation of PA by diglyceride kinase (28). Bell and Majerus (29) have recently suggested that only PI is susceptible to PI-specific phospholipase C, but not di- and triphosphoinositide.

Our observations of rapid accumulation in stimulated

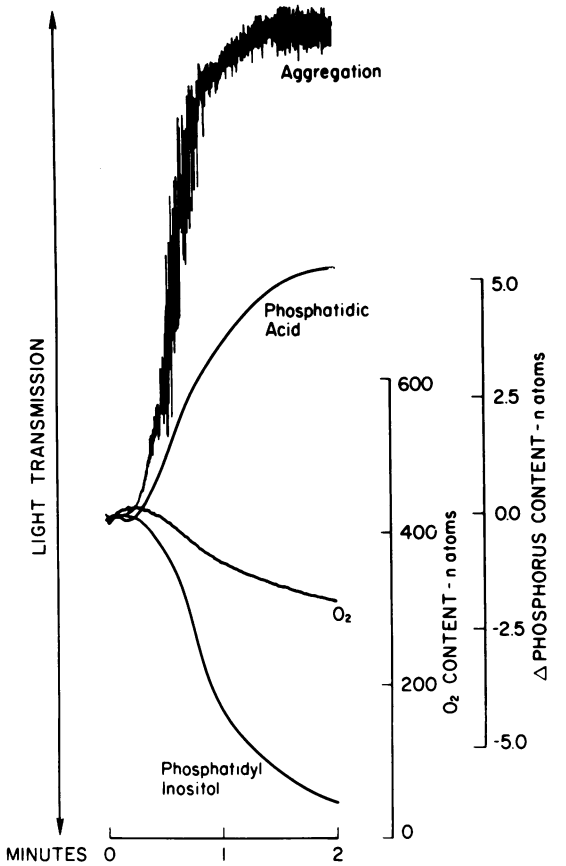

FIGURE 5 Time-course of concurrent changes in quantity of phosphatidic acid and phosphatidylinositol, as well as aggregation and oxygen consumption. Tracings of an aggregation curve and an oxygen consumption recording (O_2) are shown in composite with the phosphatidic acid and phosphatidylinositol curves derived from measurements at individual time points, i.e., 5, 10, 15, 20, 25, 30, 40, 50, 60, 90, 120 s. 100 μg collagen was added to 5×10^9 platelets. After an initial small decrease in the amount of phosphatidic acid, the time-courses had similar lag phases and inflection points. (The initial rise in the O_2 curve was due to the high oxygen content of the cold collagen suspension.)

platelets of LPE (Figs. 1 and 2; Tables I and II) and, to a lesser extent, of LPC, combined with an apparent decrease in PE and PC, indicate the presence of phospholipase A_2 activities distinct from those of the aforementioned PI-specific enzymes. We noted that LPE contained a smaller percentage of aldehydes (Table I) than was reported previously on the 1-position of platelet PE (10, 11, 25). This may suggest that the LPE was generated by a phospholipase A_2 acting more readily upon the diacyl form than upon the plasmalogen form of PE, a phenomenon also reported by Jesse and Cohen (30) in previous studies with platelet membrane preparations. The ethanolamine phosphoglycerides have a high content of arachidonate (10–12) and this was used as evidence favoring PE as a source of free arachidonate by these investigators (30). In contrast, Rittenhouse-Simmons et al. (31), using a different approach, suggested the plasmalogen form of PE as a source of arachidonate for cyclooxygenase and lipoxygenase. Partial purification of platelet phospholipase A_2 has been described (32–35). In addition, accumulation of lysophospholipids following platelet stimulation was reported in abstract form (36).

The changes in LPE and PE were completed before those in LPC and PC (Fig. 2). The changes were not due to nonspecific hydrolysis, because in unstimulated control incubations LPE or LPC did not accumulate. This difference in pattern between ethanolamine and choline phosphatides may be due to the action of phospholipase A_2 activities with a diversity of specificities, or due to changes in substrate availability as a consequence of membrane rearrangement brought about by release and/or aggregation. The latter hypothesis may be more viable since the human platelet plasma membrane has been shown to possess an asymmetric phospholipid distribution, with most of the choline-containing species facing the extracellular space, and the major portion of the noncholine phospholipids on the cytoplasmic side of the membrane (37–39). Additional support for a lag in choline phosphatide metabolism is afforded by a delay in the loss of [^{14}C]arachidonate from PC upon thrombin stimulation of prelabeled platelets (19).

Effects of inhibitors on endogenous PI-PA metabolism. Inhibition of cyclooxygenase, lipoxygenase, or thromboxane synthetase had no effect on the changes in endogenous amounts of PI and PA induced by stimulation with thrombin (Table III). This is in agreement with data recently reported by Lapetina and Cuatrecasas (4), who studied horse platelets prelabeled with [^{14}C]arachidonate.

In contrast, alterations in availability of calcium to the platelets did affect PI-PA metabolism (Table III, Fig. 3). This is probably attributable to the divalent cation requirements of the enzymes involved. On the one hand, PI-specific phospholipase C is inhibited by calcium removal (19, 27), leading to less PI breakdown. On the other, active resynthesis is stimulated because the platelet enzymes involved in this process are magnesium-and/or manganese-dependent and are in fact inhibited by calcium (28, 40–42). Resynthesis of PI implies that platelet stimulation leads to operation of the classical PI-cycle (43) in its entirety. This concept is supported by the work of Lloyd et al. (2, 3), who showed an increase in the amount of label in PI following stimulation of platelets prelabeled with [^{32}P]-phosphate.

Additional evidence for the importance of calcium is our observation that ionophore A23187 caused a drop in the PI content of platelets but, in comparison to thrombin, only a small increase in PA content (see

Results). Thus, the ionophore-induced increase in [Ca^{2+}] may activate phospholipase C (19, 27, 42), and diglyceride lipase (18), but not diglyceride kinase (28). This indicates that calcium has a multilevel regulatory role in the metabolic fate of PI and diglyceride.

It is also likely that the concentration of ionized calcium has a regulatory function in the activation of phospholipase A_2. Previous work by several investigators has emphasized the importance of calcium in the generation of free arachidonate by stimulated platelets (15, 16, 44).

Collagen-induced alterations in endogenous PI and PA content. The time-course of changes in the level of endogenous PA showed an initial decrease 10 s after collagen addition. This preceded the onset of aggregation and the oxygen burst (Fig. 5). This may indicate that PA → PI metabolism occurs momentarily to a greater extent than PI → PA conversion, resulting in an initial decrease in the amount of endogenous PA (Fig. 5). Thereafter, when the quantity of endogenous PA rises and that of PI diminishes, a diglyceride lipase (18) may become activated and yield free arachidonate for cyclooxygenase, as suggested by the O_2 burst (Fig. 5). However, because the loss in PI was equivalent to the gain in PA (Fig. 5), the pathway of PI-specific phospholipase C plus diglyceride lipase may not be the only source of free arachidonate. A phospholipase A_2 may be involved as well, since we observed an accumulation of LPE in the collagen experiments. We found that collagen stimulation leads to formation of much less thromboxane B_2 than does thrombin (unpublished observations). Thus, the relative contributions of phospholipase A_2 and PI-specific pathways may be difficult to precisely quantitate.

Our data, showing a small initial decrease in the total amount of endogenous PA, are at variance with results of studies using prelabeled rabbit, human, and horse platelets (3, 4). In these the amount of label in PA increased before other stimulus-induced changes. This apparent discrepancy may be explained by the fact that the distribution of radioactivity in prelabeled platelets (14, 5, 6) differs from the endogenous lipid distribution studied here.

Whether changes in quantity of endogenous lipid or of a radioactive pool with apparent high turnover are more indicative of platelet responses to stimuli cannot be answered at the present time.

In conclusion, our data indicate that dual pathways of phospholipid metabolism exist in stimulated platelets: a "true" phospholipase A_2 acting primarily on PE, yielding LPE as one of the products, and a pathway involving PI-specific phospholipase C, with a balance between the activities of diglyceride lipase and diglyceride kinase. Both pathways would yield free arachidonate for cyclooxygenase and lipoxygenase activities.

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