Stimulated platelets release equivalent amounts of arachidonate from phosphatidylcholine, phosphatidylethanolamine, and inositides

M. Johan Broekman[1]

Divisions of Hematology/Oncology, Departments of Medicine, New York Veterans Administration Medical Center and Cornell University Medical College, New York, NY 10010

Abstract Thrombin-induced changes in arachidonate content of platelet phospholipids were quantitated to establish the ultimate origins of this eicosanoid precursor. Fifteen seconds following thrombin addition (15 U/5 × 10^9 platelets), phosphatidylcholine lost 11.8 nmol of arachidonate and phosphatidylethanolamine lost 10.5 nmol. Arachidonate in phosphatidate, phosphatidylinositol, and phosphatidylinositol-4,5-bisphosphate combined decreased by 11.0 nmol. Increases in free and oxygenated arachidonate (41 nmol) exceeded decreases in inositides. Thus phospholipase A_2 released at least twice as much arachidonate as phospholipase C–diglyceride lipase. Phosphatidylinositol-4-phosphate levels remained unchanged upon stimulation. Therefore, increases in phosphatidylinositol-4,5-bisphosphate indicated the minimum rate of phosphorylation of phosphatidylinositol to resynthesize phosphatidylinositol-4,5-bisphosphate, following stimulus-induced breakdown by phospholipase C. Phosphatidylinositol-4,5-bisphosphate increased 1.4 nmol between 10 and 15 sec following thrombin, markedly less than phosphatidylinositol decreased (2.1 nmol). This could be due to phospholipase A_2, in addition to phospholipase C, acting directly on phosphatidylinositol to a greater extent than estimated by accumulation of lysophosphatidylinositol, degraded rapidly by lysophospholipase. Thus, upon high-dose thrombin stimulation of human platelets *A*) inositide metabolism via phospholipase C directs initial formation of *intra*cellular second messengers, and sequentially, or in parallel, *B*) arachidonate release by phospholipase A_2 supplies the larger proportion of arachidonate for syntheses of eicosanoids involved in *inter*cellular communication.—**Broekman, M. J.** Stimulated platelets release equivalent amounts of arachidonate from phosphatidylcholine, phosphatidylethanolamine, and inositides. *J. Lipid Res.* 1986. **27**: 884–891.

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Arachidonic acid is the most prevalent fatty acid in human platelets, accounting for 29% of phospholipid fatty acids (1). Palmitate, stearate, and oleate represent 20, 16, and 16%, respectively. While PC, PE, and PI represent 38, 31, and 4% of the total phospholipid, they contain 28, 48, and 11% of the total platelet arachidonate, respectively (1). Thus, of all platelet phospholipids, PI is most highly enriched in arachidonate (1–5). Upon thrombin stimulation, PI is degraded by a specific phospholipase C (6, 7). This led to an hypothesis that PI was a specific, but indirect, source of arachidonate, liberated upon platelet stimulation via sequential action of phospholipase C and diglyceride lipase (8). Previously, a phospholipase A_2-dependent pathway acting on PC and PI had been proposed (9–14). More recently, PI, as the parent compound of PIP_2, has been shown to act as a reservoir for the generation of two second messengers, diglyceride (15), and inositol-1,4,5-triphosphate. These messengers are formed from PIP_2 by activation of phospholipase C following stimulation of a variety of cell types (16), including platelets (17–25). Levels of PIP_2 in resting platelets are less than 1% of total cellular phospholipid (26), but PIP_2 is rapidly generated by sequential phosphorylation of PI (26, 27).

Although PI is the platelet phospholipid most highly enriched in arachidonate, PC and PE combine to provide a potential reservoir of arachidonate seven times that of PI (1). Mahadevappa and Holub (28) cautioned against overinterpretation of results derived from prelabeling with radi-

Abbreviations: EPA, eicosapentaenoic acid; PA, phosphatidic acid; PC, phosphatidylcholine (1-radyl-2-acyl-PC); PE, phosphatidylethanolamine (1-radyl-2-acyl-PE); PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; TLC, thin-layer chromatography. Fatty acids are designated by number of carbon atoms:number of double bonds.

[1]Established Investigator of the American Heart Association. Address for correspondence: Thrombosis Research Laboratory, Room 13025 West, New York Veterans Administration Medical Center, 408 First Avenue, New York, NY 10010.

oactive arachidonate, because this can lead to highly heterogeneous labeling of different pools. When platelets were prelabeled with arachidonate, it was initially concluded that only PC and PI released arachidonate upon stimulation (11, 12). Release of smaller quantities of radiolabeled arachidonate from PE were reported more recently (29, 30). These studies contrast with our prior investigations demonstrating accumulation of similar quantities of unlabeled lysoPC and lysoPE in thrombin-stimulated platelets (31). However, the total amount of arachidonate released from each platelet phospholipid has not been reported previously.

In the present study direct measurements of changes in endogenous arachidonate content of platelet phospholipids were carried out. The data indicate that upon thrombin stimulation of human platelets: *1*) the rise in free and oxygenated arachidonate exceeds the release of free arachidonate from inositides and PA by a factor of 3, and *2*) large quantities of arachidonate are released from both PC and PE. These results directly demonstrate for the first time that phospholipase A_2 is quantitatively more significant than the phospholipase C–diglyceride lipase pathway as a mechanism for release of arachidonate by stimulated platelets.

EXPERIMENTAL PROCEDURES

Platelet collection and processing

Washed platelet suspensions were prepared from human platelet-rich plasma as described (21, 31, 32). Cyclooxygenase activity was checked prior to platelet processing by monitoring O_2 consumption upon collagen stimulation of platelet-rich plasma (31–34). Platelets were washed twice (4°C) in Tris–citrate buffer (63 mM Tris, 95 mM NaCl, 5 mM KCl, 12 mM citric acid, pH 6.4), resuspended in 154 mM NaCl and adjusted to a platelet count of 10^{10} platelets/ml.

Experimental design

For phospholipid studies, incubations were carried out at 37°C in open tubes, with or without stirring (21, 31, 32): 0.5 ml (5×10^9) of platelets were added to 1.4 ml of buffer (75 mM Tris, 95 mM NaCl, 7 mM glucose, 3 mM KH_2PO_4, 0.35% defatted bovine albumin, pH 7.4). Stimuli were added rapidly in 0.1 ml of 154 mM NaCl after a 4-min preincubation period. Incubations were stopped by rapid addition of 7 ml of $CHCl_3$–MeOH 2:5 (v/v). To samples for PIP and PIP_2 analyses, 0.5 ml of 2 M KCl–0.1 M EDTA was added immediately after the $CHCl_3$–MeOH (21, 26).

For O_2 consumption studies, the final volume of incubations was adjusted to 1.2 ml by using 0.3 ml of platelets, 0.84 ml of buffer, and 0.06 ml of stimulus. O_2 consumption was measured (34) and expressed as nmol of O_2 utilized per 5×10^9 platelets. This is equivalent to twice the quantity of arachidonate oxygenated; the larger part of the O_2 "burst" is due to cyclooxygenation of arachidonate (33–35), consuming 2 mol of O_2/mol of arachidonate. Additional O_2 is utilized by lipoxygenation of arachidonate (utilizing 1 mol of O_2/mol of arachidonate), while a similar quantity of O_2 is utilized by enhanced mitochondrial respiration (35), which does not utilize arachidonate as substrate. Thus, for every 2 mol of O_2 consumed, 1 mol of arachidonate is oxygenated.

Lipid analyses

For analyses of lipids (other than PIP and PIP_2), incubations were extracted by a modified Bligh and Dyer method and separated by two-dimensional TLC (1, 31, 32, 36) on 0.5 mm Redi-coats-2D (Supelco, Bellefonte, PA). Samples (in $CHCl_3$–MeOH, 2:1) were spotted under N_2. Spotting tubes were rinsed twice with $CHCl_3$–MeOH, 2:1, which was also spotted to ensure quantitative transfer of the lipid extract. Solvent 1 (first dimension) was chloroform–methanol–conc. ammonia 65:25:5 (v/v/v). After drying under N_2, plates were turned 90° and developed in the second dimension with solvent 2: chloroform–acetone–methanol–acetic acid–water, 75:100:25:25:12.5 (v/v/v/v/v).

For analyses of PIP and PIP_2, lipids were extracted with 2 M KCl–0.1 M EDTA in the place of water (21, 26) and chromatographed on Whatman K5 plates (no preadsorbent) in solvent 3: chloroform–methanol–conc. ammonia–water (90:90:7:22 (v/v/v/v) (37, 21).

Lipid phosphorus was assayed as described previously (31, 32).

For fatty acid analyses, plates were dried under N_2 following the second dimensional development and stained with purified 2',7'-dichlorofluorescein or rhodamine 6G. Visualized lipids were marked and plates were dried under N_2. Marked lipid areas were scraped into tubes containing heneicosanoic acid (21:0) as internal standard, prior to addition of 2 ml of 12% BCl_3 in MeOH for preparation of fatty acid methyl esters (1, 32). For PIP_2, duplicate samples (i.e., from 10^{10} platelets) were combined in order to yield sufficient material for analyses. Fatty acid methyl esters were quantitated utilizing a Hewlett-Packard 5880A gas chromatograph, equipped with two columns (1800 × 2 mm) packed with 10% SP2340 on 100/200 mesh Chromosorb WAW. N_2 carrier gas flow was 20 ml/min. Injection port and flame ionization detector were at 235°C and 245°C, respectively. Temperature program was 5 min at 170°C, then 5°C/min to 225°C, which was maintained for an additional 15 min. Fatty acids were quantitated relative to the 21:0 added and expressed as nmol per 5×10^9 platelets. This was validated by comparing fatty acid quantitation with phosphate content of separated phospholipids

and depends on the quantitative nature of lipid extraction from incubations (32). Addition of 17:0 methyl ester after methylation of known amounts of arachidonate and 21:0 demonstrated that both fatty acids were methylated to the same extent, without degradation of arachidonate. Transmethylation of phospholipid fatty acid was > 85–90% complete. **Table 1** shows that the standard deviation of the arachidonate content of PC and PE averaged 3% of the mean.

Materials

Reagents were obtained as previously described (21, 31, 32).

RESULTS

Thrombin-induced hydrolysis of arachidonate from platelet phospholipids

The arachidonate content of five phospholipid classes was compared with that of the free fatty acids in the same incubations at several time points following stimulation (Table 1). These measurements demonstrated that in the first 15 sec of stimulation PC and PE lost approximately equal amounts of arachidonate, 11.8 and 10.5 nmol, respectively, while PI, PIP_2 and PA lost 15.5 nmol of arachidonate. During this time interval, 24.1 nmol of free arachidonate accumulated, while additional arachidonate was oxygenated. Stimulation of platelets leads to O_2 consumption in a "burst," largely by arachidonate cyclooxygenation (33–35). O_2 consumption, therefore, is a measure of the amount of arachidonate oxygenated. Thrombin induced oxygenation of 17 nmol of arachidonate/5 × 10^9 platelets during the first 15 sec, and 25 nmol during the first 30 sec (Table 1). Thus, direct measurements of endogenous arachidonate content and arachidonate oxygenation demonstrated release of a total of 41.1 nmol of arachidonate 15 sec after thrombin addition.

The arachidonate in 3–6 nmol of arachidonoyl-stearoyl-diacylglycerol accumulating 15 sec following thrombin addition (6, 8) remained esterified. Therefore it comprises part of the pool of arachidonate in inositides and PA and reduces the net amount of arachidonate hydrolyzed from inositides to 9.5–12.5 nmol. Thus, measurements of endogenous arachidonate demonstrate that upon thrombin stimulation PC, PE, and inositides each release similar quantities of arachidonate, which is then available for further metabolism, such as eicosanoid production.

Arachidonate was released in similar proportions 60 sec following thrombin; inositides and PA lost 27.0 nmol arachidonate, PC 32.1 nmol, and PE 26.1 nmol. Arachidonate in PE declined between 5 and 10 sec, then transiently increased at 15 sec (Table 1), possibly reflecting enhanced incorporation of arachidonate into plasmalogen PE (38). Ten sec and later following thrombin, the arachidonate content of both PC and PE decreased (Table 1).

These measurements of endogenous arachidonate led to two novel conclusions; *1*) PC, PE, and PI release similar amounts of endogenous arachidonate upon thrombin stimulation, *2*) Phospholipase A_2 activity (utilizing PC, PE, and possibly PI and/or PA [39–42]) releases at least twice as much arachidonate as the phospholipase C-diglyceride lipase pathway (acting only on inositides).

Effects of thrombin on arachidonate content of platelet inositides

Changes in fatty acid content of PIP_2 were very rapid; within 5 sec the endogenous levels of arachidonate and stearate in PIP_2 fell to 55–60% of baseline values (**Fig. 1**). This represented a rate of decline of 1.8 nmol/5 sec. Fatty acid content was notably higher 15 sec after thrombin addition (increasing at 1.4 nmol/5 sec), and exceeded levels of unstimulated platelets at 30 sec. These data extend prior observations in our own and other laboratories, employing multiple methodologies (17, 21, 43).

Qualitatively, the fatty acid composition of human platelet PIP_2 remained unaltered upon stimulation (**Fig. 2**), closely resembling that of PI at each time interval.

TABLE 1. Changes in arachidonate content of platelet phospholipids following thrombin stimulation[a]

Time	PC		PE		PI		PA		PIP_2		PI + PA + PIP_2	Free 20:4		Oxygenated[b] 20:4
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		Mean	SD	
sec									*nmol*					
0	203.2	3.5	361.4	6.3	66.9	5.2	0.0	0.1	4.2	0.7	71.1	0.1	0.2	0.0
5	203.7	5.7	360.2	19.3	63.7	4.2	0.5	0.3	2.4	0.4	66.6	4.6	1.4	ND[c]
10	189.3	6.7	336.3	11.6	52.1	0.9	1.7	0.5	2.1	0.1	55.9	16.4	0.5	10.0
15	191.4	6.2	350.9	4.9	50.0	1.7	2.1	0.3	3.5	0.5	55.6	24.2	0.6	17.0
30	183.9	5.0	351.6	5.9	40.1	1.2	4.8	0.2	6.0	1.6	50.9	27.5	1.5	25.0
60	171.1	8.2	335.3	0.4	30.8	2.0	7.7	0.3	5.6	0.2	44.1	26.4	2.0	37.0

[a]Fatty acids were quantitated as described in Experimental Procedures. Data represent averages (n = 3) of one experiment representative of six studies, expressed as nmol (± SD) of arachidonate per 5 × 10^9 platelets. Thrombin (0.3 U/10^8 platelets) was added after 4 min preincubation (0 time).
[b]Data derived from the time course of arachidonate oxygenation.
[c]ND; Not determined.

This resemblance is a reflection of the metabolic relationship between PIP_2 and PI (15, 16, 26, 27). As in the case of PI (1–5, 32, 43), PIP_2 consisted primarily of stearic and arachidonic acids, with a small percentage of oleate, and only trace amounts of palmitate and linoleate.

Stimulus-induced phospholipase C-mediated breakdown of inositides yields arachidonoyl-stearoyl-diacylglycerol (6, 8), which is rapidly phosphorylated to PA (27, 31, 44), with retention of fatty acid composition (32). A rapid increase in PA occurred upon thrombin stimulation (Table 1).

PIP_2 reached its nadir 5 sec after thrombin addition (Fig. 1), in contrast to PI, which showed a slight lag during the first 5 sec, declining most rapidly (11.6 nmol/5 sec) between 5 and 10 sec following stimulation (Table 1). This lag in PI decrease, as compared to PIP_2 breakdown (Fig. 1), was examined further by lowering the incubation temperature to 28°C. This slowed breakdown of PIP_2 and exaggerated the lag in PI metabolism, as measured by changes in phosphorus content (**Fig. 3**). This was not due to a generalized reduction in phosphorylation, since phosphorylation of diglyceride to PA occurred rapidly at the lower temperature (Fig. 3, middle panel). Thus, the data support a precursor–product relationship between PI and PIP_2, implied in the hypothesis that ligand–receptor interaction activates phospholipase C with PIP_2 as substrate, and is followed by stepwise phosphorylation of PI to resynthesize PIP_2 (15, 16).

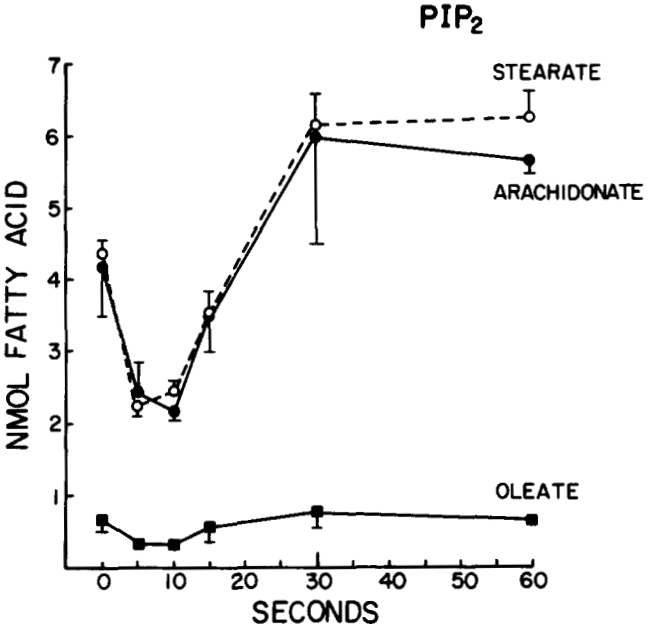

Fig. 1. Arachidonate and stearate levels in PIP_2 of human platelets exposed to thrombin (0.3 U/10^8 platelets). Data presented are means and standard deviations of triplicate determinations of PIP_2 isolated by TLC from 10 × 10^9 platelets prior to preparation of fatty acid methyl esters for gas-liquid chromatography.

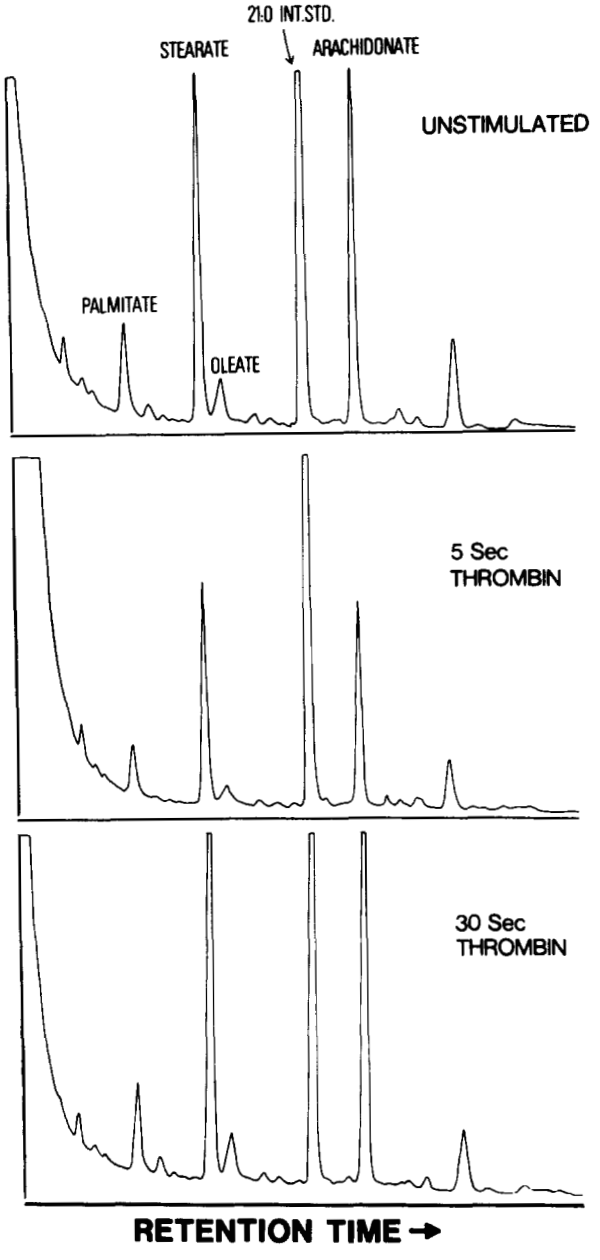

Fig. 2. Gas-liquid chromatography tracings of representative samples of fatty acid methyl esters prepared from PIP_2, obtained from resting and thrombin-stimulated human platelets (0.3 U/10^8 platelets). 21:0 INT. STD. is the internal standard of heneicosanoic acid added to silica gel scrapings prior to transmethylation (see Experimental Procedures for details). Palmitate and some stearate, as well as a fatty acid-like peak with a retention time greater than arachidonate, were also present in samples prepared from blank areas of silica gel. Stearate (47.1%), oleate (6.3%), linoleate (0.5%), and arachidonate (45.0%) were the major fatty acids (averages of six time points in triplicate: prior to, and 5, 10, 15, 30, and 60 sec after thrombin addition). Thus the fatty acid profile emphasizes the persistence of arachidonoyl-stearoyl diacylglycerol moieties in PIP_2.

Time course of free fatty acid release

Free fatty acids, bound to albumin, accumulate rapidly following thrombin addition (Table 1; cf. 32); arachidonate accumulates most rapidly, while additional free arachidonate is oxygenated (33–35). Other fatty acids in-

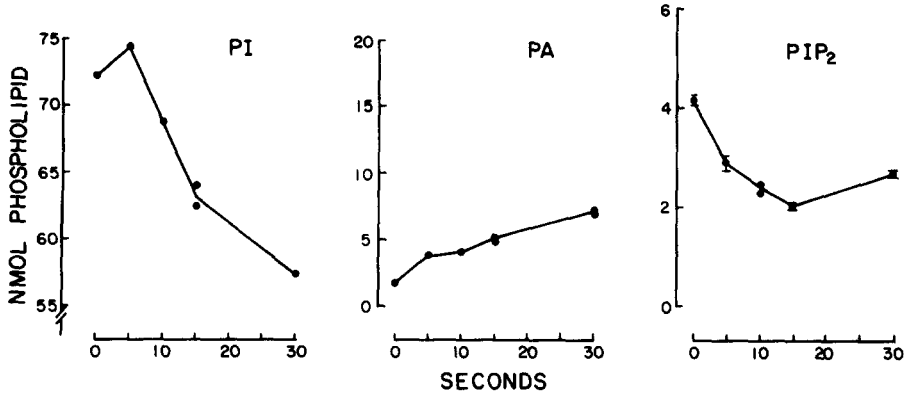

Fig. 3. Changes in platelet PIP_2, PI, and PA after thrombin stimulation (0.3 U/10^8 platelets) at 28°C. At the lower temperature, the lag of the decrease in PI is emphasized. In addition, breakdown of PIP_2 is slower than at 37°C. PA formation is still brisk, indicating that the lag in PI breakdown is not caused by a diminished platelet capacity to phosphorylate lipids.

crease somewhat more slowly. Interestingly, stearate increases at only a slightly greater rate than palmitate and oleate (32). This is important because palmitate, stearate, and oleate each account for about 16% of all platelet phospholipid fatty acids (sphingomyelin excluded, [1]). Since inositides contain little palmitate or oleate (Fig. 2; 1-5, 32), these data provide additional evidence that phospholipids other than inositides are degraded. Therefore, the phospholipase C-diglyceride lipase pathway acting on inositides (containing only 10-12% of the total platelet arachidonate [1]) is not an exclusive mechanism for release of fatty acids, including arachidonate.

DISCUSSION

Arachidonate is released from multiple endogenous sources, predominantly via phospholipase A_2

Release of arachidonate occurred to a similar extent from three phospholipid classes: PC, PE, and inositides (Table 1). Phospholipase A_2, acting principally on PC and PE (containing seven times the arachidonate of inositides [1]), predominates in this release. Decreases in arachidonate content of PC, PE, and inositides, and increases in free and oxygenated arachidonate provide two lines of support for this conclusion (Table 1).

Firstly, PC and PE together released two to three times as much arachidonate as did inositides. These data provide the first detailed analyses of sources of arachidonate released by thrombin-stimulated platelets in the presence of low quantities of albumin. That arachidonate is released predominantly by phospholipase A_2, acting on PC and PE, is supported by release of linoleate, which occurs only in trace amounts in platelet inositides (Fig. 1; refs. 1, 4, 5, 32, 45-48). Therefore linoleate was hydrolyzed from phospholipids other than inositides by phospholipase A_2. (Without albumin, fatty acids other than arachidonate are rapidly reincorporated [M. J. Broekman, unpublished observations; cf. 12].)

Secondly, released free and oxygenated arachidonate exceeded loss of arachidonate from inositides. Thus inositides are certainly not the only source of free arachidonate. Release of arachidonate from inositides, prior to their resynthesis with arachidonate from PC or PE, is not supported by the time courses of changes in PI, PC, and PE (Table 1). Moreover, other *sn*2-position fatty acids are also released (e.g., linoleate; 32), but not incorporated into inositides (Fig. 1; 32, 43, 48), indicating that inositide metabolism involves an invariant arachidonoyl-stearoyl-diacylglycerol moiety (see below). De novo synthesis of PI (46) is unlikely, since increases in stearate in PI or PIP_2 vastly exceeded increases in palmitate (Fig. 1; refs. 5, 32, 43, 48), despite accumulation of free palmitate to almost the same extent as stearate (32).

PIP levels remain constant following thrombin stimulation, as reported by this (21) and other laboratories (17, 24, 49). However, concurrent decreases in PIP and PIP_2 upon thrombin stimulation have been described (20, 43). Similarly, net resynthesis of PIP_2 occurred to a large extent (17, 21), hardly (49), or not at all (43). Under the present conditions (a constant level of PIP), the rate of phosphorylation of PI to form PIP and thence PIP_2 is not affected by net changes in PIP content. Therefore, the net rise in PIP_2 levels between 10 and 15 sec after stimulation (1.4 nmol, Fig. 1, Table 1) represents a minimum rate of phosphorylation of PI to PIP_2. This rate is a minimum, since net resynthesis of PIP_2 occurs (Fig. 1; 17, 21), but degradation by phospholipase C continues, demonstrated by accumulation of inositol triphosphate (25, 50, 51). Thus, under the present conditions, the actual flux of PI to PIP_2 is likely to be higher.

Metabolism of PIP_2 (Fig. 1; Table 1) does not account for the observed rapid decline of PI (Table 1). While phospholipase C may act on PI in addition to PIP_2 (49, 51),

inositide breakdown also occurs via phospholipase A_2 (52). We previously demonstrated rapid accumulation of lyso-PC and -PE upon platelet stimulation (31). Decreases in PC and PE (31; Table 1) exceeded increases in lyso-compounds (31), suggesting activation of lysophospholipase(s) (53) to reduce lysophospholipid levels. Therefore, phospholipase A_2 could have acted on PI (and PA) to a greater extent than previously estimated (42, 52). This further emphasizes the importance of phospholipase A_2 in the release of arachidonate from stimulated platelets.

That phospholipase A_2 is quantitatively more important for arachidonate release than phospholipase C–diglyceride lipase is supported by a recent study (49), which contrasts with earlier results (54). The more recent data indicate that 25 nmol of PI/5 × 10^9 platelets is hydrolyzed 15 sec after stimulation (49). Accounting for remaining diglyceride (6, 8) and PA, less than 20 nmol of PI/5 × 10^9 platelets can be hydrolyzed to free arachidonate (8, 43, 54). More than twice this amount is observed free and oxygenated at 15 sec (Table 1; 5).

Phospholipase D activation has been described in brain (for PC, 56) and neutrophils (for inositides, 57). However, in human platelets PA retains the characteristic fatty acid composition of inositides (32), suggesting it is *not* derived from PC or PE, while the rapid incorporation of ^{32}P into PA suggests a kinase reaction, rather than phospholipase D. Our recent results suggest activation of de novo phospholipid synthesis and phospholipase A_2 and phospholipase C upon fMet-Leu-Phe stimulation of human neutrophils (58, 59).

Inositide metabolism and arachidonate release constitute sequential or parallel events upon stimulation of platelets with high-dose thrombin. While representing separate biochemical events, phospholipase C and phospholipase A_2 can be intimately linked. Thus stimulation by weaker agonists, or low-dose thrombin, leads to activation of phospholipase C, but not phospholipase A_2 (60), possibly because $[Ca^{2+}]_i$ did not reach values high enough to activate phospholipase A_2 (29, 39, 40). A greater degree of stimulation by these weak agonists is necessary to initiate phospholipase A_2 activity, leading to release and metabolism of arachidonate (60). The converse can be also be demonstrated. Ionophore stimulation of phospholipase A_2 only leads to activation of phospholipase C if thromboxane A_2 and/or ADP become available (21–23).

Persistence of arachidonoyl–stearoyl composition of inositides

The fatty acid composition of PI and PIP_2, as well as PA accumulating after thrombin addition, persisted upon stimulation (Fig. 1, Table 1; ref 32). This preference for an invariant arachidonoyl–stearoyl diacylglycerol moiety (32, 43, 48) is emphasized also by ex vivo studies (47). Dietary supplementation with cod liver oil, rich in EPA, resulted in similar plasma free fatty acid levels of arachidonate and EPA, reflected in incorporation of EPA in platelet PC and PE. However, in contrast to in vitro studies (47, 61, 62), the EPA content of PI was *not* increased, indicating that platelets incorporate preferentially arachidonate into PI in vivo (45, 47), in agreement with the fatty acid composition of inositides (Fig. 1; ref. 32). Other blood cells (e.g., neutrophils) contain appreciably larger quantities of oleate and linoleate in PI (63) than platelets. Persistence of arachidonate and stearate in platelet inositides indicates that stimulation induces, via inositide-specific phospholipase C (6–8, 15–23, 64), formation of 2-arachidonoyl-1-stearoyl-*sn*-glycerol, but little other diglyceride species. Therefore, it is speculated that physiological activation of platelet protein kinase C may occur preferentially with 2-arachidonoyl-1-stearoyl-*sn*-glycerol.

In conclusion, the results demonstrate that arachidonate is released largely via activation of phospholipase A_2, upon platelet stimulation with a physiological agonist. More arachidonate is released from PC and PE than from inositides and PA (Table 1). The importance of arachidonate release from PC and PE is further emphasized by greater accumulation of free arachidonate and oxygenated products than accounted for by release of arachidonate from inositides.

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REFERENCES

1. Broekman, M. J., R. I. Handin, A. Derksen, and P. Cohen. 1976. Distribution of phospholipids, fatty acids, and platelet factor 3 among subcellular fractions of human platelets. *Blood.* **47:** 963–971.
2. Marcus, A. J., H. L. Ullman, and L. B. Safier. 1969. Lipid composition of subcellular particles of human blood platelets. *J. Lipid Res.* **10:** 108–114.
3. Cohen, P., and A. Derksen. 1969. Comparison of phospholipid and fatty acid composition of human erythrocytes and platelets. *Br. J. Haematol.* **17:** 359–371.
4. Mahadevappa, V. G., and B. J. Holub. 1983. Degradation of different molecular species of phosphatidylinositol in thrombin-stimulated human platelets. Evidence for preferential degradation of 1-acyl 2-arachidonoyl species. *J. Biol. Chem.* **258:** 5337–5339.
5. Skeaff, C. M., and B. J. Holub. 1985. Altered phospholipid composition of plasma membranes from thrombin-stimulated human platelets. *Biochim. Biophys. Acta.* **834:** 164–171.
6. Rittenhouse-Simmons, S. 1979. Production of diglyceride from phosphatidylinositol in activated human platelets. *J. Clin. Invest.* **63:** 580–587.
7. Mauco, G., H. Chap, and L. Douste-Blazy. 1979. Characterization and properties of a phosphatidylinositol phosphodiesterase (phospholipase C) from platelet cytosol. *FEBS. Lett.* **100:** 367–370.

8. Bell, R. L., D. A. Kennerly, N. Stanford, and P. W. Majerus. 1979. Diglyceride lipase: a pathway for arachidonate release from human platelets. *Proc. Natl. Acad. Sci. USA.* **76:** 3238–3241.
9. Schoene, N. W., and J. M. Iacono. 1975. Stimulation of platelet phospholipase A_2 activity by aggregating agents. *Federation Proc.* **34:** 257(Abstract).
10. Derksen, A., and P. Cohen. 1975. Patterns of fatty acid release from endogenous substrates by human platelet homogenates and membranes. *J. Biol. Chem.* **250:** 9342–9347.
11. Bills, T. K., J. B. Smith, and M. J. Silver. 1976. Metabolism of [^{14}C]arachidonic acid by human platelets. *Biochim. Biophys. Acta.* **424:** 303–314.
12. Bills, T. K., J. B. Smith, and M. J. Silver. 1977. Selective release of arachidonic acid from the phospholipids of human platelets in response to thrombin. *J. Clin. Invest.* **60:** 1–6.
13. Blackwell, G. J., W. G. Duncombe, R. J. Flower, M. F. Parsons, and J. R. Vane. 1977. The distribution and metabolism of arachidonic acid in rabbit platelets during aggregation and its modification by drugs. *Br. J. Pharmacol.* **59:** 353–366.
14. Lapetina, E. G., C. J. Schmitges, K. Chandrabose, and P. Cuatrecasas. 1977. Cyclic adenosine 3',5'-monophosphate and prostacyclin inhibit membrane phospholipase activity in platelets. *Biochem. Biophys. Res. Commun.* **76:** 828–835.
15. Nishizuka, Y. 1984. Turnover of inositol phospholipids and signal transduction. *Science.* **225:** 1365–1370.
16. Berridge, M. J. and R. F. Irvine. 1984. Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature.* **312:** 315–321.
17. Billah, M. M., and E. G. Lapetina. 1982. Rapid decrease of phosphatidylinositol 4,5-bisphosphate in thrombin-stimulated platelets. *J. Biol. Chem.* **257:** 12705–12708.
18. Mauco, G., H. Chap, and L. Douste-Blazy. 1983. Platelet activating factor (PAF-acether) promotes an early degradation of phosphatidylinositol-4,5-biphosphate in rabbit platelets. *FEBS. Lett.* **153:** 361–365.
19. Agranoff, B. W., P. Murthy, and E. B. Seguin. 1983. Thrombin-induced phosphodiesteratic cleavage of phosphatidylinositol bisphosphate in human platelets. *J. Biol. Chem.* **258:** 2076–2078.
20. Billah, M. M., and E. G. Lapetina. 1983. Platelet-activating factor stimulates metabolism of phosphoinositides in horse platelets: possible relationship to Ca^{2+} mobilization during stimulation. *Proc. Natl. Acad. Sci. USA.* **80:** 965–968.
21. Broekman, M. J. 1984. Phosphatidylinositol 4,5-bisphosphate may represent the site of release of plasma membrane-bound calcium upon stimulation of human platelets. *Biochem. Biophys. Res. Commun.* **120:** 226–231.
22. Rittenhouse, S. E., and W. C. Horne. 1984. Ionomycin can elevate intraplatelet Ca^{2+} and activate phospholipase A without activating phospholipase C. *Biochem. Biophys. Res. Commun.* **123:** 393–397.
23. Rittenhouse, S. E. 1984. Activation of human platelet phospholipase C by ionophore A23187 is totally dependent upon cyclo-oxygenase products and ADP. *Biochem. J.* **222:** 103–110.
24. Vickers, J. D., R. L. Kinlough-Rathbone, and J. F. Mustard. 1984. Changes in the platelet phosphoinositides during the first minute after stimulation of washed rabbit platelets with thrombin. *Biochem. J.* **219:** 25–31.
25. Vickers, J. D., R. L. Kinlough-Rathbone, and J. F. Mustard. 1984. Accumulation of the inositol phosphates in thrombin-stimulated, washed rabbit platelets in the presence of lithium. *Biochem. J.* **224:** 399–405.
26. Cohen, P., M. J. Broekman, A. Verkley, J. W. W. Lisman, and A. Derksen. 1971. Quantification of human platelet inositides and the influence of ionic environment on their incorporation of orthophosphate-^{32}P. *J. Clin. Invest.* **50:** 762–772.
27. Lloyd, J. V., E. E. Nishizawa, J. Haldar, and J. F. Mustard. 1972. Changes in ^{32}P-labelling of platelet phospholipids in response to ADP. *Br. J. Haematol.* **23:** 517–585.
28. Mahadevappa, V. G., and B. J. Holub. 1984. Relative degradation of different molecular species of phosphatidylcholine in thrombin-stimulated human platelets. *J. Biol. Chem.* **259:** 9369–9373.
29. Billah, M. M., E. G. Lapetina, and P. Cuatrecasas. 1980. Phospholipase A_2 and phospholipase C activities of platelets. Differential substrate specificity, Ca^{2+} requirement, pH dependence, and cellular localization. *J. Biol. Chem.* **255:** 10277–10231.
30. Purdon, A. D., and J. B. Smith. 1985. Turnover of arachidonic acid in the major diacyl and ether phospholipids of human platelets. *J. Biol. Chem.* **260:** 12700–12704.
31. Broekman, M. J., J. W. Ward, and A. J. Marcus. 1980. Phospholipid metabolism in stimulated human platelets: changes in phosphatidylinositol, phosphatidic acid, and lysophospholipids. *J. Clin. Invest.* **66:** 275–283.
32. Broekman, M. J., J. W. Ward, and A. J. Marcus. 1981. Fatty acid composition of phosphatidylinositol and phosphatidic acid in stimulated platelets. Persistence of arachidonyl–stearyl structure. *J. Biol. Chem.* **256:** 8271–8274.
33. Pickett, W. C., and P. Cohen. 1976. Mechanism of the thrombin-mediated burst in oxygen consumption by human platelets. *J. Biol. Chem.* **251:** 2536–2538.
34. Bressler, N. M., M. J. Broekman, and A. J. Marcus. 1979. Concurrent studies of oxygen consumption and aggregation in stimulated human platelets. *Blood.* **53:** 167–178.
35. Akkerman, J. W. N., and H. Holmsen. 1981. Interrelationships among platelet responses: studies on the burst in proton liberation, lactate production, and oxygen uptake during platelet aggregation and Ca^{2+} secretion. *Blood.* **57:** 956–966.
36. Turner, J. D., and G. Rouser. 1970. Precise quantitative determination of human blood lipids by thin-layer and triethylaminoethylcellulose column chromatography. I. Erythrocyte lipids. *Anal. Biochem.* **38:** 423–436.
37. Schacht, J. 1978. Purification of polyphosphoinositides by chromatography on immobilized neomycin. *J. Lipid. Res.* **19:** 1063–1067.
38. Rittenhouse-Simmons, S., F. A. Russell, and D. Deykin. 1976. Transfer of arachidonic acid to human platelet plasmalogen in response to thrombin. *Biochem. Biophys. Res. Commun.* **70:** 295–301.
39. Jesse, R. L., and R. C. Franson. 1979. Modulation of purified phospholipase A_2 activity from human platelets by calcium and indomethacin. *Biochim. Biophys. Acta.* **575:** 467–470.
40. Apitz-Castro, R. J., M. A. Mas, M. R. Cruz, and M. K. Jain. 1979. Isolation of homogeneous phospholipase A_2 from human platelets. *Biochem. Biophys. Res. Commun.* **91:** 63–71.
41. McKean, M. L., J. B. Smith, and M. J. Silver. 1981. Formation of lysophosphatidylcholine by human platelets in response to thrombin. Support for the phospholipase A_2 pathway for the liberation of arachidonic acid in platelets. *J. Biol. Chem.* **256:** 1522–1524.
42. Billah, M. M., E. G. Lapetina, and P. Cuatrecasas. 1981.

Phospholipase A_2 activity specific for phosphatidic acid. A possible mechanism for the production of arachidonic acid in platelets. *J. Biol. Chem.* **256:** 5399–5403.
43. Mauco, G., C. A. Dangelmayer, and J. B. Smith. 1984. Inositol lipids, phosphatidate and diacylglycerol share stearoylarachidonoylglycerol as a common backbone in thrombin-stimulated human platelets. *Biochem. J.* **224:** 933–940.
44. Lapetina, E. G., and P. Cuatrecasas. 1979. Stimulation of phosphatidic acid production in platelets precedes the formation of arachidonate and parallels the release of serotonin. *Biochim. Biophys. Acta.* **573:** 394–402.
45. Mahadevappa, V. G., and B. J. Holub. 1982. The molecular species composition of individual diacyl phospholipids in human platelets. *Biochim. Biophys. Acta.* **713:** 73–79.
46. Prescott, S. M., and P. W. Majerus. 1981. The fatty acid composition of phosphatidylinositol from thrombin-stimulated human platelets. *J. Biol. Chem.* **256:** 579–582.
47. Schacky, C. v., W. Siess, S. Fischer, and P. C. Weber. 1985. A comparative study of eicosapentaenoic acid metabolism by human platelets in vivo and in vitro. *J. Lipid Res.* **26:** 457–464.
48. Broekman, M. J. 1984. Platelet phosphatidylinositol 4,5-bisphosphate consists of the arachidonyl-stearyl species. Its thrombin-induced turnover uses arachidonyl-stearyl phosphatidylinositol and produces arachidonyl-stearyl phosphatidic acid. *Circulation.* **70 (II):** 57 (Abstract #226).
49. Wilson, D. B., E. J. Neufeld, and P. W. Majerus. 1985. Phosphoinositide interconversion in thrombin-stimulated human platelets. *J. Biol. Chem.* **260:** 1046–1051.
50. Watson, S. P., R. T. McConnell, and E. G. Lapetina. 1984. The rapid formation of inositol phosphates in human platelets by thrombin is inhibited by prostacyclin. *J. Biol. Chem.* **259:** 13199–13203.
51. Rittenhouse, S. E., and J. P. Sasson. 1985. Mass changes in myoinositol trisphosphate in human platelets stimulated by thrombin. Inhibitory effects of phorbol ester. *J. Biol. Chem.* **260:** 8657–8660.
52. Billah, M. M., and E. G. Lapetina. 1982. Formation of lysophosphatidylinositol in platelets stimulated with thrombin or ionophore A23187. *J. Biol. Chem.* **257:** 5196–5200.
53. Trugnan, G., G. Bereziat, M. C. Manier, and J. Polonovski. 1979. Phospholipase activities in subcellular fractions of human platelets. *Biochim. Biophys. Acta.* **573:** 61–72.
54. Bell, R. L., and P. W. Majerus. 1980. Thrombin-induced hydrolysis of phosphatidylinositol in human platelets. *J. Biol. Chem.* **255:** 1790–1792.
55. Smith, J. B., C. Dangelmaier, and G. Mauco. 1985. Measurement of arachidonic acid liberation in thrombin-stimulated human platelets. Use of agents that inhibit both the cyclooxygenase and lipoxygenase enzymes. *Biochim. Biophys. Acta.* **835:** 344–351.
56. Hattori, H., and J. N. Kanfer. 1985. Synaptosomal phospholipase D potential role in providing choline for acetylcholine synthesis. *J. Neurochem.* **45:** 1578–1584.
57. Cockcroft, S. 1984. Ca^{2+}-dependent conversion of phosphatidylinositol to phosphatidate in neutrophils stimulated with fMet-Leu-Phe or ionophore A23187. *Biochim. Biophys. Acta.* **795:** 37–46.
58. Serhan, C. N., M. J. Broekman, H. M. Korchak, A. J. Marcus, and G. Weissman. 1982. Endogenous phospholipid metabolism in stimulated neutrophils. Differential activation by FMLP and PMA. *Biochem. Biophys. Res. Commun.* **107:** 951–958.
59. Wynkoop, E. M., M. J. Broekman, H. M. Korchak, A. J. Marcus, and G. Weissman. 1986. Phospholipid metabolism in human neutrophils activated by N-formyl-methionyl-leucyl-phenylalanine. Degranulation is not required for release of arachidonic acid: studies with neutrophils and neutrophil-derived cytoplasts. *Biochem. J.* **236:** 829–837.
60. Siess, W., P. C. Weber, and E. G. Lapetina. 1984. Activation of phospholipase C is dissociated from arachidonate metabolism during platelet shape change induced by thrombin or platelet-activating factor. Epinephrine does not induce phospholipase C activation or platelet shape change. *J. Biol. Chem.* **259:** 8286–8292.
61. Hirai, A., T. Terano, T. Hamazaki, J. Sajiki, S. Kondo, A. Ozawa, T. Fujita, T. Miyamoto, Y. Tamura, and A. Kumagai. 1982. The effects of the oral administration of fish oil concentrate on the release and the metabolism of [^{14}C]arachidonic acid and [^{14}C]eicosapentaenoic acid by human platelets. *Thromb. Res.* **28:** 285–298.
62. Ahmed, A. A., V. G. Mahadevappa, and B. J. Holub. 1983. The turnover of (1-^{14}C)eicosapentaenoic acid among individual phospholipids of human platelets. *Nutr. Res.* **3:** 673–680.
63. Mueller, H. W., J. T. O'Flaherty, D. G. Greene, M. P. Samuel, and R. L. Wykle. 1984. 1-*O*-Alkyl-linked glycerophospholipids of human neutrophils: distribution of arachidonate and other acyl residues in the ether-linked and diacyl species. *J. Lipid. Res.* **25:** 383–388.
64. Rittenhouse, S. E. 1983. Human platelets contain phospholipase C that hydrolyzes polyphosphoinositides. *Proc. Natl. Acad. Sci. USA.* **80:** 5417–5420.