Arachidonate cannot be released directly from diacyl-*sn*-glycero-3-phosphocholine in thrombin-stimulated platelets

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The origin of the arachidonate released from platelets on stimulation with thrombin was investigated by comparing the specific activities of released arachidonate and of arachidonoyl-containing phospholipids using rat platelets prelabelled with arachidonate. Quantification of the released arachidonate was determined in the presence of BW 755 C, a dual cyclo-oxygenase/lipoxygenase inhibitor, which was found not to modify the arachidonate mobilization between the platelet phospholipids. The phospholipid molecular species were analysed by h.p.l.c. of diradylglycerol benzoate derivatives of diacyl, alkylacyl and alkenylacyl classes. The labelled/unlabelled arachidonate ratio varied greatly in the phospholipids depending on whether an ether or acyl bond was present in *sn*-1 position of the glycerol, on the length and degree of unsaturation of this fatty chain and on the polar head group. Between 15 s and 5 min of stimulation by thrombin, the released arachidonate kept a constant specific activity which was considerably lower than the specific activity of diacyl-GPC. The specific activity of the released arachidonate was intermediate between the specific activities of the 16:0-20:4 and 18:0-20:4 species of diacyl-GPI and diacyl-GPE, and corresponded to the mean specific activity of alkylacyl-GPC. The data indicate that the released arachidonate cannot come directly from diacyl-GPC, and that two phospholipids in particular can act as direct precursors of the released arachidonate. These are (1) the alkylacyl-GPC and (2) the diacyl-GPE whose hydrolysis would induce an arachidonate transfer from diacyl-GPC.

INTRODUCTION

Thromboxane A_2 and other active metabolites synthesized from arachidonic acid during platelet aggregation [1] are considered to play an important role in thrombosis and inflammatory reactions. The changes in labelling observed when fatty acid-prelabelled platelets were stimulated with thrombin prompted Bills *et al.* [2] to hypothesize that arachidonate was selectively released from phosphatidylcholine and phosphatidylinositol through the action of phospholipase A_2 . Due to different turnover rates of arachidonate in the platelet phospholipids, radiolabelling experiments of the glycerol backbone or of the arachidonate moiety [3–6] and mass analysis [7–9] resulted in conflicting observations concerning the origin of released arachidonic acid. The role of phosphatidylcholine as the main arachidonate precursor has been questioned because of the small amount of arachidonate in this phospholipid compared with others (especially phosphatidylethanolamine). Phosphatidylinositol was shown to contribute a maximum of 15% of the total arachidonate released in human platelets stimulated with high doses of thrombin [10,11]. Resolution of phospholipids into their molecular species allowed Takamura *et al.* [8] and Purdon *et al.* [9] to observe that at least three phospholipids, namely diacyl-GPC, diacyl-GPI and diacyl-GPE, contributed to arachidonate release when human platelets were stimulated with high thrombin concentrations. These studies showed clearly that in diacyl-GPC only the arachidonoyl-containing

species were hydrolysed, further arguing for specificity of the releasing pathway.

We recently observed, using rat platelet sonicates, that platelet phospholipase A_2 was considerably more active on phosphatidylethanolamine than on phosphatidylcholine, and that the Ca^{2+} -dependent phospholipid hydrolysis was not specific for the fatty acids esterifying phospholipids [12]. Rabbit platelet phospholipase A_2 also shows no selectivity for arachidonate-containing molecular species *in vitro* [13,14]. Thus, when considering experiments *in vitro*, this phospholipase A_2 does not appear to be the most likely candidate to specifically release arachidonate primarily from phosphatidylcholine. Furthermore, we have previously observed a specific transfer of arachidonate from diacyl-GPC to ether phospholipids and to diacyl-GPE in resting [15] and stimulated [5] rat platelets. This transfer reaction remained specific for arachidonate when assayed *in vitro* [16–19]. To account for the specificity of arachidonate release, we suggested that following stimulation by thrombin, diacyl-GPE, which is mainly composed of arachidonoyl species, would be hydrolysed first. Then the acyl-lyso-GPE produced could induce in turn a specific transfer of arachidonate from diacyl-GPC to diacyl-GPE.

In this paper, we took advantage of (1) the different turnovers of arachidonate between the various molecular species; and (2) the possibility of blocking arachidonate oxidation using the compound BW 755 C, to compare the specific activity of the released arachidonic acid with

the specific activities of phospholipid molecular species containing arachidonate. The data indicate that there is no direct relationship between acyl-arachidonoyl-GPC and the released arachidonate.

MATERIALS AND METHODS

Platelet preparation and [14 C]arachidonate incorporation

Rat platelets were obtained from 2-month-old Wistar rats by cardiac puncture and isolated by the procedure described by Ardlie *et al.* [20] using Tyrode buffer, pH 6.5, containing 0.25% gelatin. Acetylsalicylic acid (0.1 mM) was added to the first platelet suspension for 15 min. The platelets were then centrifuged and resuspended in the same buffer. [14 C]Arachidonic acid (0.8 μ Ci; 56 mCi/mmol; Amersham) complexed to human serum albumin was added to 1 ml of the platelet suspension (1×10^9 platelets/ml). The platelets were incubated at 37 °C for 30 min, then washed twice with Tyrode buffer, pH 6.5, to remove excess exogenous arachidonic acid and resuspended in Tyrode buffer, pH 7.4, containing 0.8 mM- Ca^{2+} .

Platelet stimulation and arachidonate release

Platelets suspended in Tyrode buffer, pH 7.4, were allowed to stand for 20 min at 37 °C before stimulation. Under standard conditions, platelets were stimulated with 2 units of bovine thrombin/ml (Sigma) in the presence of 100 μ M-BW 755 C (Wellcome Research Laboratories, Beckenham, Kent, U.K.) to inhibit arachidonate oxygenation. As measured by the decrease in absorbance using a dual channel aggregometer, platelet aggregation was not modified by the addition of compound BW 755 C to the platelet suspension 1 min before addition of thrombin. The reaction was stopped by adding chloroform/methanol (1:2, v/v). Arachidic acid was added as internal standard and the lipids extracted by the method of Bligh & Dyer [21]. Solvents were removed under a stream of N_2 and the lipids were resuspended in chloroform/methanol (1:1, v/v). The lipid extract was divided into two parts which were spotted on a t.l.c. plate. Separation of free fatty acids was carried out by migration in system I: first with isopropylene ether/acetic acid (19:1, v/v) and, after drying, in the same direction with petroleum ether/ethyl ether/acetic acid (90:30:1, by vol.). The spot corresponding to free arachidonic acid was scraped from the plate and either counted for radioactivity or methylated with BF_3 in KOH/methanol to measure arachidonate mass by g.l.c. [22]. Fatty acid methyl esters were separated using a Carbowax 20 M capillary column operating between 200 and 220 °C, and the mass of arachidonate was calculated from the arachidic acid added as an internal standard. The specific activity of the released arachidonate was expressed as d.p.m./nmol.

Phospholipid analysis

The lipid extracts from stimulated and non-stimulated platelets were fractionated in system II: chloroform/methanol/water (65:25:4, by vol.) in order to verify the [14 C]arachidonate mobilization from the phospholipids upon thrombin stimulation. Radioactive spots were then scraped and counted.

Phospholipid molecular species analysis

Specific activities of the phospholipid molecular species

were determined by the method of Blank *et al.* [23]. Phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine were separated by two-dimensional t.l.c. The lipid extract from 4×10^9 platelets was run first in tetrahydrofuran/acetone/methanol/water (50:20:40:8, by vol.) and in the second dimension in chloroform/methanol/water (65:25:4, by vol.). The phospholipids, detected by autoradiography or dichlorofluorescein, were scraped from the plate and extracted from the powder by the method of Bligh & Dyer [21]. The phospholipids were dissolved in diethyl ether and subjected to phospholipase C attack. Phospholipase C (20 i.u.) from *Bacillus cereus* (Boehringer-Mannheim) in phosphate buffer, pH 7.1, was added to the phospholipid solution. After shaking for 3 h at room temperature, more than 90% of phosphatidylcholine and phosphatidylethanolamine was hydrolysed, based on phospholipid radiolabelling. Phosphatidylserine hydrolysis required incubation at 30 °C for 20 h. About 50% hydrolysis of phosphatidylinositol was obtained on incubation in the presence of 200 i.u. phospholipase C at 30 °C for 20 h. Diacylglycerols were then converted to diacylglycerobenzoate derivatives according to Blank *et al.* [23] and resolved into diacyl, alkylacyl and alkenylacyl derivatives by t.l.c. in benzene/hexane/diethyl ether (50:45:4, by vol.). The benzoate derivatives were extracted from the powder and the molecular species were analysed by h.p.l.c. on a 5 μ m Lichosphere RP-18 analytical column. Separation was carried out by isocratic elution with acetonitrile/propan-2-ol and the molecular species were monitored at 230 nm. As checked by Blank *et al.* [23], the molar absorbance measured in acetonitrile/propan-2-ol at 230 nm was identical for the different molecular species of diradyl benzoates. Thus the molar amounts of the components can be determined directly from absorbance at 230 nm. Radioactivity was counted on-line using a Radiomatic Flow-One scintillation counter. Specific activities of arachidonate-containing molecular species were expressed in d.p.m./nmol.

RESULTS

Effect of compound BW 755 C on arachidonate oxygenation and on arachidonate mobilization from rat platelet phospholipids

When rat platelets are stimulated with thrombin, there is transfer of arachidonate from diacyl-GPC to ether-GPC and ether-GPE, and to diacyl-GPE, concurrent with arachidonate release [5]. The compound BW 755 C has been shown to inhibit both cyclo-oxygenase and lipoxygenase activities in human platelets without inhibiting arachidonate release [24]. To verify that this compound efficiently inhibited arachidonate oxygenation in rat platelets without modifying arachidonate transfer reactions between phospholipids, arachidonate-prelabelled rat platelets were stimulated with thrombin in the absence or in the presence of 100 μ M-BW 755 C. When platelets were preincubated with aspirin, cyclo-oxygenase products were not produced. The radioactivity released in the absence of BW 755 C was recovered as a single spot co-migrating on t.l.c. with the lipoxygenase product 12-hydroxyeicosatetraenoic acid. When the platelets were preincubated with BW 755 C, more than 80% of the radioactivity released from phospholipids was recovered as free arachidonate.

Fig. 1. [^{14}C]Arachidonate mobilization upon thrombin stimulation

Prelabelled rat platelets suspended in Tyrode buffer, pH 7.4, containing 0.8 mM- Ca^{2+} were stimulated with 2 units of thrombin/ml. The reactions were stopped by addition of chloroform/methanol (1:2, v/v), and the lipids were separated on t.l.c. plates using solvent system II (see the Materials and methods section). Data are expressed as percentage change in total d.p.m. relative to non-stimulated platelets and are the means $\pm$ S.E.M. of six different experiments. ■, Phosphatidylinositol; ◆, phosphatidic acid; ●, phosphatidylcholine; ▲, phosphatidylethanolamine; ○, arachidonic acid.

Fig. 2. BW 755 C did not modify arachidonate mobilization

The platelet suspension was incubated with 2 units of thrombin/ml for 5 min. When added, BW 755 C was at a concentration of 100 μM . The lipid fractions were separated on t.l.c. plates with solvent system II (see the Materials and methods section). Data are means $\pm$ S.D. of two different determinations. AA, arachidonate; PE, phosphatidylethanolamine; PA, phosphatidic acid; TG, triacylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine.

spot migrating slightly above phosphatidylcholine. Fig. 1(a) shows that the appearance of this radioactive product closely corresponded to the decrease in phosphatidylinositol labelling, at least during the first 2 min of stimulation. This radioactive spot did not appear when platelets were stimulated with ionophore A23187, which did not induce any decrease in phosphatidylinositol labelling under our incubation conditions (results not shown). This arachidonate-labelled product which is closely related to phosphatidylinositol turnover probably corresponds to phosphatidic acid and accounts for the decrease in [^{14}C]arachidonate observed in phosphatidylinositol. As previously observed, the radioactivity liberated from phospholipids and the increase in labelling of phosphatidylethanolamine counterbalanced the decrease in labelling of phosphatidylcholine (Fig. 1b).

This arachidonate mobilization following thrombin stimulation was not modified by the presence of the cyclo-oxygenase and lipoxygenase inhibitor BW 755 C. Fig. 2 shows that the appearance of phosphatidic acid and the increase in labelled phosphatidylethanolamine induced by thrombin were identical whether BW 755 C was present or not.

Comparison between the specific activity of the released arachidonate and the specific activities of arachidonoyl-containing phospholipids

[^{14}C]Arachidonate-prelabelled platelets suspended in Tyrode buffer, pH 7.4, for 20 min were stimulated with thrombin in the presence of BW 755 C in order to determine the specific activity of released arachidonic acid. Alternatively, prelabelled platelets were extracted to determine the specific activities of arachidonoyl-containing phospholipids. These specific activities varied slightly from one experiment to another due to various factors: platelet preparation, accurate amount of

In this study, to separate the different phospholipids and the released arachidonic acid, we used a neutral solvent system that allowed us to observe, following thrombin stimulation, the appearance of a radioactive

Table 1. Arachidonoyl species distribution in rat platelet phospholipids

Molecular species were separated as their benzoylated diacylglycerols and quantified by absorbance at 230 nm as indicated under Materials and methods. Arachidonoyl species are expressed as a percentage of total molecular species, and are means $\pm$ S.E.M. of three to six different preparations. Arachidonate levels (nmol/ 10^9 platelets) were calculated from total amount of phospholipid (245 ± 25 nmol/ 10^9 platelets) and from phospholipid distribution (diacyl-GPC, $37.2 \pm 2.7\%$; alkylacyl-GPC, $2.8 \pm 0.55\%$; diacyl-GPE, $8.8 \pm 0.84\%$; alkenylacyl-GPE, $14.5 \pm 1.1\%$; diacyl-GPI, $5.6 \pm 1.8\%$).

Arachidonoyl species	Arachidonoyl species as % total species				
	Diacyl-GPC	Diacyl-GPE	Diacyl-GPI	Alkylacyl-GPC	Alkenylacyl-GPE
20:4-20:4	0.4 ± 0.2	0.3 ± 0.2	< 1	—	—
18:2-20:4	0.9 ± 0.5	1.5 ± 0.1	< 1	—	—
18:1-20:4	0.6 ± 0.3	3.6 ± 0.3	3.9 ± 1.1	11.5 ± 3.9	19.5 ± 2.4
16:0-20:4	5.3 ± 0.9	8.5 ± 0.4	5.8 ± 1.4	6.9 ± 0.2	22.5 ± 3.4
18:0-20:4	4.2 ± 1.5	46.4 ± 3.7	76.7 ± 3.3	5.0 ± 0.1	20.8 ± 1.7
Total 20:4	11.4	60.0	86.4	25.6	67.3
nmol of 20:4/ 10^9 platelets	10.4	12.9	11.8	1.7	23.9

Table 2. Specific activities of arachidonoyl species in resting platelets

Benzoyl derivatives of molecular species separated by h.p.l.c. were quantified by absorbance at 230 nm and counted on-line as indicated under Materials and methods. Data represent the values from the same experiment as in Table 3 and are representative of three other experiments.

Arachidonoyl species	Specific activity (d.p.m./nmol)				
	Diacyl-GPC	Diacyl-GPE	Diacyl-GPI	Alkylacyl-GPC	Alkenylacyl-GPE
20:4-20:4	17900	24800	> 50000	—	—
18:2-20:4	15200	8600	> 14000	6400	640
18:1-20:4	17600	5400	15000	3700	460
16:0-20:4	13900	4200	8900	3900	470
18:0-20:4	10500	1010	2800	3200	350
Total 20:4 species	13450	2270	5600	3800	440

[14 C]arachidonic acid added in the platelet preparation and, particularly, exact duration of washings. As it was necessary to closely compare the specific activities of phospholipids to the specific activity of released arachidonate, it was not possible to average the results. Consequently, data presented hereafter correspond to one experiment which is representative of four different experiments.

Specific activities of arachidonoyl-containing phospholipid molecular species

Individual phospholipids resolved into diacyl, alkylacyl and alkenylacyl subclasses were subjected to h.p.l.c. analysis and separated as their molecular species. These species were quantified by u.v. absorption and their radioactivity counted on-line using a scintillation counter. Table 1 gives the mass content of arachidonoyl species in the phospholipids whose radiolabelling was shown to change when platelets were stimulated with thrombin [5]. In diacyl phospholipids, the two major molecular species are 16:0-20:4 and 18:0-20:4. Their

relative amounts are almost identical in diacyl-GPC, while 18:0-20:4 is considerably more abundant than 16:0-20:4 in diacyl-GPI and diacyl-GPE. Species with unsaturated fatty acids in position 1, namely the 20:4, 18:2 and 18:1 species, are minor components in diacyl phospholipids.

In the ether phospholipids, the distribution is more regular than in the diacyl phospholipids, due primarily to a higher content of 18:1-20:4 and also of 18:2-20:4 species. There is no 20:4-20:4 in the ether phospholipids. It is to be noticed that the proportion of arachidonoyl species in choline phospholipids was 11 % and 25 % for diacyl and alkylacyl classes respectively, whereas the proportion of arachidonoyl species in the other phospholipids was higher than 60 %. From the content of phospholipid classes in rat platelet, we calculated the amount of arachidonate in each phospholipid class: the three diacyl phospholipids, namely diacyl-GPC, diacyl-GPE and diacyl-GPI, contained almost identical amounts of arachidonate; the content of arachidonate in alkenylacyl-GPE was two-fold greater than in diacyl

Table 3. Time course of arachidonic acid release induced by thrombin

Following platelet stimulation with 2 units of thrombin/ml in the presence of BW 755 C (100 μ M), free arachidonic acid was quantified by g.l.c. and radioactivity assayed from t.l.c. as indicated under Materials and methods. Data represent values from the same experiment as in Table 2. This experiment is representative of three other experiments.

Time after stimulation	Arachidonic acid	
	Total released (nmol/10 ⁹ platelets)	Specific activity (d.p.m./nmol)
15 s	1.12	3525
30 s	1.41	3575
60 s	1.81	3670
2 min	2.05	3640
5 min	2.03	3705

phospholipids, and alkylacyl-GPC contained about one-sixth of the amount of arachidonate as diacyl phospholipids.

Every molecular species containing arachidonate was labelled in our conditions and every phospholipid class presented a special labelling pattern. The specific activities of the different molecular species in each phospholipid class are given in Table 2. The values for the different diacyl-GPC species were rather close except for the 18:0-20:4 species which was somewhat lower than the others. In diacyl-GPE and diacyl-GPI, large differences depending on the fatty acid in *sn*-1 position were observed. The greater the unsaturation of the fatty acid in *sn*-1 position, the higher was the specific activity of the molecular species. The 18:0-20:4 species had a considerably lower specific activity than the other molecular species, the value for the 16:0-20:4 remaining close to that for the unsaturated species. In the ether phospholipids, differences between the various molecular species were lower than in diacyl phospholipids. Comparing all phospholipid classes, the 16:0-20:4 species turnover rate was in the order diacyl-GPC > diacyl-GPI > diacyl-GPE > alkylacyl-GPC > alkenylacyl-GPE. This order was modified for the 20:4-20:4 species whose specific activity was higher in diacyl-GPI and diacyl-GPE than in diacyl-GPC, and also for the 18:0-20:4 species whose specific activity was higher in alkylacyl-GPC than in diacyl-GPE and diacyl-GPI. Finally, mean specific activity calculated from total arachidonate mass and total counts in each phospholipid class was in the order diacyl-GPC > diacyl-GPI > alkylacyl-GPC > diacyl-GPE > alkenylacyl-GPE.

Time course and specific activity of released arachidonate

The mass of arachidonate was measured by g.l.c. and radioactivity counted from t.l.c. separation. Table 3 shows that arachidonate was released after 15 s of thrombin stimulation; then the release increased slowly to reach 2.3 nmol/10⁹ platelets after 5 min. The mean $\pm$ S.E.M. ($n = 7$) of released arachidonic acid was 2.45 ± 0.35 nmol/10⁹ platelets after 5 min. Radioactivity recovered in free arachidonate paralleled free mass closely

such that the specific activity of the released arachidonic acid remained constant throughout the 5 min thrombin stimulation.

In the experiment shown in Tables 2 and 3, the mean specific activity of released arachidonate was 3623 ± 73 d.p.m./nmol, while mean specific activities of the different phospholipid classes ranged from 13450 d.p.m./nmol for acyl-arachidonoyl-GPC to 440 d.p.m./nmol for alkenyl-arachidonoyl-GPE.

DISCUSSION

When platelets are stimulated, the arachidonate released from phospholipids is very rapidly metabolized. To estimate the amount of arachidonate released from phospholipids in order to calculate its specific activity, we used the compound BW 755 C which has been shown to block arachidonate transformation both by cyclo-oxygenase and by lipoxygenase in human platelets [24]. Our data show that more than 80% of the released radioactivity was recovered as free arachidonic acid when rat platelets were stimulated with thrombin in the presence of BW 755 C. We verified that this compound had no effect on arachidonate mobilization and release from phospholipids as follows: (1) the appearance of labelled phosphatidic acid and the increase in phosphatidylethanolamine labelling were not modified by the presence of BW 755 C; and (2) the radioactivity released as free arachidonic acid and arachidonate derivatives in the presence of BW 755 C was identical to the released radioactivity in its absence. Thus this inhibitor, which blocks the bulk of arachidonate transformation without modifying the mechanisms of its release, has been utilized in this study to quantify the release of arachidonate on thrombin stimulation.

The release of arachidonate increased with time, up to 2.45 ± 0.35 nmol following 5 min stimulation with 2 units/ml of thrombin. The rate of arachidonate release was lower than in human platelets [4,24]. This is surprising if we consider the very high phospholipase A₂ activity of rat platelets [25,26]. Radioactivity in free arachidonate paralleled free mass closely throughout the time course. Thus from 15 s to 5 min, free arachidonate kept a constant specific activity.

On measuring mass and amount of radiolabelling in every arachidonoyl-containing molecular species, we observed that the specific activities depended on whether an ether or acyl bond was present at the *sn*-1 position of the glycerol moiety, on the length and unsaturation of this fatty chain and on the polar head group of the phospholipid. The selectivities concerning arachidonate incorporation induced differences in the specific activities as great as the time of labelling was short. Then, transfer reactions tended to decrease these differences [15]. In order to obtain specific activities as different from each other as possible, platelets were labelled for only 30 min with [¹⁴C]arachidonic acid. As transfer reactions were occurring during platelet washings, in order to compare the specific activities of phospholipids and released arachidonate, platelets were extracted at exactly the same time as other platelets were stimulated.

Comparison of specific activities in acyl-arachidonoyl-GPC and in released arachidonate clearly excludes a direct relationship between them. The mean specific activity of acyl-arachidonoyl-GPC was 3–4-fold higher than that of the released arachidonate. Even the specific

activity of 18:0-20:4 molecular species, which had the lowest specific activity in acyl-arachidonoyl-GPC, was 2-fold higher than that of the released arachidonate. These data indicate that the arachidonic acid liberated from phospholipids when platelets are stimulated with thrombin cannot derive directly from acyl-arachidonoyl-GPC hydrolysis.

Considering mean specific activity, the alkyl-arachidonoyl-GPC could be a direct precursor of released arachidonate because it is the only phospholipid whose arachidonate specific activity corresponded to the specific activity of the released arachidonate. However, only 25% of alkylacyl-GPC molecular species are arachidonate species (Table 1) and the total amount of arachidonate in alkyl-arachidonoyl-GPC is less than 2 nmol/10⁹ platelets, while 80% of the released arachidonate amounted to 2.45 ± 0.35 nmol/10⁹ platelets after 5 min stimulation with thrombin. Besides, it does not appear likely that important alkyl-arachidonoyl-GPC breakdown might occur upon platelet stimulation with thrombin [5]. These observations do not support alkyl-arachidonoyl-GPC as a main supplier of the released arachidonate. This is not in contradiction with PAF-acether formation from alkyl-arachidonoyl-GPC upon platelet stimulation with thrombin, because synthesis of this potent biologically active phospholipid can be accounted for by a low rate of alkyl-arachidonoyl-GPC breakdown [27,28].

The specific activity of released arachidonate was intermediate between that of 16:0-20:4 and 18:0-20:4 in diacyl-GPI and in diacyl-GPE. Thus these two phospholipids could account for the released arachidonate, but this implies some preferential hydrolysis of 18:0-20:4 species in diacyl-GPI and some preferential hydrolysis of the species different from 18:0-20:4 in diacyl-GPE. Such specificities have never been demonstrated for any phospholipase A₂.

In this study we observed the appearance of radiolabelled phosphatidic acid concurrently with the decrease in phosphatidylinositol labelling. The phosphatidic acid then slowly disappeared. The formation of phosphatidic acid could reflect, in rat as in human platelets stimulated with thrombin, a control mechanism shifting the metabolism of diacylglycerols to phosphatidic acid [29] and preventing arachidonate release by diacylglycerol lipase [30,31]. It is thus very likely that phosphatidylinositol contributes only a minor part to the arachidonate release in rat as in human platelets [10,11] stimulated with high doses of thrombin.

In the predominant diacyl-GPE 18:0-20:4 species and then in diacyl-GPE as a whole, the specific activity at the time of the stimulation is slightly lower than that of released arachidonate. However, arachidonate can be transferred from diacyl-GPC to ether phospholipids [32-35] and to diacyl-GPE [15,24], and these transfers are activated when platelets are stimulated with thrombin [5,6,36]. In arachidonate-prelabelled rat platelets, a 2% increase in diacyl-GPE labelling was observed after 1 min of stimulation with thrombin [5]. Studies *in vitro* demonstrated that the ATP-independent transfers were specific for arachidonate over other fatty acids and were induced by the presence of lysoderivatives [16-19]. Rat platelet phospholipase A₂ was shown to selectively hydrolyse diacyl-GPE [12] and this hydrolysis was masked in the presence of coenzyme A which induced an arachidonate transfer from diacyl-GPC to diacyl-GPE

[37]. Thus initial diacyl-GPE hydrolysis following a rise in Ca²⁺ due to thrombin stimulation appears to promote a rapid arachidonate transfer from diacyl-GPC, accounting for the 2% increase in diacyl-GPE labelling observed after 1 min [5]. Mean diacyl-GPE specific activity could then become sufficiently close to that of released arachidonate such that diacyl-GPE could be a direct precursor. The specific arachidonoyl species breakdown observed in human platelet diacyl-GPC [8,9] supports the specific transfer of arachidonate from diacyl-GPC to diacyl-GPE.

A slow transfer of [¹⁴C]arachidonate to alkenylacyl-GPE also occurred in stimulated platelets. However, alkenylacyl-GPE had a specific activity considerably lower than that of released arachidonate, and this slow transfer could not sufficiently increase [¹⁴C]arachidonate content in alkenylacyl-GPE to account for the high and constant specific activity of the released arachidonate.

If we cannot completely exclude the possibility that the released arachidonate might originate from the mixture of high and low specific activity pools, the differences in phospholipase A₂ activity towards the various classes of phospholipids [12] do not favour this hypothesis. It appears more likely from the present data that diacyl-GPC is not a direct precursor of arachidonate, and that two other phospholipids mainly act as direct precursors of released arachidonate: the alkylacyl-GPC, whose mean specific activity correlated well with that of released arachidonate, and diacyl-GPE following [¹⁴C]arachidonate transfer from diacyl-GPC. Diacyl-GPI was primarily recycled through phosphatidic acid.

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