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ASYMMETRY OF ARACHIDONIC ACID METABOLISM IN THE PHOSPHOLIPIDS OF THE HUMAN PLATELET MEMBRANE AS STUDIED WITH PURIFIED PHOSPHOLIPASES

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(1) Human platelets were incubated with high density lipoproteins (HDL) doubly labelled with either free [^{14}C]arachidonate / [^{3}H]arachidonoylphosphatidylcholine or free [^{14}C]oleate / [^{3}H]oleoylphosphatidylcholine. Whereas [^{14}C]arachidonate was incorporated at a 10–15-times higher rate than [^{14}C]oleic acid, the exchange of both species of phosphatidylcholine occurred to the same extent. In both cases, free ^{3}H-labelled fatty acids were generated during the labelling procedure, indicating phospholipase A_2 hydrolysis. A redistribution of radioactivity to other phospholipids was noted after exchange of [^{3}H]arachidonoylphosphatidylcholine only. (2) The exchange of phosphatidylcholine to platelets was confirmed using [^{14}C]choline-labelled dipalmitoyl- and 1-palmitoyl-2-arachidonoylphosphatidylcholines. (3) Non-lytic degradation of platelet phospholipids by phospholipases revealed that free fatty acids were incorporated at the inside of the cells, whereas exchange was taking place on the platelet outer surface. However, 2-arachidonoylphosphatidylcholine displayed a more rapid movement towards the cell inside. The above findings suggest a topological asymmetry for the two pathways (acylation and exchange) of fatty acid renewal in platelets. The possible mechanisms and physiological relevance of the translocation of the external arachidonic acid pool across the membrane are discussed.

Introduction

Arachidonic acid is known to play a major role in the regulation of platelet functions as a precursor to various biologically active molecules like thromboxane A_2 [1]. This fatty acid is present almost totally in the 2-position of platelet glycerophospholipids and its release, which might involve various kinds of lipases [2–13], is a prerequisite to its further conversion by cyclooxygenase and lipoxygenase [14]. Several studies dealt with the mechanism of arachidonic acid renewal in platelet glycerophosphatides [14–18]. Two pathways have been described: the first one (Lands pathway) involves the esterification of acceptor lysophospholipids by the acyl-coenzyme A derived from either endogenous or exogenous arachidonic acid [14–17] and the second, the exchange of intact PC molecules, occurs between plasma lipoproteins and the platelet membrane [18]. Both pathways might participate in the renewal of arachidonate in platelet phospholipids [17,18].

Phospholipases have been widely used to detect the transverse distribution of phospholipids in various biological membranes [19–22]. In this respect, confirming a previous report by Schick et al. [23], we described an asymmetric distribution of phospholipids in the platelet plasma membrane, characterized by the predominance of choline-containing phospholipids in the outer half and by the almost exclusive location of anionic phospholipids

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Abbreviations: PC, phosphatidylcholine; HDL, high density lipoproteins.

(phosphatidylserine and -inositol) and phosphatidylethanolamine in the inner leaflet [21]. As a consequence of phospholipid asymmetry, arachidonic acid was further shown to display a non-homogeneous distribution, since only 6% of the total arachidonate present in the platelet is located in the outer surface [22].

Studies by Renooij et al. [24–26] indicated that the Lands pathway occurs in the inner half of the erythrocyte membrane, whereas the exchange with the plasma lipoproteins takes place on the outer face of the red cell membrane. Furthermore, the two pools of phospholipids present on both sides of the membrane are susceptible to a slow equilibration process involving a flip-flop mechanism [25,26].

The existence of such an asymmetry in the platelet membrane, besides its physiological significance, could offer a means to label specifically the minor external pool of arachidonic acid and to compare its behaviour with that of the large inner pool during platelet activation.

The present study was thus undertaken in order to determine the sidedness of arachidonic acid renewal in the platelet membrane phospholipids using purified phospholipases. The results were compared to those obtained with oleic acid, which is not a precursor of eicosanoids.

Materials and Methods

Purified phospholipase A_2 from bee venom and sphingomyelinase C from *Staphylococcus aureus* were kindly donated by Professor R.F.A. Zwaal [26]. All radioactive materials ([9,10(n)-^{3}H]oleic acid (5.7 Ci/mmol), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (120 Ci/mmol), dipalmitoyl-L-α-phosphatidyl[*N-methyl*-^{3}H]choline (77 Ci/mmol), [1-^{14}C]arachidonic acid (54 mCi/mmol), [1-^{14}C]oleic acid (57.4 mCi/mmol), dipalmitoyl-L-α-phosphatidyl[*N-methyl*-^{14}C]choline (58 mCi/mmol)) were from the Radiochemical Centre, Amersham, U.K. Indomethacin and soybean lipoxygenase (EC 1.13.11.12) were from Sigma Chemical Co., St. Louis, MO. Dipalmitoylphosphatidylcholine and arachidonoyl chloride were purchased from Interchim, Montluçon, France. Bovine serim albumin (30%, w/v) was from the Transfusion Centre.

Preparation of radioactive substrates

1-Acyl-2-[^{3}H]oleoyl-sn-glycero-3-phosphocholine and 1-acyl-2-[^{3}H]arachidonoyl-sn-glycero-3-phosphocholine. Rat liver microsomes were prepared for acylation by either [^{3}H]oleic acid (1 mCi) or [^{3}H]arachidonic acid (250 μCi) as described in Ref. 28. After lipid extraction, the 2-[^{3}H]acylphosphatidylcholine purified by thin-layer chromatography [29] was eluted with chloroform/methanol (1 : 1, v/v) and then stored at −20°C, under nitrogen. Thin-layer chromatography controls showed no contamination by other lipids. The specific activities of [^{3}H]arachidonoylphosphatidylcholine and of [^{3}H]oleoylphosphatidylcholine were $3 \cdot 10^6$ and $9 \cdot 10^6$ dpm/μmol, respectively.

1-Palmitoyl-2-arachidonoyl-sn-glycero-3-phospho[N-methyl-^{3}H]choline. The specific activity of 1,2-dipalmitoyl-*sn*-glycero-3-phospho[*N-methyl*-^{3}H]choline was adjusted to 10^7 dpm/μmol by isotopic dilution with unlabelled L-dipalmitoylphosphatidylcholine and then submitted to the action of *Crotalus adamanteus* venom phospholipase A_2. After conversion to its $CdCl_2$ adduct, the lysophosphatidylcholine was then acylated with arachidonoyl chloride as described by Chakrabarti and Khorana [30]. Alternatively, 1-palmitoyl-*sn*-glycero-3-phospho[*N-methyl*-^{14}C]choline was reacylated using free arachidonic acid in the presence of microsomes.

Labelling of HDL with radioactive substrates

Human high density lipoproteins (d = 1.063–1.21 g/ml) were isolated according to the method of Ayrault-Jarrier et al. [31]. Purity of the preparation was ascertained by polyacrylamide gel electrophoresis. The labelling procedure was similar to that of Béréziat et al. [18] with the following adaptations: 10 μmol [^{3}H]arachidonoyl- or [^{3}H]oleoylphosphatidylcholine and the corresponding ^{14}C-labelled free fatty acid (150 nCi) were co-sonicated in 2 ml of 33 mM phosphate buffer, pH 7.4. Liposomes obtained were centrifuged for 20 min at 105 000 × g and the supernatant containing monolamellar liposomes was incubated for 2 h at 37°C with 6 mg HDL protein. HDL were then spun down for 18 h at 105 000 × g and were resuspended in 2 ml of Tyrode buffer (pH 7.4) containing 0.35% (w/v) bovine serum

albumin and 0.25 mM EGTA [32]. About 80–85% of HDL lipid phosphorus was in PC. The same procedure was used for the labelling of HDL with diacyl-*sn*-glycero-3-phospho[*N-methyl*-^{3}H]choline.

Platelet isolation

Platelets were isolated from platelet concentrates obtained from the Transfusion Centre. The storage length never exceeded 17 h. Alternatively, fresh human blood collected on acid/citrate/dextrose as an anticoagulant was used [33]. In both cases, platelet suspensions were prepared according to the method of Ardlie et al. [32]. Final suspensions were in a calcium-free Tyrode buffer (pH 7.4), containing 0.2 mM EGTA, 10 mM glucose and 0.35% (w/v) bovine serum albumin. They were adjusted to 10 mg platelets/ml suspension (wet weight), which corresponds to 0.2–0.25 μmol/ml as lipid phosphorus. The whole procedure was performed at room temperature in siliconized glassware (Sigmacote, Sigma).

Incubation conditions

Platelet suspensions (2 ml) were incubated with labelled HDL (0.25 ml, 1–1.2 μmol phospholipids) for various times, at 37°C in a shaking, thermostatically controlled water bath. In some experiments, in which only the acylation by exogenous fatty acid was studied, platelets were incubated either with [^{3}H]oleic acid (2 μCi) or with [^{14}C]arachidonic acid (0.025 μCi) without HDL. At the end of the incubation, cells were separated by centrifugation (1500 × *g* for 10 min), and the supernatant containing HDL was collected for further lipid analysis. Platelets were then washed twice with a calcium-free Tyrode buffer (pH 6.5) containing 0.35% bovine serum albumin (w/v) and finally suspended in a Tyrode buffer (pH 7.35) lacking albumin, and containing 11 mM glucose, 1 mM $MgCl_2$, the calcium concentration being reduced to 0.25 mM.

Treatment of intact platelets with phospholipases

In order to discriminate between the labelled phospholipids of the outer surface and of the cell inside, platelets were submitted to the sequential action of phospholipase A_2 from bee venom and of sphingomyelinase C from *S. aureus* as previously described [22]. Platelets were first preincubated for 10 min with indomethacin (20 μM), used as an anti-aggregant, and with soybean lipoxygenase (110 U/ml). After incubation with the phospholipases for 60 min, an aliquot of the cell suspension was centrifuged at 1500 × *g* for 8 min and the supernatant was assayed for lactate dehydrogenase activity. Only pellets where cell lysis remained less than 8% were kept for further analysis. The remaining cell suspension was cooled at 0–4°C and EDTA (15 mM) was immediately added to block the phospholipase activities. Control incubations with no enzyme were carried out in the same way.

Lipid analysis

Lipids were extracted according to the method of Bligh and Dyer [34] after acidification with 0.01 ml formic acid per ml platelet suspension. The different phospholipids, free fatty acids and the other neutral lipids were separated by a two-dimensional thin-layer chromatography on silica gel F 254, 0.25 mm thickness (Merck), using the two solvent systems of Broeckhuyse [35]. The lipid spots were visualized by exposure to iodine vapour and were then analysed for their radioactivity or phosphorus content.

Analytical determination

Radioactivity was determined in an Intertechnique liquid scintillation spectrometer (model SL 4000) with automatic quenching correction. Lipid phosphorus was assayed by a modification [36] of the procedure of Fiske and SubbaRow [50], after destruction with 70% $HClO_4$ at 190°C. Lactate dehydrogenase was measured as previously described [21] using the method of Wroblewski and La Due [37]. Protein was determined by the method of Lowry et al. [38] using bovine serum albumin as standard.

Results

(1) Distribution of radioactivity in the lipids of HDL

HDL were labelled by incubation with liposomes containing the radioactive lipids, and were then reisolated by centrifugation. About 60% of the label initially present in the medium was recovered with the lipoprotein. Using phosphatidylcholines labelled either on the 2-position with

[^{3}H]oleic/[^{3}H]arachidonic acid, or on the choline moiety, over 95% of the HDL label was found in PC, and no appreciable degradation of the phospholipid was detected. When a ^{14}C-labelled free fatty acid was added to the liposomes, it was recovered as such in the labelled reisolated HDL.

(2) Time course of oleic acid and arachidonic acid incorporation into platelet phospholipids

The kinetics of exchange of [^{3}H]oleoyl- and [^{3}H]arachidonoylphosphatidylcholine from HDL to platelets are compared in Figs. 1A and 2A. A plateau of exchange was more rapidly reached with oleoylphosphatidylcholine. However, the amount of radioactive PC transferred to platelets at the end of a 120 min incubation was comparable using either substrate; e.g., 10–20 nmol/1000 nmol platelet phospholipids, since the specific activity of [^{3}H]oleoylphosphatidylcholine in HDL was 3–4-fold higher than that of the [^{3}H]arachidonoyl species. Actually, this represents a very low fraction, 0.8–1.2%, of the [^{3}H]PC initially present

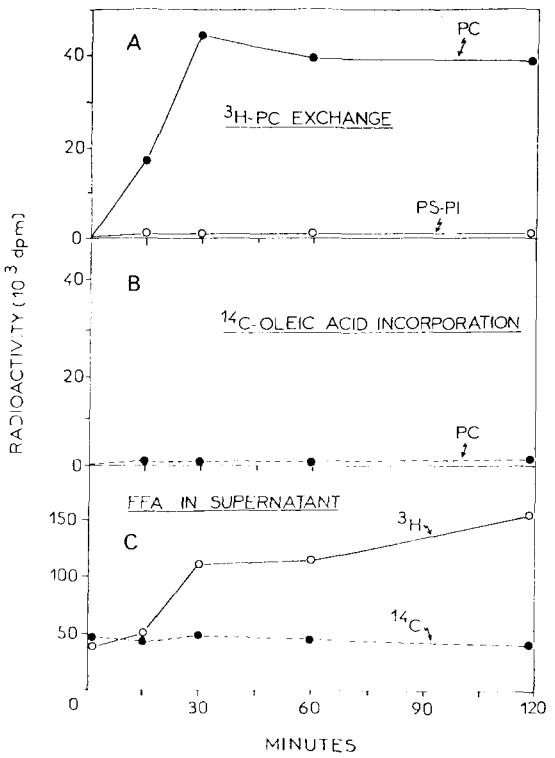

Fig. 1. Time course of oleic acid incorporation into platelet phospholipids from [^{14}C]oleate and from [^{3}H]oleoylphosphatidylcholine. Platelets were incubated with doubly labelled HDL ([^{3}H]oleoylphosphatidylcholine, $5.5 \cdot 10^6$ dpm; and [^{14}C]-labelled free oleic acid, $4.6 \cdot 10^4$ dpm). Results are expressed as a function of time for: (A) [^{3}H]oleoylphosphatidylcholine exchanged to cells; (B) [^{14}C]oleic acid incorporated to cells. Only PC (45% of the total incorporated label) is represented here for more clarity. Other labelled phospholipids were phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine (12 and 10% of the total label, respectively. See Table II.) (C) ^{3}H- or ^{14}C-labelled free oleic acid present in the post-incubation supernatant. Typical experiment representative of three different experiments. FFA, free fatty acids.

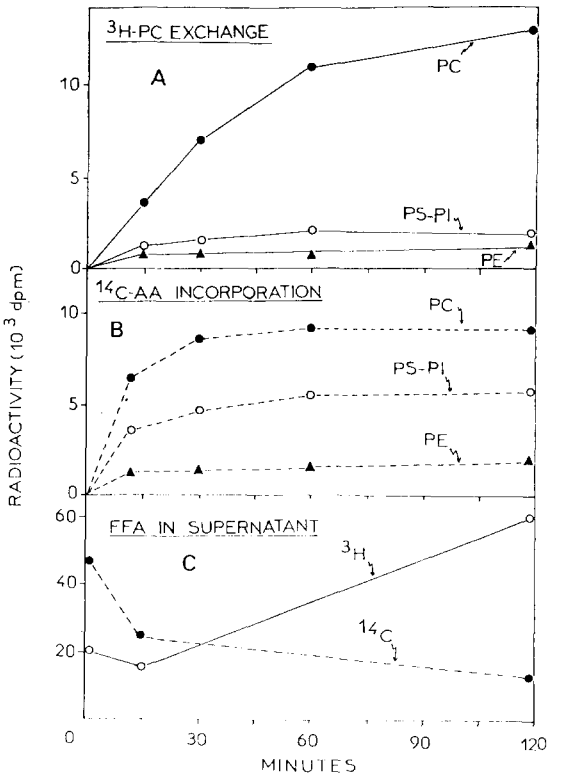

Fig. 2. Time course of arachidonic acid incorporation into platelet phospholipids from [^{14}C]arachidonate and from [^{3}H]arachidonoylphosphatidylcholine. Platelets were incubated with doubly labelled HDL ([^{3}H]arachidonoylphosphatidylcholine, $2.7 \cdot 10^6$ dpm; and ^{14}C-labelled free arachidonic acid, $4.8 \cdot 10^4$ dpm). Results are expressed as a function of time for: (A) [^{3}H]arachidonoylphosphatidylcholine exchanged to cells; (B) [^{14}C]arachidonic acid incorporated into cells; (C) ^{3}H- or ^{14}C-labelled free arachidonic acid present in the post-incubation supernatant. Typical experiment representative of four different experiments. PS, phosphatidylserine, PI, phosphatidylinositol; PE, phosphatidylethanolamine; FFA, free fatty acids.

in the HDL preparations. A similar extent of exchange was recorded using PC labelled on their polar head group.

In contrast, the rates of incorporation of oleic and arachidonic acid when presented in a free form were revealed to be quite different from each other (Figs. 1B and 2B). Using an equivalent concentration of labelled fatty acid (of the same specific activity), the amount of cell-associated radioactivity, at all incubation times, was 10–15-times greater for arachidonate than for oleate labelling. Besides platelet PC, phosphatidylserine and phosphatidylinositol were found to be most active in incorporating arachidonic acid, especially when considering that they represent only 15–16% of the total cell phospholipids. Phosphatidylethanolamine was labelled to a lesser extent.

Similar results were obtained when the radioactive fatty acids were used dispersed in an albumin/Tyrode buffer with no HDL present. It is noteworthy that, confirming previous data from Bériziat et al. [18], some degree of redistribution of the radiolabel among cell phospholipids was also noted following exchange of [^{3}H]arachidonoylphosphatidylcholine. This was not observed with the oleoylspecies.

As free arachidonate is readily taken up by platelets, the amount of labelled free fatty acids in HDL was monitored over the incubation period and compared for both kinds of doubly labelled HDL (Figs. 1C and 2C). While the content of ^{14}C-labelled free fatty acid decreased in the post-incubation supernatants, thus paralleling its cellular uptake, that of the ^{3}H-labelled homologue was enhanced. This behaviour was repeatedly observed. In both cases, control labelled HDL incubated alone for 60–120 min showed no consistent changes in the distribution of radioactive lipids (data not shown). This observation suggests a continuous generation of [^{3}H]arachidonic/[^{3}H]oleic acid, following incubation with platelets. It is to be noted that the amounts of such generated ^{3}H-labelled free fatty acids after a 120 min incubation are 4–5-times that of the [^{3}H]PC exchanged to cells, during the same period.

(3) Non-lytic degradation by exogenous phospholipases of doubly labelled platelets

To explore the cell sidedness of the two processes studied here (i.e., [*acyl*-^{3}H]PC exchange/^{14}C-labelled fatty acid incorporation), platelets that had been doubly labelled for 15 min with HDL were further submitted to the non-lytic action of exogenous phospholipases. As previously shown [20,21], this allows a total degradation of phospholipids in the outer layer of the platelet membrane.

TABLE I

NON-LYTIC DEGRADATION BY EXOGENOUS PHOSPHOLIPASES OF DOUBLY LABELLED (OLEIC ACID) PLATELETS

Platelets were incubated for 15 min with [^{3}H]oleoylphosphatidylcholine- and [^{14}C]oleic acid-labelled HDL. Distribution of cell lipid radioactivity was determined after incubation for 60 min in the absence or presence of phospholipases under non-lytic conditions. Results are expressed as percent of total lipid radioactivity for [^{14}C]oleic acid labelling, [^{3}H]oleoylphosphatidylcholine labelling and represent means ± S.E. of three to five experiments.

		Controls	Phospholipase treatment
^{14}C	Phosphatidylcholine	44.5 ± 3.0	34.3 ± 2.0
	Phosphatidylserine ± phosphatidylinositol	12.7 ± 1.6	8.2 ± 1.9
	Phosphatidylethanolamine	8.8 ± 0.7	7.5 ± 1.0
	Neutral lipids	16.2 ± 2.1	10.7 ± 2.7
	Free fatty acids	16.6 ± 4.0	37.1 ± 3.9
^{3}H	Phosphatidylcholine	83.2 ± 2.7	11.5 ± 1.3
	Lysophosphatidylcholine	1.4 ± 0.1	11.6 ± 1.6
	Neutral lipids	2.5 ± 0.5	1.5 ± 0.5
	Free fatty acids	9.1 ± 1.5	72.4 ± 2.8

TABLE II

NON-LYTIC DEGRADATION BY EXOGENOUS PHOSPHOLIPASES OF DOUBLY LABELLED (ARACHIDONIC ACID) PLATELETS

Platelets were incubated for 15 min with [^{3}H]arachidonoylphosphatidylcholine- and [^{14}C]arachidonic acid-labelled HDL. Distribution of cell lipid radioactivity was determined after incubation for 60 min in the absence or in the presence of phospholipases under non-lytic conditions. Results are expressed as percent of cell lipid radioactivity for [^{14}C]arachidonic acid labelling, [^{3}H]arachidonoylphosphatidylcholine labelling and represent means ± S.E. of four to seven experiments.

		Controls	Phospholipase treatment
^{14}C	Phosphatidylcholine	50.2 ± 2.0	41.6 ± 2.3
	Phosphatidylserine + phosphatidylinositol	27.2 ± 2.0	23.1 ± 1.7
	Phosphatidylethanolamine	9.6 ± 0.9	9.0 ± 0.5
	Neutral lipids	4.8 ± 1.7	3.6 ± 1.4
	Free fatty acids	5.7 ± 2.2	20.3 ± 4.1
^{3}H	Phosphatidylcholine	80.3 ± 2.4	40.8 ± 11.2
	Phosphatidylserine + phosphatidylinositol	10.1 ± 3.1	12.0 ± 2.3
	Phosphatidylethanolamine	3.1 ± 1.6	5.4 ± 0.8
	Neutral lipids	2.6 ± 0.4	4.9 ± 3.4
	Free fatty acids	2.7 ± 1.4	31.2 ± 10.9

As shown in Tables I and II, oleic and arachidonic acids differed in their incorporation into the various cell lipids. A substantial accumulation as free fatty acid (16%) or in the neutral lipids (16%) was noted for oleate but not for arachidonate (about 5%). However, in both cases, the platelet-labelled phospholipids were poorly hydrolysed by the subsequent action of exogenous phospholipases: 16 and 13.5%, respectively, of the [^{14}C]oleic and [^{14}C]arachidonic acid cell radioactivity were released from phospholipids upon enzymatic treatment, and were recovered as free fatty acids.

In contrast, 86% of the [^{3}H]oleoylphosphatidylcholine acquired through exchange was accessible to phospholipase action (Table I). It is noteworthy that some of the ^{3}H radioactivity released from phospholipids was recovered as lyso compounds, reflecting that part of the [^{3}H]oleoylphosphatidylcholine initially labelled at the C_1-position. After exchange of [^{3}H]arachidonoylphosphatidylcholine (Table II), a redistribution of radioactivity among cell phospholipids was evident, and about 50% of the labelled PC was degraded by exogenous phospholipase A_2.

As shown in Table III, the percentage of labelled PC degradation by the phospholipases was not essentially different upon labelling the platelets for 15 or 120 min, with either HDL preparation. Yet, the exchanged [^{3}H]PC exhibited a slightly lesser accessibility after 120 min.

In these experiments, phospholipid degradation by exogenous phospholipases was determined

TABLE III

NON-LYTIC DEGRADATION OF RADIOACTIVE PC IN PLATELETS INCUBATED FOR VARIOUS TIMES WITH DOUBLY LABELLED HDL

In the case referred to as oleic acid, platelets were labelled with HDL containing [^{14}C]oleic acid and [^{3}H]oleoylphosphatidylcholine. In the case referred to as arachidonic acid, platelets were labelled with HDL containing [^{14}C]arachidonic acid and [^{3}H]arachidonoylphosphatidylcholine. Results are given as percentage of hydrolysis upon treatment with bee venom phospholipase A_2 and *S. aureus* sphingomyelinase and are the means ± S.E. of three to seven experiments.

		Time of labelling	
		15 min	120 min
Oleic acid	^{3}H	86 ± 2	74 ± 11
	^{14}C	23 ± 3	28 ± 7
Arachidonic acid	^{3}H	49 ± 12	38 ± 8
	^{14}C	17 ± 3	22.5 ± 5

comparatively to controls incubated for 60 min in the absence of enzymes. However, in three experiments, controls were compared to non-incubated samples. It was found that 19.9 ± 9.3% of [^{3}H]arachidonoylphosphatidylcholine was hydrolysed, whereas [^{3}H]oleoylphosphatidylcholine degradation was 5.5 ± 3.5%. In contrast, [^{14}C]arachidonoylphosphatidylcholine remained unchanged (0 ± 7.9% hydrolysis), while [^{14}C]oleoylphosphatidylcholine decreased by 22.9 ± 6.3%.

(4) Non-lytic degradations of platelet phospholipids upon labelling with [^{3}H]- or [^{14}C]cholinephosphatidylcholines

HDL were prepared, labelled on the PC polar head group with [^{3}H]- or [^{14}C]methylcholine. Two kinds of molecules were incorporated into HDL : dipalmitoyl- and 1-palmitoyl-2-arachidonoylphosphatidylcholine. The latter was either biosynthetically obtained or chemically synthesized as described in Materials and Methods.

An exchange of PC towards platelets occurred to an extent comparable with that observed with other labels. About 80% of the newly incorporated dipalmitoylphosphatidylcholine remained available to phospholipase action, irrespective of the time of cell labelling (Table IV). By contrast, the degradation of the cell-transferred 1-palmitoyl-2-arachidonoylphosphatidylcholine was dependent on the time of incubation with HDL and showed a decrease for prolonged labelling times (Table IV).

Discussion

The purpose of this study was to determine the membrane sidedness of the two pathways involved in the renewal of arachidonic acid in platelet phospholipids, i.e., the acylation or Lands pathway and the exchange pathway. The methodology was based on the treatment of intact platelets with a combination of bee venom phospholipase A_2 and *S. aureus* sphingomyelinase C under non-lytic conditions. Such treatment is thought to degrade selectively all the phospholipids present on the platelet outer surface and led to the proposal of an asymmetric distribution of phospholipids between the two leaflets of the platelet plasma membrane [21,22].

The same experimental model was previously applied to the red cell membrane: after labelling the cells with either ^{14}C-labelled fatty acids (Lands pathway) or plasma lipoproteins bearing ^{32}P-labelled phospholipids (exchange pathway), Renooij et al. [24,25] found that only the [^{32}P]phospholipids were significantly accessible to the phospholipase degradation. It was thus concluded that the fatty acids were predominantly incorporated in the inner leaflet of the membrane, whereas the

TABLE IV

PHOSPHOLIPASE TREATMENT OF CHOLINE-LABELLED PLATELETS

Platelets were incubated with [^{3}H]- or [^{14}C]methylcholinephosphatidylcholine-labelled HDL for the times indicated. 1-Palmitoyl-2-arachidonoylphosphatidylcholine contained exclusively arachidonic acid at the C_2-position when chemically synthesized or a predominance of polyunsaturated species when biosynthetically prepared. Cells were further treated by exogenous phospholipases. Results are expressed as percent of labelled-PC hydrolysis and are the means (± S.E.) of three individual experiments or individual values.

Molecular species	Incubation time (min)	Hydrolysis of labelled phosphatidylcholine (%)
Dipalmitoylphosphatidylcholine	15	77 and 82
	30	73 and 81
	120	80 and 80
1-Palmitoyl-2-arachidonoylphosphatidylcholine		
chemically synthesized	15	79.5 ± 2.9
	120	56.5 ± 6.7
biochemically synthesized	30	56 and 69
	120	50 and 53

exchange process was taking place in the outer half of the membrane.

Phospholipases can be used as biochemical probes of membrane phospholipid sidedness, as long as no rapid transmembrane movement of phospholipids takes place. In this respect, it is important to recall that the transmembrane exchange of PC is very slow in the red cell membrane from human, whereas its half-time in the rat is about 7 h [39]. This is much longer than the incubation time with phospholipases used in the present study. As to the platelets, Bevers et al. [40] recently described a rapid transfer of phosphatidylserine from inner to outer leaflet occurring in the platelet membrane, under very specific conditions of activation with calcium ionophore or with a combination of thrombin plus collagen. This effect was certainly avoided in our study, since only resting platelets were used.

Our results clearly show that upon labelling platelet phospholipids with either oleic or arachidonic acid, most of the label remains inaccessible to the phospholipase degradation, indicating that the fatty acids are predominantly incorporated at the platelet inside, i.e., in the inner leaflet of the plasma membrane and (or) in other intracellular membranes. A preliminary experiment of subcellular fractionation revealed that about 60% of the label was located in the plasma membrane, whatever the fatty acid used. This could be explained by the presence in platelets of phospholipid-exchange protein [41].

As previously described, indomethacin was added to platelets during phospholipase treatment in order to avoid platelet aggregation. In this metabolic investigation, the addition of an excess of soybean lipoxygenase was also found to be necessary, since preliminary experiments had shown that some of the radioactive arachidonic acid released by phospholipase A_2 was reincorporated into phospholipids, mostly phosphatidylethanolamine. In the presence of exogenous lipoxygenase, this effect was no longer observed, since hydroxyl derivatives of arachidonic acid are poorer substrates for reacylation into platelet phospholipids than the parent fatty acid [42].

Upon labelling the platelets by the exchange with HDL of PC bearing [^{3}H]oleic or [^{3}H]arachidonic acid in the 2-position, phospholipase treatment revealed that only oleic acid remained highly accessible to the enzyme attack, whereas arachidonic acid displayed a more internal location. The latter fatty acid was also the only one displaying a redistribution to other phospholipids. A similar difference was previously reported by Béréziat et al. [18] between arachidonic and linoleic acid. At that time of the study, such a peculiar behaviour of 2-arachidonoylphosphatidylcholine could be suspected to have an artifactual origin. Indeed, around 1% of the total radioactivity of HDL preparations is present in the free fatty acid fraction. Probably due to a specific arachidonoyl-Co A synthetase [17], arachidonic acid is very actively incorporated into platelet phospholipids (about 10–15-times quicker than oleic acid, cf. Figs. 1B and 2B). So the labelling of other phospholipids and the more internal localization of [^{3}H]arachidonic acid might be due to those traces of non-esterified arachidonic acid present in all preparations used.

For that reason, most of the exchange experiments were performed using a double-labelling procedure by adding a tracer dose of ^{14}C-labelled fatty acid to the HDL suspension. For comparison with arachidonic acid, oleic acid was preferred to linoleic acid, which is only available with a ^{14}C label. This model should have allowed an estimation of the proportion of platelet phospholipids having incorporated [^{3}H]arachidonic acid via the Lands pathway, since the ^{14}C label could be used as an internal standard. However, such an estimation was revealed to be impossible since, as shown in Figs. 1C and 2C, there was a continuous generation of [^{3}H]arachidonic acid (as well as [^{3}H]oleic acid) from HDL-PC, so that the ratio $^3H/^{14}C$ in HDL-free fatty acids did not remain constant. Such a phospholipase A_2-like hydrolysis of HDL-PC was only found in the presence of platelets and never exceeded 3% of hydrolysis after a 120 min incubation. These cells are known to contain active phospholipases A_2 [2–6], but our data do not allow us to conclude whether PC was hydrolysed after transfer to the platelets or whether a phospholipase A_2 was released from the cells, as shown previously for sheep platelets [43].

Also, reincubation of platelets labelled after exchange revealed the same kind of phospholipase A_2 activity, which was found to be the most active

towards [^{14}C]oleoylphosphatidylcholine and towards [^{3}H]arachidonoylphosphatidylcholine, which still remains difficult to explain.

To avoid the drawback encountered with the fatty acid labelling of PC, the exchange experiments were repeated with [^{14}C]choline-labelled PC. In this case, both dipalmitoyl- and 1-palmitoyl-2-arachidonoylphosphatidylcholines showed an external location after a short incubation time (15 min). This evidences the fact that the previous experiments with [^{3}H]arachidonate-labelled PC led to an artifactual incorporation of the free fatty acid at the inside of the cells. However, upon longer labelling times, arachidonoylphosphatidylcholine showed a more internal location, whereas dipalmitoylphosphatidylcholine was always exposed to the cell exterior. This would indicate a translocation process to the inner leaflet of the platelet membrane specific for arachidonoylphosphatidylcholine. A more rapid flip-flop rate has been indeed described for the most unsaturated PC species in the rat erythrocyte membrane [25,26]. But in the latter case, phospholipid movement occurred in both senses, which is different from platelets. One cannot also exclude that the phospholipase A_2 previously revealed by the release of [^{3}H]oleic or arachidonic acid might have been more active towards arachidonoylphosphatidylcholine, the lysophosphatidylcholine formed being then reacylated at the inside of the platelets [44–46].

In conclusion, our study shows that, like in the erythrocyte, incorporation of fatty acids in all glycerophospholipids takes place at the inside of the platelet, whereas the exchange of intact PC molecules with the plasma lipoproteins involves the outer leaflet of the surface membrane. This allows elimination of a receptor-mediated internalization process of HDL as a mechanism of PC incorporation into platelets. This also provides evidence for the presence in the platelets of various phospholipid pools, as recently suggested by Holmsen et al. [47] and might account for the discrepancies encountered between various groups as to the role of PC, degraded by phospholipase A_2 [6] and the role of phosphatidylinositol, degraded by phospholipase C [7–10] and phospholipase A_2 [13], in the release of arachidonic acid upon platelet activation. The procedure used in most of the studies reported in the literature used arachidonic acid as a radioactive precursor, which leads to the labelling of only internal phospholipid pools.

This work also gives some indication that arachidonoylphosphatidylcholine species present on the platelet outer surface might be rapidly translocated to the inside of the cell by a mechanism which remains to be elucidated. Comparing our results on arachidonic acid distribution in the platelet membrane to those of Schick et al. [48] showing an identical fatty acid composition in external and internal platelet phosphatidylethanolamines, we have calculated that the 6% of total cell arachidonic acid present on the platelet surface should belong to the external phosphatidylethanolamine [49]. As a consequence, the PC molecules forming the external leaflet of the plasma membrane would be devoid of arachidonate. This is at variance with the erythrocyte, which has been shown to display a symmetrical distribution of PC molecular species between both halves of the membrane [26]. Because of its possible physiological significance, such a suspected non-random transverse distribution of PC molecular species in the platelet plasma membrane would justify further experimental work as well as the mechanism of a specific translocation of arachidonoylphosphatidylcholine to the platelet inside.

Our present results extend those of Béréziat et al. [18] showing that platelet-lipoprotein exchange of PC might represent a likely way for renewal of platelet arachidonate. They suggest that specific internalization of arachidonoylphosphatidylcholine, probably involving a deacylation step with a reacylation in other glycerophospholipids, might participate in maintaining arachidonate at the inside of the cell, essentially in the large phosphatidylethanolamine pool.

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References

1 Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2994–2998

2 Trugnan, G., Bérézíat, G., Manier, M.C. and Polonovski, J. (1979) Biochim. Biophys. Acta 573, 61–72
3 Apitz-Castro, R., Cruz, M., Mas, M. and Jain, M.K. (1981) Thromb. Res. 23, 347–354
4 Jesse, R.L. and Franson, R.C. (1979) Biochim. Biophys. Acta 575, 467–470
5 Kannagi, R., Koizumi, K. and Masuda, T. (1981) J. Biol. Chem. 256, 1177–1184
6 McKean, M.L., Smith, J.B. and Silver, M.J. (1981) J. Biol. Chem. 256, 1522–1524
7 Mauco, G., Chap, H., Simon, M.F. and Douste-Blazy, L. (1978) Biochimie 60, 653–661
8 Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580–587
9 Mauco, G., Chap, H. and Douste-Blazy, L. (1979) FEBS Lett. 100, 367–370
10 Bell, R., Kennerly, D.A., Stanford, N. and Majerus, P.W. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 3238–3241
11 Chau, L.Y. and Tai, H.H. (1981) Biochem. Biophys. Res. Commun. 100, 1688–1695
12 Billah, M.M., Lapetina, E.G. and Cuatrecasas, P. (1981) J. Biol. Chem. 256, 5399–5403
13 Billah, M.M. and Lapetina, E.G. (1982) J. Biol. Chem. 257, 5196–5200
14 Bills, T.K., Smith, J.B. and Silver, M.J. (1976) Biochim. Biophys. Acta 424, 303–314
15 Bills, T.K., Smith, J.B. and Silver, M.J. (1977) J. Clin. Invest. 60, 1–6
16 Chambaz, J., Bérézíat, G., Pépin, D. and Polonovski, J. (1979) Biochimie 61, 127–130
17 Wilson, D.B., Prescott, SM. and Majerus, P.W. (1982) J. Biol. Chem. 257, 3510–3515
18 Bérézíat, G., Chambaz, J., Trugnan, G., Pépin, D. and Polonovski, J. (1978) J. Lipid Res. 19, 495–500
19 Zwaal, R.F.A., Roelofsen, B. and Colley, C.M. (1973) Biochim. Biophys. Acta 300, 159–182
20 Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D. and Van Deenen, L.L.M. (1973) Biochim. Biophys. Acta 323, 178–183
21 Chap, H.J., Zwaal, R.F.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146–164
22 Perret, B., Chap, H.J. and Douste-Blazy, L. (1979) Biochim. Biophys. Acta 556, 434–446
23 Schick, P.K., Kurika, K.S. and Chacko, G.K. (1976) J. Clin. Invest. 57, 1221–1226
24 Renooij, W., Van Golde, L.M.G., Zwaal, R.F.A., Roelofsen, B. and Van Deenen, L.L.M. (1974) Biochim. Biophys. Acta 363, 287–292
25 Renooij, W., Van Golde, L.M.G., Zwaal, R.F.A. and Van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53–58
26 Renooij, W. and Van Golde, L.M.G. (1977) Biochim. Biophys. Acta 470, 465–474
27 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83–96
28 Waite, M. and Van Deenen, L.L.M. (1967) Biochim. Biophys. Acta 137, 498–517
29 Skipski, V.P., Peterson, F.F. and Barclay, M. (1964) Biochem. J. 90, 374–378
30 Chakrabarti, P. and Khorana, H.G. (1975) Biochemistry 14, 5021–5023
31 Ayrault-Jarrier, M., Levy, G., Wald, R. and Polonovski, J. (1963) Bull. Soc. Chim. Biol. 45, 349–360
32 Ardlie, N.G., Packham, M.A. and Mustard, J.F. (1970) Br. J. Haematol. 19, 7–17
33 Aster, R.H. and Jandl, J.H. (1964) J. Clin. Invest. 43, 843–855
34 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–918
35 Broeckhuyse, R.M. (1969) Clin. Chim. Acta 23, 457–461
36 Böttcher, C.J.F., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203–204
37 Wroblewski, F. and La Due, J.S. (1955) Proc. Soc. Exp. Biol. 90, 210–215
38 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
39 Van Meer, G., Poorthuis, B.J.H.M., Wirtz, K.W.A. Op den Kamp, J.A.F. and Van Deenen, L.L.M. (1980) Eur. J. Biochem. 103, 283–288
40 Bevers, E.M., Comfurius, P., Van Rijn, J.L.M.L., Hemker, H.C. and Zwaal, R.F.A. (1982) Eur. J. Biochem. 122, 429–436
41 Laffont, F., Chap, H., Soula, G. and Douste-Blazy, L. (1981) Biochem. Biophys. Res. Commun. 102, 1366–1371
42 Stenson, W.F. and Parker, C.W. (1979) Prostaglandins 18, 285–292
43 Vadas, P. and Hay, J.B. (1980) Life Sci. 26, 1721–1729
44 Cohen, P., Derksen, A. and Van den Bosch, H. (1970) J. Clin. Invest. 49, 128–139
45 Elsbach, P., Pettis, M. and Marcus, A. (1971) Blood 37, 675–683
46 Joist, J.H., Dolezel, G., Lloyd, J.V. and Mustard, J.F. (1976) Blood 48, 199–211
47 Holmsen, H., Dangelmaier, C.A. and Holmsen, H.K. (1981) J. Biol. Chem. 256, 9393–9396
48 Schick, P.K., Schick, B.P., Brandeis, G. and Mills, D.C.B. (1981) Biochim. Biophys. Acta 643, 659–662
49 Chap, H., Perret, B., Mauco, G. Plantavid, M., Laffont, F., Simon, M.F. and Douste-Blazy, L. (1982) Toxicon 20, 291–298
50 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–400