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ASYMMETRIC DISTRIBUTION OF ARACHIDONIC ACID IN THE PLASMA MEMBRANE OF HUMAN PLATELETS

A DETERMINATION USING PURIFIED PHOSPHOLIPASES AND A RAPID METHOD FOR MEMBRANE ISOLATION

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Summary

1. Non-lytic degradation of human platelet phospholipids have been performed using a combination of bee venom phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) and *Staphylococcus aureus* sphingomyelinase C (sphingomyelin choline phosphohydrolase). Under these conditions, 25.4% of total phospholipids are degraded and 6.4% of total platelet arachidonic acid is released.

2. A new method for rapid isolation of platelet plasma membrane is described, based on the use of [^{3}H]concanavalin A as a membrane marker and of self-generating gradients of Percoll. Plasma membranes are enriched 5.2 fold in lectin marker and 0.43 in *N*-acetyl-β-D-glucosaminidase, the main contaminant. This method allows to estimate that 57% of the total cell phospholipids and 61% of the total arachidonic acid content are located in the plasma membrane.

3. The distribution of phospholipids and arachidonic acid between the two leaflets of the plasma membrane has been deduced by using these values and those obtained from non-lytic treatment of intact platelets by phospholipases. It is concluded that 45% of plasma membrane phospholipids, comprising 93% of sphingomyelin, 45% of phosphatidylcholine, 9% of phosphatidylserine, 16% of phosphatidylinositol and 20% of phosphatidylethanolamine form the outer half of the human platelet plasma membrane. These phospholipids appear to bear only 10% of the total membrane arachidonic acid.

Introduction

Previous studies using purified phospholipases [1—3] or chemical labels [4,5] have shown an asymmetric distribution of phospholipids in both erythrocyte and platelet membranes. It has been suggested that membrane asymmetry of the blood cells might regulate haemostasis by keeping the procoagulant anionic phospholipids masked to the plasma coagulation factors [6,7].

Another consequence of the platelet membrane asymmetry might concern arachidonic acid. This fatty acid plays a key role in the platelet function as a precursor of thromboxanes and prostaglandins [8—11]. Because of its unique location in the phosphoglycerides, its conversion by cyclooxygenase depends on the activation of platelet phospholipase(s) [12,13]. A great heterogeneity is observed between different phospholipid classes when considering their arachidonic acid content [14—16]. It could thus be asked whether the non-random organization of phospholipids between the two leaflets of the platelet plasma membrane would not imply an asymmetric distribution of platelet arachidonic acid. Since this fatty acid is totally present in the 2-position of glycerophospholipids [14,17], extensive degradation of phospholipids from intact cells by phospholipase A_2 and sphingomyelinase C should release all the arachidonic acid present on the platelet surface. This experimental model was previously used to determine the phospholipid distribution in the plasma membrane of red cells [1,2] and of pig platelets [3].

Because of the presence of numerous intracellular organelles inside the platelet, the phospholipid distribution at the level of the plasma membrane itself can be determined only if one knows the proportion of total phospholipids located in the plasma membrane. For pig platelets, this calculation was performed by isolating membranes from sphingomyelinase C-treated and non-treated platelets [3]. But the human platelet membrane could not be investigated by the same procedure because of the too high requirement in human blood. Furthermore, most of the current methods reported so far for platelet plasma membrane isolation involve centrifugation steps on density gradients for times as long as 3—15 h (for a recent review, see Ref. 18). Such procedures might be unsuitable in certain lipid studies, owing to the risk of oxidation of long chain unsaturated fatty acids. Centrifugation times required for subcellular fractionation are much reduced when using self-generating gradients of silica particles coated with polyvinylpyrollidone (Percoll).

The present study deals with the determination of phospholipid and arachidonic acid distribution in the human platelet membrane. This is based on the use of non-lytic degradation of platelet phospholipids by phospholipases, coupled to the analysis by gas-liquid chromatography of the fatty acids released under this treatment. Furthermore, a simple and rapid method for isolation of human platelet plasma membranes is described. Specific labelling of the plasma membrane by tritiated concanavalin A is used to estimate which proportions of the total cell phospholipids and of the total arachidonic acid content are located in the membrane. These results allow to describe an asymmetric distribution of both phospholipids and arachidonic acid.

Materials and Methods

Purified phospholipase A_2 from bee venom and sphingomyelinase C from *Staphylococcus aureus* were kindly donated by Prof. R.F.A. Zwaal [19]. Eicosatetraynoic acid was a gift from Hoffman La Roche, Basel, Switzerland. [^{3}H]acetylconcanavalin A and 5-hydroxy[^{14}C]tryptamine creatine sulphate (serotonin) were from the Radiochemical Center, Amersham, U.K. Percoll (polyvinylpyrollidone-coated silica particles) was from Pharmacia Fine Chemicals, Uppsala, Sweden.

Platelet isolation

Human blood was collected from healthy volunteer donors using acid/citrate/dextrose as an anticoagulant [20]. Platelets were isolated by differential centrifugations and washed twice in a calcium-free Tyrode buffer (pH 6.5) according to Ardlie et al. [21]. They were finally suspended either in a Tyrode buffer for the incubations with phospholipases or in a Tris/KCl buffer for lysis and further fractionation. The whole procedure was performed at room temperature in siliconized glassware (Siliclad).

Treatment of intact platelets with phospholipases

Platelet suspensions (10 mg/ml, wet weight, corresponding to $5—5.5 \cdot 10^8$ cells/ml) were submitted to the sequential action of phospholipase A_2 from bee venom and sphingomyelinase C from *S. aureus* as previously described [3] with the following modifications: incubation medium was 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; platelets were preincubated for 10 min with eicosatetraynoic acid (30 μM) used as an antiaggregant and phospholipase A_2 from bee venom (14 IU/100 mg platelet) replaced the *Naja naja* enzyme.

After 60 min incubation, a part of the cell suspension was centrifuged at $1500 \times g$ for 8 min and the supernatant was assayed for lactate dehydrogenase activity. The pellets were used for phospholipid analysis.

Treatment of lysed platelets with phospholipase A_2

Platelets were suspended in a Tris-HCl buffer (25 mM, pH 7.35) containing 0.25 mM $CaCl_2$ and lysed by sonication (six times 10 s) as described by Baenziger and Majerus [22]. Incubations in the presence of bee venom phospholipase A_2 (28 IU/100 mg platelets) were carried out at 37°C for 90 min with gentle stirring.

Phospholipid analysis

Lipids were extracted according to Reed et al. [23] after inhibition of the phospholipase activities by 15 mM EDTA. Phospholipids were then separated by two dimensional thin-layer chromatography on Silicagel F 254-coated plates, 0.25 mm thickness (Merck) using the solvent of Skipski et al. [24] in the first direction and the solvent II of Anderson et al. [25] in the second direction. The different spots were analysed for their phosphorus content by a modification [26] of the Fiske and Subbarow method. The percentage of phospholipid

hydrolysis was determined using lysophosphatidylcholine + phosphatidylcholine as an internal standard.

Fatty acid analysis

For fatty acid analysis, appropriate volumes of the platelet suspensions were immediately submitted to lipid extraction [27] after inhibition of the phospholipase activities by EDTA (15 mM final concentration). The non-esterified fatty acids were isolated by thin-layer chromatography [28] and methylated as in [29]. For the measurement of total arachidonic acid content, the lipid extract of a non-incubated platelet suspension was directly submitted to the methylation procedure. In preliminary experiments comparing dipalmitoyl phosphatidylcholine and palmitic acid, it was verified that both esterified and non-esterified fatty acids were methylated to the same extent. Gas-liquid chromatography was performed at 170—195°C (2°C/min from the time of sample injection) with an Intersmat analyser (model IGC 12FL) on a column packed with 10% diethylene glycol succinate on 80—100 mesh chromosorb W, with a nitrogen flow rate of 30 ml/min. Arachidonic acid concentrations were calculated using heneicosanoic acid as internal standard. This was added to the suspensions just prior to lipid extraction. To avoid oxidation of unsaturated fatty acids, the whole procedure was carried out under nitrogen.

Platelet membrane preparation

Labelling of the cells by [^{14}C]serotonin and [^{3}H]concanavalin A. The platelet-rich plasma obtained by centrifugation of the total blood was incubated with [^{14}C]serotonin (40 nCi/ml) for 30 min at room temperature. Non-incorporated serotonin was removed by washing. Platelets suspended in the second washing buffer (12 mg/ml) were then labelled with tritiated concanavalin A (100 nCi/ml) for 40 min at room temperature. After discarding the excess of unbound concanavalin A by washing twice, the supernatants showed a very low and constant amount of residual labelled lectin (3—5% of a total). Total bound concanavalin A represented 45% of the lectin amount used for the labelling procedure.

Platelet disruption. Cells were finally suspended at a concentration of 28—30 mg/ml (wet weight) in cold Tris-HCl buffer (25 mM, pH 7.35) containing 100 mM KCl and 10 mM ATP/$CaCl_2$. This will be referred to as lysis buffer. The whole procedure was then carried out at 4°C. Platelets were sonicated three times 10 s as described by Baenziger and Majerus [22]. An aliquot of the whole homogenate was kept for enzyme, radioactivity and total lipid phosphorus measurements.

Subcellular fractionation. Homogenate was rid of cellular debris by centrifugation (1000 × *g* for 10 min). The supernatant was mixed with appropriate volumes of Percoll: usually 6 ml of supernatant were diluted with a mixture containing 10.32 ml of Percoll, 12 ml of twice-concentrated lysis buffer and 1.68 ml of water, giving a starting density of 1.05 and an osmolarity identical to that of the lysis buffer.

The centrifugation was performed in a fixed angle rotor (Beckman 60 Ti) at 79 000 × *g* for 15 min in cellulose nitrate tubes. Fractions of 2 ml were collected by pipetting from the top and immediately assayed for radioactiv-

ity. Those showing the highest content in radiolabelled lectin were diluted three times in the lysis buffer and centrifuged at 105 000 × *g* for 120 min. By this way, Percoll beads sedimented and formed a cushion at the bottom of the tube, while biological material remained collected and lightly adhesive at the surface of this cushion. Supernatants were removed and membranes were resuspended by gentle sucking and blowing using a Pasteur capillary pipette. The Percoll cushion remained stuck to the tube.

Radioactivity, enzyme and protein assays

Radioactivity was determined with a Packard liquid scintillation spectrometer (model 544) with automatic quenching correction, using Instagel/toluene (50/50, v/v) as a scintillation liquid.

Lactate dehydrogenase was measured as previously described [3] using the method of Wroblewski and La Due [30]. *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) was assayed according to Day et al. [31] and the same method was applied to the determination of phosphodiesterase (EC 3.1.4.1) activity using bis(*p*-nitrophenyl)phosphate (Sigma) as a substrate and 0.1 M ammonium acetate buffer as incubation medium. Succinate : cytochrome *c* reductase (EC 1.3.99.1) was assayed by the method of Tisdale [32]. Protein was determined by the method of Lowry et al. [33] using bovine serum albumin as standard.

Results

Arachidonic acid release from phospholipase-treated platelets

The composition of fatty acids released from platelet phospholipids upon non-lytic treatment by phospholipase A_2 and sphingomyelinase C is given in Table I. As expected, most of them are unsaturated since they are removed from the 2-position of phosphoglycerides through the phospholipase A_2 action. However, the presence of significant amounts of palmitic and stearic acids (14.7% and 9.2%, respectively) indicates that disaturated phospholipid species should be present on the platelet surface. Noteworthy, low amounts of such

TABLE I

COMPOSITION OF FATTY ACIDS RELEASED DURING NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 AND SPHINGOMYELINASE C IN INTACT PLATELETS

Results are the mean (±S.D.) of four experiments and represent the molar percentage of total fatty acids released.

Fatty acids	Percentage of total
16 : 0	14.7 (±2.9)
16 : 1	13.5 (±5.0)
18 : 0	9.2 (±2.1)
18 : 1	14.3 (±1.1)
18 : 2	10.3 (±3.7)
18 : 3	4.0 (±1.4)
20 : 4	15.0 (±4.7)
22 : 1	8.5 (±3.7)
Others	10.3 (±3.0)

TABLE II

ARACHIDONIC ACID RELEASE DURING NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 AND SPHINGOMYELINASE C IN INTACT PLATELETS

The values are expressed in nmol/10^9 platelets. The numbers in parenthesis refer to the percentage of total platelet arachidonic acid.

Experiment number	Total arachidonic acid	Arachidonic acid released	
		Control cells	Phospholipase-treated cells
1	210	2.1 (1.2%)	17.3 (8.2%)
2	210	1.9 (0.9%)	14.0 (6.7%)
3	208	1.2 (0.6%)	8.8 (4.2%)
4	160	2.6 (1.6%)	13.0 (8.1%)
5	180	traces	8.2 (4.6%)

molecular species have been already reported in the red cell membrane by Van Golde et al. [34].

Because of the strong specificity of phospholipase A_2, a direct comparison between accessible and non-accessible fatty acids can be undertaken only for those showing an exclusive location in the 2-position of glycerophospholipids. This is the case of arachidonic acid and the results of Table II show that this fatty acid is scarcely released during non-lytic incubations with phospholipases (6.4% ± 1.9, mean ± S.D. of total). In the same time, 25.4% of total platelet phospholipids, containing essentially sphingomyelin and phosphatidylcholine, were hydrolysed by phospholipases under these conditions (see also further Table V).

On the opposite, in the gas-liquid chromatograms of total phospholipids isolated from lysed cells treated by bee venom phospholipase A_2, the arachidonic acid peak had disappeared or was hardly detectable and could not be quantified.

Platelet membrane purification

Upon short sonication periods, almost total lysis of platelets was performed (95% of lactate dehydrogenase became soluble). However, intracellular organelles were relatively preserved from disruption, since only 9.5% and 18.2% of *N*-acetyl-β-D-glucosaminidase and serotonin, respectively, appeared in the supernatants. When considering membrane markers, the amount of uncoupled concanavalin A slightly increased after cell lysis (11% versus 5% in intact cells), while 27.5% of solubilization was measured for phosphodiesterase.

A preliminary fractionation was then necessary to get rid of cellular debris. After centrifugation at $1000 \times g$ for 10 min, the supernatant was slightly enriched in membrane material, since 44% of concanavalin A was recovered, versus 27% and 25% for *N*-acetyl-β-D-glucosaminidase and serotonin, respectively.

This supernatant was then mixed with appropriate volumes of Percoll. In this technique, the beads of colloidal silica sediment in the gravity field according to their size. By centrifugation in a fixed-angle rotor, one can obtain self-generating density gradients, where each subcellular particle equilibrates at its own density [35].

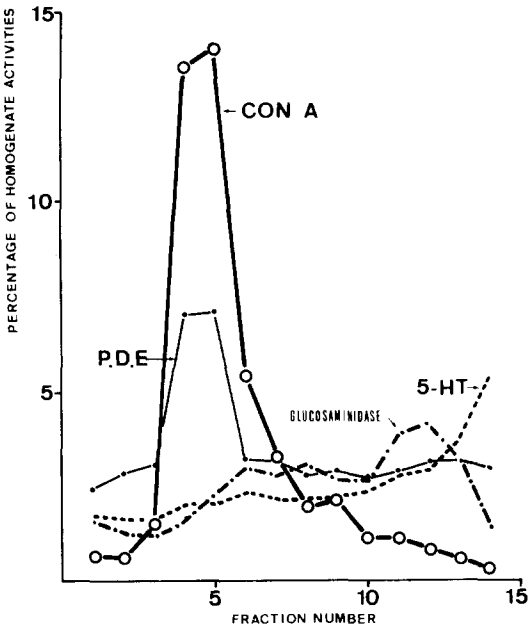

Fig. 1. Distribution of tritiated concanavalin A, serotonin and enzymatic activities on Percoll gradients. The different values for each marker are represented as percentages of total homogenate activities and are the results from a typical experiment. Similar profiles were obtained in eight experiments. Abbreviations: Con A, [^{3}H]concanavalin A; 5-HT, [^{14}C]serotonin; P.D.E., phosphodiesterase; glucosaminidase, *N*-acetyl-β-D-glucosaminidase.

Fig. 1 represents the partition of different markers along such a gradient. As shown by concanavalin A and phosphodiesterase, membranes equilibrated with a sharp focusing at the lowest densities and were found in two fractions (generally 4 and 5). On the opposite, serotonin was located at the bottom of the tube. *N*-Acetyl-β-D-glucosaminidase showed a major peak in the heaviest fractions and a slighter increase in fraction 5 giving some contamination of membrane material. Because of the need of highly pure plasma membranes in our studies, usually only the lightest non-contaminated membrane fraction (No. 4) was kept for further analysis. Noteworthy, at a variance with other conventional methods, the lactate dehydrogenase activity was distributed all over the gradient (not shown here), since the homogenate was uniformly mixed with Percoll medium.

The purest membrane fraction was then separated from both Percoll beads and soluble material by centrifugation. The relative specific activities of several markers in this fraction are summed up in Table III. Membranes exhibit a 5.2 fold enrichment in lectin. The relative specific activity is lower for phosphodiesterase, which was more solubilized. On the contrary, contaminations by succinate : cytochrome *c* reductase, *N*-acetyl-β-D-glucosaminidase and serotonin were not significant or very low.

An additional support in favor of membrane purity is brought by the phospholipid composition, since the molar ratio of sphingomyelin, a major phos-

TABLE III

RELATIVE ACTIVITIES OF ENZYMES, LECTIN AND SEROTONIN IN PLASMA MEMBRANES

Results are expressed as mean ± S.D. For further details, see the text. n.d., not detectable.

Marker	Number of experiments	Homogenate	Plasma membranes	Relative specific activities
[^{3}H]Concanavalin A	4	35 840 * (± 2835)	187 100 * (± 28 000)	5.2
N-Acetyl-β-D-glucosaminidase	4	1.09 ** (± 0.19)	0.47 ** (± 0.21)	0.43
[^{14}C]Serotonin	4	40 536 * (± 10 490)	865 * (± 1240)	0.02
Phosphodiesterase	3	0.097 ** (± 0.022)	0.28 ** (± 0.05)	2.9
Succinate: cytochrome *c* reductase	2	0.25 **	n.d.	n.d.

* dpm/mg.
** μmol/h per mg.

pholipid in plasma membranes [36], increases from 17.8% in the whole platelet to 23.4% in the membrane (Table V).

Phospholipid distribution in the plasma membrane

Concanavalin A, used as a membrane marker, provides a good reference to estimate the phospholipid content of the plasma membrane. So, for previously labelled homogenized cells, one can easily determine the amount of lectin bound/μmol of total phospholipids (expressed as *A* in dpm/μmol, in Table IV). The same is done with purified plasma membranes (*B*). The ratio *A*/*B* represents the percentage of total phospholipids present in the plasma membrane, which is 57% for human platelets.

Using this ratio and the data from non-lytic degradation, one is able to determine the percentage of hydrolysis inside the plasma membrane for each phospholipid class. This can be done because all the results are expressed in percentages of total phospholipids and details of this method are reported elsewhere [3]. For instance, sphingomyelin hydrolysis represents 12.4% of total platelet phospholipids in intact cells. The corresponding degradation in the plasma

TABLE IV

ESTIMATION OF THE PHOSPHOLIPID CONTENT OF THE PLASMA MEMBRANE

The values *A* and *B* are expressed in dpm/μmol of phospholipids and are the mean ± S.D. of eight experiments.

$$A = \frac{\text{Membrane-bound concanavalin A}}{\text{Total platelet phospholipids}} = 142\,800 \text{ dpm/μmol } (\pm 24\,360)$$

$$B = \frac{\text{Membrane-bound concanavalin A}}{\text{Total membrane phospholipids}} = 250\,900 \text{ dpm/μmol } (\pm 29\,600)$$

$$\frac{A\text{: Total membrane phospholipids}}{B\text{: Total platelet phospholipids}} = 0.57$$

TABLE V

NON-LYTIC DEGRADATION OF PHOSPHOLIPIDS BY PHOSPHOLIPASE A_2 (BEE VENOM) AND SPHINGOMYELINASE C (*S. AUREUS*) IN HUMAN PLATELETS

The values are expressed as percentage of total phospholipid (mean ± S.D.). The numbers in parentheses refer to the percent degradation of the corresponding phospholipid classes. Results are from six experiments.

	Total platelet		Plasma membrane	
	Phospholipid composition	Hydrolysis	Phospholipid composition	Hydrolysis *
Sphingomyelin	17.8 ± 0.8	12.4 ± 0.8	23.4 ± 0.7	21.7 (92.7)
Phosphatidylcholine	38.4 ± 1.6	9.0 ± 1.4	35.0 ± 1.7	15.8 (45.1)
Phosphatidylserine	11.2 ± 0.9	0.5 ± 0.7	9.9 ± 0.2	0.9 (9.1)
Phosphatidylinositol	5.0 ± 1.3	0.5 ± 0.3	5.6 ± 0.6	0.9 (16.0)
Phosphatidylethanolamine	27.6 ± 2.0	3.0 ± 0.8	26.1 ± 1.4	5.3 (20.3)
Total phospholipid	100	25.4	100	44.6

* Calculated as described in the text and in Ref. 3.

membrane is given by the ratio 12.4/0.57 and is thus equal to 21.7% of total membrane phospholipids. As sphingomyelin represents 23.4% of total membrane phospholipids, the percentage of degradation of this class in the plasma membrane is thus 92.7%. This was applied to each phospholipid class and the results were compared to the phospholipid composition of the human platelet plasma membrane (results are summarized in Table V). It is concluded that the phospholipids degraded under non-lytic conditions by phospholipases represented nearly the half of membrane phospholipids. This fraction was essentially made of sphingomyelin and phosphatidylcholine, while anionic phospholipids remained masked to the phospholipase action.

Arachidonic acid partition in the plasma membrane

The same method of calculation as used for membrane phospholipid determination has been applied to estimate which proportion of total arachidonic acid is present in the plasma membrane. This is done by measuring the arachidonic acid content in both purified plasma membranes and whole homogenate. The results indicated a slightly higher specific content of this fatty acid in the membrane fraction when compared to the homogenate (0.403 μmol ± 45/μmol of phospholipids versus 0.376 μmol ± 28/μmol of phospholipids, respectively). So we can estimate at 61% the proportion of total arachidonic acid belonging to the plasma membrane. By substracting the 6.4% of total cell arachidonic acid released by phospholipase A_2 under non-lytic conditions, one can conclude that nearly 90% of the platelet membrane arachidonic acid is spared in the inner leaflet.

Discussion

Taking advantage of the exclusive location of arachidonic acid in the 2-position of glycerophospholipids, the cellular distribution of this fatty acid could be investigated by measuring the amounts of arachidonic acid released upon

non-lytic degradation of platelet phospholipids by bee venom phospholipase A_2 and *S. aureus* sphingomyelinase C. As no further hydrolysis occurs upon more prolonged incubations, and as the remaining phospholipids become available to enzymatic attack only upon cell lysis, this method is thought to discriminate between interior and exterior phospholipids [1—3].

So the 6% of total arachidonic acid released under these conditions should represent the fraction of this fatty acid located on the platelet surface, since all of it becomes accessible to phospholipase A_2 in lysed platelets. Total recovery of free arachidonic acid was enabled by the use of eicosatetraynoic acid, which impairs any transformation of arachidonic acid by cyclooxygenase and lipooxygenase [8]. Noteworthy, the corresponding degradation of total phospholipids in intact cells was the same (25%) as previously reported [3].

The validity of this experimental model has been questioned by several authors [37—39]. However, the results of phospholipid degradation obtained with intact human erythrocytes using purified phospholipases are generally concordant [1,2,19,40—42], whereas yet unexplained discrepancies exist for the microsomal membranes [38,43—45]. In the case of platelets, our findings are in good agreement with those of Schick et al. [5], based on the use of trinitrobenzene sulphonic acid as a chemical probe for aminophospholipids. Contradictory data were obtained by Otnaess and Holm [46] and the possible reasons for such a discrepancy have been already discussed [3].

One must be aware that phospholipids and arachidonic acid not exposed to phospholipase attack in intact platelets belong to both inner leaflet of the plasma membrane and to other intracellular membranes. So, to determine their distribution at the level of the plasma membrane itself, it is necessary to evaluate their respective proportion present in this membrane. The method described in this study allowed a rapid isolation of purified platelet plasma membrane. As previously observed [3,22], the homogenization procedure by short sonication periods provides an almost total cell lysis without altering significantly intracellular organelles. The presence of CaATP in the lysis buffer improved the recovery of membrane material in the $1000 \times g$ supernatant. Interestingly, Abramowitz et al. [47] reported that 10 mM CaATP causes dissociation of both actomyosin complexes and actin-like protein polymers, and our observation might indicate that depolymerization of contractile proteins might help to a more efficient platelet disruption. The $1000 \times g$ centrifugation step appeared to be essential to reach a good purity in membrane fractions and it avoided occasional clumping occurring on the Percoll gradients upon fractionation of a whole homogenate.

Radioactive concanavalin A provides a good and convenient membrane marker since it specifically binds to membrane glycoproteins [48] and since it remains almost entirely particulate after sonication. However, this is only valid if no redistribution of concanavalin A occurs between plasma membrane and cellular organelles during the membrane isolation. This possibility appears rather unlikely, since concanavalin A is present on the Percoll gradient as a single homogeneous peak. This nicely coincides with the phosphodiesterase peak, which has been previously described as a good plasma membrane marker [49,50]. Actually, any concanavalin A redistribution would imply the presence of minor peaks, corresponding to granular markers.

The different enrichments shown in Table III are very comparable to those obtained with other methods [49—51]. As discussed by Sixma and Lips [18], a maximal purification factor of 5 should be expected during platelet membrane isolation. If one assumes that the purified membrane fraction is representative of the whole platelet membrane, our calculation method gives a good estimation of the total platelet phospholipids and arachidonic acid present in the plasma membrane. Concerning phospholipids, similar results were obtained with pig platelets by other methods [3]. This estimation allows to describe the distribution of phospholipids and arachidonic acid between the two halves of the plasma membrane. This is highly asymmetric for all the phospholipids, except phosphatidylcholines, which are almost equally shared between the two leaflets. This distribution essentially resembles that previously reported for human erythrocytes [1,2] and pig platelets [3]. It might explain the selective location of arachidonic acid in the membrane inside, since the arachidonate-rich phospholipid classes (phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol) are mainly situated at the internal face of the membrane. Noteworthy, such a partition had been suggested by Derksen and Cohen [52] but no direct experimental evidence was yet available. A preferential location of tetraenoic phosphatidylcholine species at the inside of the membrane might be unprobable, as already shown for the red cell membrane by Renooij and van Golde [53]. Although our results only concern arachidonic acid, they may be compared to those of Sandra and Pagano [54], who showed a predominance of unsaturated fatty acids in the inner leaflet of LM cell membranes.

Concluding remarks

The present study gives another example of phospholipid asymmetry in a biomembrane. In spite of differences coming from the phospholipid composition or the specialization of each kind of membrane, the phospholipid disposition in membranes of flowing blood cells seems to obey to a general rule: one of the main character is the almost exclusive location of anionic phospholipids in internal structures. Evidence has been recently given for the role displayed by phospholipid asymmetry in the regulation of blood coagulation [6,7].

Another physiological meaning of phospholipid asymmetry appears in the present study. It is shown that arachidonic acid is mostly located at the inside of the cell, in both intracellular membranes and in the inner leaflet of the plasma membrane. One could thus expect that the arachidonic acid used as a precursor for prostaglandin and thromboxane synthesis is coming from intracellular phospholipids. As histochemical studies have located the site of prostaglandin formation in the dense tubular system [55], the particular distribution of arachidonic acid in internal structures might enable a more efficient coupling between the release and the transformation of arachidonic acid.

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References

1 Zwaal, R.F.A., Roelofsen, B. and Colley, C.M. (1973) Biochim. Biophys. Acta 300, 159—182
2 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
3 Chap, H., Zwaal, R.F.A. and Van Deenen, L.L.M. (1977) Biochim. Biophys. Acta 467, 146—164
4 Bretscher, M.S. (1972) J. Mol. Biol. 71, 523—528
5 Schick, P.K., Kurica, K.B. and Chacko, G.K. (1976) J. Clin. Invest. 57, 1221—1226
6 Zwaal, R.F.A., Comfurius, P. and Van Deenen, L.L.M. (1977) Nature 268, 360-362
7 Zwaal, R.F.A. (1978) Biochim. Biophys. Acta 515, 163—205
8 Hamberg, M. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3400—3404
9 Hamberg, M., Svensson, J. and Samuelsson, B. (1974) Proc. Natl. Acad. Sci. U.S. 71, 3824—3828
10 Hamberg, M., Svensson, J. and Samuelsson, B. (1975) Proc. Natl. Acad. Sci. U.S. 72, 2994—2998
11 Moncada, S., Gryglewski, R., Bunting, S. and Vane, J.R. (1976) Nature 263, 663—665
12 Bills, I.K., Smith, J.B. and Silver, M.J. (1976) Biochim. Biophys. Acta 424, 303—314
13 Pickett, W.C. and Cohen, P. (1976) J. Biol. Chem. 251, 2536—2538
14 Marcus, A.J., Ullman, H.L. and Safier, L.B. (1969) J. Lipid Res. 10, 108—114
15 Nordöy, A. and Lund, S. (1968) Scand. J. Clin. Lab. Invest. 22, 328—338
16 Cohen, P. and Derksen, A. (1969) Br. J. Haematol. 17, 359—371
17 Iacono, J.M., Zellner, D.C., Paoletti, R., Ishikawa, T., Frigeni, V. and Fumigalli, R. (1973—1974) Haemostasis 5, 165—175
18 Sixma, J.J. and Lips, J.M.M. (1978) Thromb. Haemostasis 39, 328—337
19 Zwaal, R.F.A., Roelofsen, B., Comfurius, P. and Van Deenen, L.L.M. (1975) Biochim. Biophys. Acta 406, 83—96
20 Aster, R.H. and Jandl, J.H. (1964) J. Clin. Invest. 43, 843—855
21 Ardlie, N.G., Packham, M.A. and Mustard, J.F. (1970) Br. J. Haematol. 19, 7—17
22 Baenziger, N.L. and Majerus, P.W. (1974) Methods Enzymol. 31, 149—155
23 Reed, C.F., Swisher, S.N., Marinetti, G.V. and Ede, G.V. (1960) J. Lab. Clin. Med. 56, 281—289
24 Skipski, V.P., Peterson, R.F. and Barclay, M. (1964) Biochem. J. 90, 374—378
25 Anderson, R.E., Maude, M.B. and Feldman, G.L. (1969) Biochim. Biophys. Acta 187, 345—353
26 Böttcher, C.J.F., Van Gent, C.M. and Pries, C. (1961) Anal. Chim. Acta 24, 203—204
27 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—918
28 Derksen, A. and Cohen, P. (1973) J. Biol. Chem. 248, 7396—7403
29 Stoffel, W., Chu, F. and Ahrens, E.H. (1959) Anal. Chem. 31, 307—308
30 Wroblewski, F. and La Due, J.S. (1955) Proc. Soc. Exp. Biol. 90, 210—215
31 Day, H.J., Holmsen, H. and Hovig, T. (1969) Scand. J. Haematol. Suppl. 7, 3—35
32 Tisdale, H.D. (1967) Methods Enzymol. 10, 213—215
33 Lowry, O.H., Rosebrough, N.J., Farr, N.L. and Randall, N.J. (1951) J. Biol. Chem. 193, 265—275
34 Van Golde, L.M.G., Tomasi, V. and Van Deenen, L.L.M. (1967) Chem. Phys. Lipids 1, 282—293
35 Pertoft, H., Laurent, T.C., Laas, T. and Kagedal, L. (1978) Anal. Biochem. 88, 271—282
36 Emmelot, P., Bos, C.J., van Hoeven, R.P. and van Blitterswijk, W.J. (1974) Methods Enzymol. 31, 75—90
37 Martin, J.K., Luthra, M.G., Wells, M.A., Watts, R.P. and Hanahan, D.J. (1975) Biochemistry 14, 5400—5408
38 Sundler, R., Sarcione, S.L., Alberts, A.W. and Vagelos, P.R. (1977) Proc. Natl. Acad. Sci. U.S. 74, 3350—3354
39 Adamich, M. and Dennis, E.A. (1978) J. Biol. Chem. 253, 5121—5125
40 Gul, S. and Smith, A.D. (1974) Biochim. Biophys. Acta 367, 271—281
41 Kahlenberg, A., Waker, C. and Rohrlick, R. (1974) Can. J. Biochem. 52, 803—806
42 Haest, C.W.M. and Deuticke, B. (1976) Biochim. Biophys. Acta 436, 353—365
43 De Pierre, J.W. and Dallner, G. (1975) Biochim. Biophys. Acta 415, 411—472
44 Higgins, J.A. and Dawson, R.M.C. (1977) Biochim. Biophys. Acta 470, 342—356
45 Van de Besselaar, A.M.H.P., de Kruijff, B., van den Bosch, H. and van Deenen, L.L.M. (1978) Biochim. Biophys. Acta 510, 242—255
46 Otnaess, A.B. and Holm, T. (1976) J. Clin. Invest. 57, 1419—1425
47 Abramowitz, J., Stracher, A. and Detwiler, T. (1972) Biochem. Biophys. Res. Commun. 49, 958—963
48 Kaplan, K.L. and Nachman, R.L. (1975) Thromb. Res. 7, 847—859

49 Barber, A.J. and Jamieson, G.A. (1970) J. Biol. Chem. 245, 6357—6365
50 Taylor, D.G. and Crawford, N. (1976) Biochim. Biophys. Acta 426, 688—696
51 Rittenhouse-Simmons, S. and Deykin, D. (1976) Biochim. Biophys. Acta 426, 688—696
52 Derksen, A. and Cohen, P. (1975) J. Biol. Chem. 250, 9342—9347
53 Renooij, W. and van Golde, L.M.G. (1977) Biochim. Biophys. Acta 470, 465—474
54 Sandra, A. and Pagano, R.E. (1978) Biochemistry 18, 332—338
55 Gerrard, J.M., White, J.G., Rao, G.H.T. and Townsend, D.W. (1976) Am. J. Pathol. 83, 283—297