Turnover of phospholipid linoleic and arachidonic acids in human platelets from plasma lecithins

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Abstract Incubation of platelet-rich plasma with high density lipoproteins labeled either with 1-acyl-2-[1-^{14}C]-arachidonoyl-*sn*-glycero-3-phosphocholine or 1-acyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine showed the existence of a pool of phosphatidylcholine in platelets, which rapidly exchanges with the phosphatidylcholine in plasma. The labeled linoleic and arachidonic acids from the respective labeled phosphatidylcholines were found in the other glycerophospholipids. These transacylation processes were much more active with the arachidonoyl-labeled phosphatidylcholine than with linoleoyl-labeled phosphatidylcholine. Arachidonic acid was mainly found in the phosphatidylinositol.

Supplementary key words phosphatidylcholine exchange

In human platelets, arachidonic acid is the major fatty acid (25–35% of total fatty acids (1)); it represents about 42%, 32%, 23%, and 12% of the fatty acids of PI, PE, PS, and PC, respectively (2). Hamberg et al. (3, 4) showed that arachidonic acid was the precursor of the thromboxanes, powerful platelet aggregating agents, and we showed that local thromboses were followed by an important drop in platelet arachidonic acid level (5). These observations underline the major role of arachidonic acid in platelet physiology. Thus, the mechanisms involved in its release and the constitution of its cellular pool are of primary importance.

Numerous data have already shown that palmitic, stearic, oleic, and linoleic acids can be used by platelets (6–10). Recently, Bills, Smith, and Silver (10, 11) showed that arachidonic and 8,11,14-eicosatrienoic acids were preferentially taken up by platelets. Platelets are able to synthesize fatty acids from acetate de novo (6), since they have the necessary malonyl CoA synthetase and fatty acid synthetase (12). [^{14}C]Glycerol was preferentially incorporated into PA, PI, and PC (13). Several studies showed that de novo synthesis of phospholipids was much slower than fatty acid incorporation (6, 8). The eventuality of a deacylation–reacylation cycle was then taken into account (8). The observation by Derksen and Cohen (14) that platelet membranes incubated at 37°C at pH 9.5 with 10 mM Ca^{2+} were able to release arachidonic acid suggested the existence of a phospholipase A_2. Jesse and Cohen (15) suggested that PE was the arachidonic acid precursor; however thrombin is able to increase the release of arachidonic acid specifically from PC and PI (11, 16). Our preliminary work showed that, in human platelet homogenates, 1-[1-^{14}C]stearoyl-2-acyl-*sn*-glycero-3-phosphocholine was hydrolyzed to give the 1-[1-^{14}C]-stearoyl-*sn*-glycerol-3-phosphocholine (17). Human platelets are able to reacylate lysolecithin (18). This is of particular importance in view of the maintenance of a low level of free arachidonic acid in platelets. Lysolecithins, and especially 1-acyl-lysolecithins, inhibit aggregation (19, 20). Due to the uptake of plasma phospholipids, 35% of lysolecithin and 25% of lecithin of rabbit platelets appear to be renewed within 6 hr (21).

In the present study an exchange between plasma 2-linoleoyl-lecithin and 2-arachidonoyl-lecithin and those of platelets and the preferential utilization by 2-arachidonoyl-lecithin for the transacylation processes will be demonstrated.

MATERIALS AND METHODS

Materials

Egg phosphatidylcholine (PC), 99% pure, was purchased from Serva (Heidelberg). Arachidonic acid,

Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LDL, low density lipoprotein; HDL, high density lipoprotein; PRP, platelet-rich plasma; PFP, platelet-free plasma; PPO, 2,5-diphenyloxazole; POPOP, 1,4-di[2-(5-phenyloxazolyl)]-benzene; DEGS, diethylglycolsuccinate; 2-arachidonoyl-lecithin, 1-acyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; 2-linoleoyl-lecithin, 1-acyl-2-linoleoyl-*sn*-glycero-3-phosphocholine.

ATP, coenzyme A, and *Crotalus* venom phospholipase A_2 were from Sigma Chemical Co (St Louis, MO). TLC plates (F.1500) were from Schleicher and Schüll (FRG). Linoleic and stearic acids were from Calbiochem (San Diego, CA). [1-^{14}C]Stearic acid (51 mCi/mmol) was obtained from the Commissariat à l'Energie Atomique (Gif s/Yvette). The above mentioned fatty acids were 99% pure. [1-^{14}C]Linoleate (61 mCi/mmol) and [1-^{14}C]arachidonate (55 mCi/mmol) were purchased from Amersham (Arlington Heights, IL) at 98% purity. Unopettes were from Becton Dickinson (Rutherford, NJ).

Synthesis of 1-acyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine (2-[1-^{14}C]linoleoyl-lecithin) and 1-acyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (2-[1-^{14}C]arachidonoyl-lecithin)

Egg lecithin was hydrolyzed by means of snake venom phospholipase A_2 and reacylated with either [1-^{14}C]linoleic acid or [1-^{14}C]arachidonic acid using rat liver microsomes (22, 23). The specific activity of 1-^{14}C-labeled fatty acids was adjusted with the appropriate amounts of unlabeled arachidonic or linoleic acids. Lipids were then extracted (24), chromatographed on a silicic acid column (25), and stored in chloroform under N_2.

After treatment of the labeled phosphatidylcholine with snake venom, it was confirmed that the degree of labeling was 95% in position 2. Gas–liquid chromatography indicated that linoleic and archidonic acids represented, respectively, 48% and 49% of total fatty acids of labeled lecithins. The specific radioactivity of 2-[1-^{14}C]arachidonoyl-lecithin and 2-[1-^{14}C]linoleoyl-lecithin were, respectively, 5.8×10^6 dpm/μmol and 16×10^6 dpm/μmol.

Labeling of HDL by specific lecithins

High density lipoproteins (HDL) were prepared according to Ayrault-Jarrier et al. (27). 2-[1-^{14}C]-Linoleoyl-lecithin (23 μmol) or 2-[1-^{14}C]arachidonoyl-lecithin (16 μmol), after evaporation of the chloroform, were resuspended in 100 μl of ether–methanol 1:1 (v/v). Two ml of phosphate buffer (22 mM) was added and the phospholipids were sonicated. After elimination of solvents in vacuo, 13 mg/ml HDL was added in the same buffer. Incubation was carried out for 1 hr at 37°C. HDL was then sedimented for 18 hr at 105,000 *g* and collected in the lower 1.4 ml of the centrifuge tube. The fatty acids and lysoderivatives formed during the incubation from the lecithins were present in the supernatant solution. Purity of HDL was controlled by a radioautogram of a two-dimensional immunoelectrophoretogram (28). Radioactivity was found at the level of precipitating lines. Moreover, 90% of the radioactivity and 90% of lipid phosphorus were found associated with the HDL; all radioactivity was recovered in PC. This confirms that there was incorporation and not simply an exchange. Such preparations of labeled HDL contained 9 mg/ml of protein and either 12 μmol/ml of 2-arachidonoyl-lecithin or 18 μmol/ml of 2-linoleoyl-lecithin.

Platelet preparation

The Centre National de Transfusion Sanguine provided us with fresh blood collected from young male donors (20–30 years, A+ group) exhibiting normal platelet aggregation. None of the donors was given any drug known to affect plasma or platelet lipid metabolism. Samples used were those having normal cholesterol, triglyceride, and phospholipid levels with a normal lipoprotein electrophoresis pattern.

Blood samples were collected in anticoagulant ACD solution, as described by Aster and Jandl (29). All experiments were carried out using either plastic vials or silicone-treated flasks. The platelet-rich plasma (PRP) was obtained after centrifugation at 100 *g* for 10 min. Platelets were pelleted at 2000 *g* for 10 min at 15°C. The pellet was then washed twice with 15 ml of 15 mM Tris buffer, pH 7.4; NaCl, 0.15 M; KCl, 5 mM; and EDTA, 1.5 mM. Platelets were counted after a 100-fold dilution in a Malassez cell. Proteins were measured according to Lowry et al. (34) and lipids were extracted for analysis after the final washing.

Incubation procedure

In order to study the exchange of lecithins between plasma and platelets, 0.1 ml of either 2-[1-^{14}C]arachidonoyl-lecithin-labeled HDL or [1-^{14}C]linoleoyl-lecithin-labeled HDL was added to 5 ml of PRP. Specific radioactivity in PRP of 2-arachidonoyl-lecithin was 2.95×10^6 dpm/μmol (0.49 μmol/ml). For 2-linoleoyl-lecithin specific radioactivity was 6.98×10^6 dpm/μmol (1.06 μmol/ml). Incubations were carried out at 37°C with mild agitation in plastic vials in order to avoid platelet aggregation. PRP content was 1.7×10^8 platelets/ml, i.e., 0.25 mg of platelet proteins. After the indicated time of incubation, the vials were cooled to 0°C. Platelets were not aggregated as seen by phase contrast microscopy. The vials were immediately centrifuged at 4°C for 15 min at 2000 *g*. Platelets were then washed and pelleted, as described above.

Lipid extraction and analysis

Platelet pellets were extracted by chloroform–methanol 2:1 (v/v). Plasma samples were extracted

using the technique of Bligh and Dyer (30). Phospholipids were chromatographed on thin-layer silica gel G plates. When one-dimensional chromatograms were run, the plates were developed in chloroform–methanol–water 65:25:4 (v/v). For two-dimensional separation, the plates were first developed as described above. The plates were then redeveloped as described by Dod and Gray (31) using tetrahydrofuran–methylal–methanol–2 N NH_4OH 60:30:30:6 (v/v) in the second dimension.

Neutral lipids were separated as described by Skipski (32) on silica gel plates. The first migration was run for 10 cm using petroleum ether–diethyl ether–acetic acid 90:10:1 (v/v). After drying the plate in vacuo, plates were developed in the same direction for 15 cm with isopropyl ether–acetic acid 96:4 (v/v).

Spots were revealed by treating the plates with iodine vapor, ninhydrin, and by radioautography. Internal standards were chromatographed under the same conditions.

Phospholipids were hydrolyzed with CH_3OH–KOH (0.5 N) for 5 min; this was followed by a BF_3–methanolic transmethylation for 5 min at 60°C under dry N_2 (33). Fatty acids were measured by means of gas–liquid chromatography. A 3-m column, with 4% DEGS on Gas-Chrom Q, 100–120 mesh, operated at 170°C was used; detection was by flame ionization.

Lipid phosphorus was measured according to Rouser, Siakotes, and Fleischer (35). The radioactivity content of the spots was measured using an Intertechnique liquid scintillation counter. The following scintillation medium was used: POPOP, 0.3 g; PPO, 5 g; toluene, 1 liter; Triton X 100, 0.5 liter, and water, 0.1 liter. Quenching correction was evaluated by means of the channel ratio method (36).

RESULTS

After a 5-min incubation, one-dimensional electrophoresis clearly indicated that the radioactivity was distributed within the two major plasma lipoproteins, thus indicating a rapid exchange between the phospholipids bound to the HDL and those of plasma lipoproteins. Over the course of the incubation, the ratio of labeled HDL/labeled LDL did not vary. After 2 hr of incubation, the lipids from the platelet-free plasma (PFP) were extracted. Ninety-seven percent of the radioactivity was recovered in PC, 0.8% in esterified cholesterol, and 0.5% in free fatty acids. Residual radioactivity was found in LPC. The uptake of plasma linoleoyl- or arachidonoyl-lecithins was calculated by dividing total platelet radioactivity by the specific radioactivity of plasma linoleoyl- or arachidonoyl-lecithins. Results are expressed in nmol of lecithin incorporated by 10^9 platelets. The quantity of linoleic or arachidonic acid incorporated in different platelet phospholipids from a specific phosphatidylcholine was obtained by dividing radioactivity found in this phospholipid by the specific activity of the corresponding fatty acid in phosphatidylcholine.

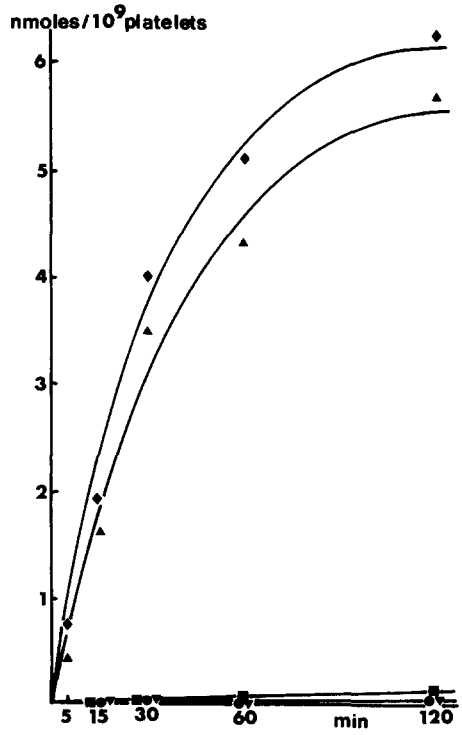

Fig. 1. 2-Linoleoyl-lecithin exchange between plasma and platelets. Incubation procedure is indicated in Materials and Methods. Results are expressed in nmol per 10^9 platelets. Total 2-linoleoyl-lecithin exchange, ● —— ◆. Incorporation from 2-linoleoyl-lecithin in PC, ▲ —— ▲; PI, ■ —— ■; PS, ● —— ●; and PE, ▼ —— ▼.

Figs. 1 and 2 show that the uptake of the two specific lecithins by platelets was of the same order of magnitude; 6.2 nmol of 2-linoleoyl-lecithin and 4.6 nmol of 2-arachidonoyl-lecithin were incorporated in 10^9 platelets after 2 hr. The difference of incorporation between these two fatty acids may be explained by a greater oxidation of archidonate than of linoleate in platelets. Ninety percent of the 2-[1-^{14}C]linoleoyl-lecithin was recovered in lecithin after 2 hr, 6% in the lysolecithin, and 4% in three other glycerophospholipids (PE, PS, and PI) (Fig. 1). However when 2-arachidonoyl-lecithin was added as a complex with HDL, only 52% of the total radioactivity was recovered in the PC after 2 hr. The remainder was found in PI (21%), PE (6%), PS (4%), and LPC (17%) (Fig. 2).

After 2 hr, 40 nmol of plasma 2-linoleoyl-lecithin and 17 nmol of plasma 2-arachidonoyl-lecithin were

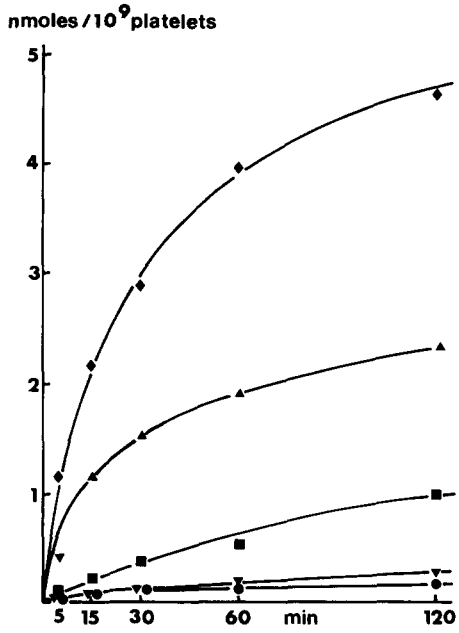

Fig. 2. 2-Arachidonoyl-lecithin exchange between plasma and platelets. Incubation procedure is indicated in Materials and Methods. Results are expressed in nmol per 10^9 platelets. Total 2-arachidonoyl-lecithin exchange, ◆ —— ◆. Incorporation from 2-arachidonoyl-lecithin in PC, ▲ —— ▲; PI, ■ —— ■; PS, ● —— ●; and PE, ▼ —— ▼.

found in 1 μmol of platelet PC (**Table 1**). Under such conditions, in 1 μmol of PI, 5.5 nmol of linoleic acid, and 71 nmol of arachidonic acid came from plasma lecithins. The contribution of fatty acids from plasma lecithin to fatty acids in PE and PS was much lower; 6.5 and 5.8 nmol/μmol of arachidonic acid and 0.64 and 0.76 nmol/μmol of linoleic acid were in PE and PS, respectively.

Fig. 3 shows the percentage incorporation of fatty acids in the different molecular species of phospholipids, either from free fatty acids or from the exchanged lecithins, after a 15-min incubation.

Thirteen percent, 7%, and 3% of arachidonoyl-PI, arachidonoyl-PC, and linoleoyl-PC were renewed from free fatty acids while 3%, 5.5%, and 14%, respectively, were renewed from lecithins. Eighty-seven percent and 24% of the very small amount of linoleoyl-PI in platelets were renewed from FFA or lecithins.

Arachidonoyl-PE and PS were renewed to a lesser extent: 1.5% or 1.3%, respectively, from FFA, and 0.7% or 0.3% from lecithins. There was almost no renewal of linoleoyl-PE and PS.

DISCUSSION

There are some differences between our results and those of Joist et al. (21) who studied the exchange between plasma and platelet phospholipids in rabbits; 2.5% of rabbit platelet PC was labeled after 6 min and 5.5% after 3 hr while, in our experiment, 0.3% of human platelet PC was labeled after 5 min and 4% or 1.7% after two hr for the two molecular species. These differences might have been due to the different plasma and platelet phospholipids in humans and rabbits and to differences in fatty acid compositions. Moreover the ^{32}P labeling that was used by Joist et al. did not take into account the transacylation processes. We showed that human platelets are able to exchange their phospholipids with those of lipoproteins as do erythrocytes (37) and cultured human cells (38). Our work does not enable us to identify which lipoprotein is involved since, after a 5-min incubation, the labeled phospholipids were found equally distributed in HDL and LDL. In fact a very rapid phospholipid exchange is possible between the two lipoproteins (39). Linoleic acid coming from lecithin is very poorly transferred to the other phospholipids. However, 2-arachidonoyl-lecithin can be involved in a very active transfer process since we found [1-^{14}C]arachidonate in platelet PI, PE, and PS after 5 min.

TABLE 1. Specific incorporation of linoleic and arachidonic acids from plasma lecithins in the major platelet glycerophospholipids

		5′	15′	30′	60′	120′	*a*
Linoleic acid	PC	3 ± 0.5	11.5 ± 2	25 ± 1	29 ± 1.5	40 ± 2	71–121
	PE		0.53 ± 0.42	0.74 ± 0.03		0.64 ± 0.11	42
	PS		0.28 ± 0.24	0.46 ± 0.15	0.56 ± 0.51	0.76 ± 0.30	0–33
	PI	0.55 ± 0.15	2.2 ± 0.7	4.8 ± 0.3	4.3 ± 0.8	5.5 ± 0.3	7–25
Arachidonic acid	PC	2.8 ± 0.6	8.1 ± 1.6	10.5 ± 0.5	14 ± 1	17 ± 1	121–143
	PE	1.2 ± 0.3	1.5 ± 0.3	3.2 ± 0.7	4.1 ± 0.3	6.5 ± 0.2	302
	PS	1.1 ± 0.3	1.8 ± 0.4	2.8 ± 0.7	3.6 ± 0.3	5.8 ± 0.7	167–227
	PI	6.8 ± 1.4	16 ± 1	23 ± 10	38 ± 3	71 ± 3	321–428

[a] Molecular species (total homogenate) nmol/μmol PL. Values calculated from data in references 1 and 2. Experimental procedure is indicated in Materials and Methods.

Results are expressed in nmol fatty acids incorporated per μmol of phospholipids: $\frac{\text{incorporation in each phospholipid}}{\text{quantity of concerned phospholipid}}$.

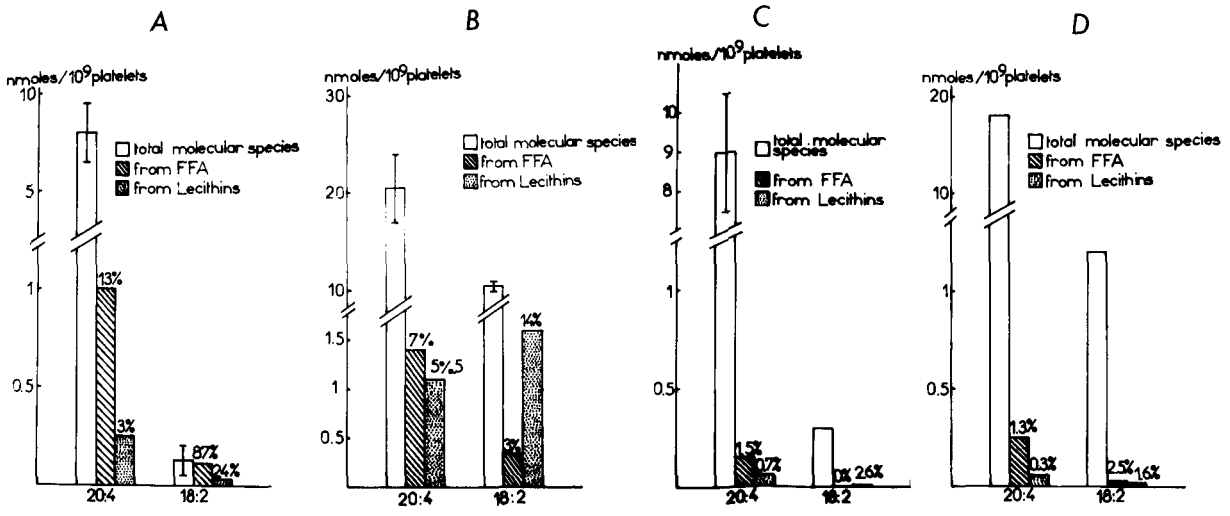

Fig. 3. Comparison of the linoleic and arachidonic renewal in platelet phospholipids from plasma free fatty acids and lecithins. The quantities of molecular species are calculated from data in references 1 and 2. Incorporation values from lecithins are obtained after 15 min of incubation (incubation procedure is indicated in Materials and Methods). Incorporation values from free fatty acids are obtained after 15 min of incubation of 5 ml of PRP with 1 μCi of either [1-^{14}C]linoleic or [1-^{14}C]arachidonic acids as described by Bills, Smith, and Silver (10). Panel *A*, phosphatidylinositol; panel *B*, phosphatidylcholine; panel *C*, phosphatidylserine; panel *D*, phosphatidylethanolamine.

We can conclude from our experiments that platelets possess a phosphatidylcholine pool that rapidly exchanges with the plasma lipoproteins. As it can be shown in a semilog representation for plasma lecithin exchange as a function of incubation time, this pool exchanges completely within 20–30 min. The pool size was 2.9 nmol/10^9 platelets for arachidonoyl lecithin and 3.8 nmol/10^9 platelets for linoleoyl lecithin, i.e., 2% and 2.8%, respectively, of total lecithins. Fourteen to sixteen percent of arachidonoyl-lecithins and 20–35% of linoleoyl-lecithins are implicated in this rapidly exchangeable pool. Most probably this pool is composed of the phospholipids constituting the external leaflet of the platelet plasma membrane.

The data expressed in Fig. 3 were obtained after a 15-min incubation period during which the rapidly exchangeable pool was not filled and the transfer processes towards other compartments were negligible.

The ratio of arachidonoyl-lecithin/linoleoyl-lecithin was 1.5–2 in platelets. The constancy of this ratio results from a dual mechanism: *a*) the exchange between the lecithin from plasma and platelets and *b*) the esterification of free fatty acids. The exchange processes favor the accumulation of linoleate in lecithin while the transacylation mainly concerns arachidonoyl-lecithin.

Arachidonoyl- and linoleoyl-PI were mainly renewed from FFA (four times more than from lecithins). Arachidonoyl PC was equally renewed from FFA and lecithins while linoleoyl PC was renewed 4.5 times more from lecithins than from FFA.

The molecular species of PS and PE containing arachidonic acid were mainly renewed from FFA while linoleoyl PS was mainly renewed from plasma lecithins. In the case of linoleoyl PE, the two processes were very weak and of equal importance.

Our preliminary results on platelet homogenates (17) confirmed the presence of a phospholipase A_2, whose existence was indirectly deduced by several authors (11, 14, 40). Moreover, platelets are able to acylate lyso-PC (18). Human platelets should possess the enzymatic equipment necessary for a rapid incorporation of arachidonic acid in PC and PI despite the fact that the plasma level of arachidonic acid remains unchanged. The rates of incorporation are much lower in PS and in PE despite the higher relative amount of arachidonate in both phospholipids. This can be explained either by the presence of phospholipases and transacylases more specific for PC and PI or by a different cellular localization for these phospholipids. In the latter case, it implies a topological vicinity of rapidly exchangeable pool of PC and PI.

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