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The activities of acyl-CoA: 1-acyl-lysophospholipid acyltransferase(s) in human platelets

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The activities of acyl-CoA: 1-acyl-lysophospholipid acyltransferases (EC 2.3.1.23) have been studied in human platelet lysates by using endogenously formed [^{14}C]acyl-CoA from [^{14}C]fatty acid, ATP and CoA in the presence of 1-acyl-lysophosphatidyl-choline (lysoPC), -ethanolamine (lysoPE), -serine (lysoPS) or -inositol (lysoPI). Linoleic acid as fatty acid substrate had the highest affinity to acyl-CoA: 1-acyl-lysophospholipid acyltransferase with lysoPC as variable substrate, followed by eicosapentaenoic acid (EPA) and arachidonic acid (AA). The activity at optimal conditions was 7.4, 7.3 and 7.2 nmol/min per 10^9 platelets with lysoPC as substrate, with linoleic acid, AA and EPA respectively. EPA and AA were incorporated into all lyso-forms. Linoleic acid was also incorporated into lysoPE at a high rate, but less into lysoPS and lysoPI. DHA was incorporated into lysoPC and lysoPE, but only slightly into lysoPI and lysoPS. Whereas incorporation of all fatty acids tested was maximal for lysoPC and lysoPI at 200 and 80 μM respectively, maximal incorporation needed over 500 μM for lysoPE and lysoPS. The optimal concentration for [^{14}C]fatty acid substrates was in the range 15–150 μM for all lysophospholipids. Competition experiments with equimolar concentrations of either lysoPC and lysoPI or lysoPE resulted in formation of [^{14}C]PC almost as if lysoPI or lysoPE were not added to the assay medium.

INTRODUCTION

The phospholipid composition in platelets is mainly determined at the time of thrombopoiesis, whereas the composition of molecular species is remodelled during circulation after thrombopoiesis (Masuda *et al.*, 1991).

Long-chain fatty acids bound to albumin are rapidly taken up by human platelets (Spector *et al.*, 1970). The formation of acyl-CoA esters from non-esterified fatty acids and CoA is the initial step in fatty acid utilization in the cells. As acyl-CoAs are believed to have general damaging effects in membranes, they are most likely not accumulated, but are rapidly incorporated into platelet phospholipids, oxidized or hydrolysed. The metabolic process directing the various fatty acids into the different phospholipid classes in the platelets is not fully known. Neufeld *et al.* (1983) demonstrated that fatty acid esterification by intact platelets was similar to acyl-CoA synthesis by platelet membranes. We have recently described that the acyl-CoA reaction is almost identical for fatty acids from $C_{12:0}$ to $C_{22:6,n-3}$ under optimal conditions, but the maximum enzyme activities are different (Bakken *et al.*, 1991*a*).

The presence of an acyl-CoA: lysophosphatidylcholine acyltransferase in platelets was described by McKeen *et al.* (1982). Later it was shown that the enzyme is enriched in the intracellular membrane fraction (McKeen *et al.*, 1986), and it was postulated that the remodelling pathway ('Lands pathway'; Lands & Crawford, 1976) was an important one for the incorporation of unsaturated fatty acids into platelet phospholipids.

Human platelets exhibit a certain specificity for incorporation of polyunsaturated fatty acids in terms of uptake and glycerophospholipid subclasses (Hajarine & Lagarde, 1986). Laposata *et al.* (1987) later described that labelled arachidonic acid (AA) incubated with human platelets was rapidly taken up and incorporated selectively into phospholipids within the dense tubular system.

It is well known that the fatty acid pattern in blood platelet membranes is of great importance for the metabolic functions of platelets. The membrane fluidity, which is determined by the relative content of the different phospholipid classes and cholesterol, is an important parameter in the responsiveness of the platelets (Stubbs, 1989), and affects the functions of receptors and enzymes embedded in the membrane. AA, liberated from the phospholipids, is converted into endoperoxides and thromboxane A_2, which activate platelets, whereas eicosapentaenoic acid (EPA) gives rise to thromboxane A_3 and inhibits the effect of thromboxane A_2 (Fischer & Weber, 1983, 1984). Thus the amount of EPA or AA in platelet phospholipids is of great importance for thromboxane synthesis.

Although intensively studied, no definite conclusions concerning the determining steps of fatty acid incorporation into platelet phospholipids have been drawn. McKeen *et al.* (1982) concluded that most likely the specificity of acyl-CoA synthetases was the determining factor, and Wilson *et al.* (1982) concluded that platelets contained an acyl-CoA synthetase (EC 6.2.1.3) specific for $C_{20:4,n-6}$ and other prostanoid precursors. Recently we found (Bakken *et al.*, 1991*a*) only one long-chain acyl-CoA synthetase in human platelets with a broad specificity and high specific activities for fatty acids from $C_{12:0}$ to $C_{22:6,n-3}$.

The present work was therefore undertaken to study the incorporation of fatty acids from endogenously formed acyl-CoA into the different lysophospholipid classes in platelets. The use of endogenously formed acyl-CoA has several advantages: it resembles conditions *in vivo*, there are no detergent effects of added acyl-CoA, and also it allows the use of a wide range of fatty acids. We here report the activities of acyl-CoA: 1-acyl-lysophospholipid acyltransferases (EC 2.3.1.23) in platelet

Abbreviations used: AA, arachidonic acid ($C_{20:4,n-6}$); EPA, eicosa-5,8,11,14,17-pentaenoic acid ($C_{20:5,n-3}$); DHA, docosa-4,7,10,13,16,19-hexaenoic acid ($C_{22:6,n-3}$); DTT, dithiothreitol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; lysoPC, 1-acyl-lysophosphatidylcholine; lysoPE, 1-acyl-lysophosphatidylethanolamine; lysoPS, 1-acyl-lysophosphatidylserine; lysoPI, 1-acyl-lysophosphatidylinositol; PLA_2, phospholipase A_2.

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lysates, using 1-acyl-lysophosphatidyl-choline, -inositol, -ethanolamine and -serine as lysophospholipid substrates, and $C_{20:4,n-6}$, $C_{20:5,n-3}$, $C_{22:6,n-3}$, $C_{18:2,n-6}$, $C_{18:1,n-9}$ and $C_{16:0}$ as endogenously derived acyl-CoA substrates.

We also demonstrate that the polyunsaturated fatty acids are exclusively incorporated into the *sn*-2 position in lysoPC.

MATERIALS AND METHODS

Reagents

[1-^{14}C]Palmitic acid ($C_{16:0}$), [1-^{14}C]oleic acid ($C_{18:1,n-9}$), [1-^{14}C]linoleic acid ($C_{18:2,n-6}$), [1-^{14}C]AA ($C_{20:4,n-6}$), [1-^{14}C]EPA ($C_{20:5,n-3}$) and 1-[1-^{14}C]palmitoyl-lysoPC were purchased from Amersham International (Amersham, Bucks, U.K.). [1-^{14}C]DHA ($C_{22:6,n-3}$) was purchased from New England Nuclear (Boston, MA, U.S.A.). Specific radioactivities of all labelled fatty acids were about 55 mCi/mmol. Ultima Gold from Packard was used as scintillator. AA, EPA and DHA were from Biomol (Plymouth Meeting, PA, U.S.A.). ATP, CoA, palmitic, oleic and linoleic ($C_{18:2,n-6}$) acids, 1-acyl-lysophospholipids (containing primarily stearate and palmitate, except for lysoPS, containing stearate), dithiothreitol (DTT), Triton X-100, EDTA, Hepes and phospholipase A_2 (PLA_2; EC 3.1.1.4; 760 units/mg of protein) were purchased from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of the highest purity commercially available. Silica gel t.l.c. plates were from Schleicher & Schuell (Dassel, Germany).

Preparation of platelet lysates

Blood platelets were prepared from samples from healthy donors, as described previously (Holmsen *et al.*, 1977; Bakken & Farstad, 1989). The washed and resuspended platelets were sonicated for 3 × 5 s in an ice-bath, or frozen and thawed twice. The lysates were kept at −80 °C and portions were thawed just before use.

Estimation of the number of platelets

The number of platelets was determined in a Coulter Counter (Model S-plus III; Coulter Electronics, Hialeah, FL, U.S.A.).

Assay of acyl-CoA:1-acyl-lysophospholipid acyltransferase in human blood platelets

The acyl-CoA:1-acyl-lysophospholipid acyltransferase activity in platelets was determined by measuring the formation of [^{14}C]phospholipid (PL) from 1-acyl-lysophospholipid and endogenously formed [1-^{14}C]acyl-CoA from [^{14}C]fatty acid, CoA and ATP in the presence of Mg^{2+} and endogenous acyl-CoA synthetase. The assay was optimized for substrate, cofactor and platelet concentrations. The standard incubation medium contained 50 mM-Hepes buffer, pH 7.4, 5 mM-DTT, 3.5 mM-$MgCl_2$, 2 mM-ATP, 100 μM-CoA, 15–150 μM-[1-^{14}C]fatty acid, 200 μM-lysoPC (or 200 μM-lysoPI, 400–600 μM-lysoPS or 100–600 μM-lysoPE) and platelet lysate corresponding to (75–100) × 10^6 platelets in a final volume of 250 μl. LysoPC and lysoPI were dissolved in water, and lysoPE and lysoPS were dissolved in 20–50 mM-KOH and sonicated for 3 × 15 s just before use, or dissolved in KOH and warmed at 37 °C before adding water.

Incubations were carried out at 37 °C for 10 min, which is within the time giving a constant reaction rate. The reaction was terminated by addition of chloroform/methanol (1:2, by vol.). The lipids were extracted by the procedure of Bligh & Dyer (1959), and a 400 μl portion of the chloroform layer, containing the lipids, was evaporated under N_2. The lipids were separated by development in one-dimensional t.l.c. on silica-gel plates with chloroform/methanol/acetic acid/water (25:15:4:2, by vol.) as the solvent phase (Murase *et al.*, 1988). The lipids were identified by co-chromatography by standards of PC, PI, PS and PE. Butylated hydroxytoluene (0.05 %, w/v) was added to the organic solvents at all stages of extraction and chromatography to minimize autoxidation of unsaturated fatty acids. The t.l.c. plates were sprayed with I_2 in light petroleum (b.p. 40–60 °C) to make the spots visible. The plates were then cut into pieces, and the radioactivity was counted in a Packard Tri-Carb model 2450 instrument after 2 days for the PE and PS samples, and after 5 days for the PC and PI samples. The specific radioactivities of the fatty acid substrates were 600–1000 c.p.m./nmol.

Since lysoPE and lysoPS are insoluble in water, and the suspensions turned cloudy within 1 h after they were dissolved in KOH and sonicated or warmed, an experiment was performed to investigate whether the measured transferase activity was constant within the time necessary for the experiment. Leaving the platelet suspensions containing all substrates and cofactors on an ice-bath, samples were taken out for incubation at 37 °C every 15 min for 1 h. The measured activity was decreased by about 5 %, indicating that the lysoPE and lysoPS in the solutions were still available as substrates, and meaning less than 5 % decrease in the measured activity in our experiments, which never exceeded $\frac{3}{4}$ h. The transferase activity was decreased by 20 % when the platelet suspension was kept at room temperature with 200 μM-lysoPC, CoA, ATP, DTT and $MgCl_2$ under optimal conditions. The platelet suspensions were therefore kept on ice until all incubations were performed.

The assay of acyltransferase activity was very reproducible, the difference between parallel measurements being within ±5 % for all substrates tested.

1-[1-^{14}C]Palmitoyl-lysoPC was used as acceptor for unlabelled endogenously formed acyl-CoAs in a few experiments and compared with 1-palmitoyl-lysoPC as acceptor for endogenously formed [1-^{14}C]acyl-CoAs.

Assay with PLA_2

To ensure that the formed phospholipids from the acyl-CoA:1-acyl-lysophospholipid acyltransferase assay were labelled in the *sn*-2 position, we added PLA_2 to the incubation medium. The experiment was performed as above, but after 10 min of incubation the pH was increased to 8.0 with KOH, $CaCl_2$ was added to a final concentration of 5 mM and then 15 units of PLA_2 was added (modified method; Laychock *et al.*, 1986; Rubin *et al.*, 1990). This mixture was incubated for 1 h at 37 °C. The reaction was stopped, and the lipids were extracted and separated on silica-gel t.l.c. plates as above. A parallel sample measuring the formation of labelled phospholipid was used as control, both to make sure that the phospholipid was formed and to calculate the degradation of the phospholipid after addition of PLA_2.

Degradation of the separated 1-acyl-2-[1-^{14}C]palmitoyl-PC with PLA_2

To check that palmitate also was exclusively incorporated into the *sn*-2 position of the PC, the 1-acyl-2-[1-^{14}C]palmitoyl-PC spot on the silica-gel t.l.c. plate was cut out and extracted with chloroform once, and then twice with chloroform/methanol. The collected extracts were dried in a stream of N_2. The residue was dissolved in 60 μl of diethyl ether and vigorously vortex-mixed for a few minutes. Then 50 mM-Tris buffer, pH 8.5, 5 mM-$CaCl_2$ and 15 units of PLA_2 were added, and the mixture was vortex-mixed for 4–5 min. The suspension was incubated at 37 °C for 75 min in a closed tube (corked) in a shaking water bath. The diethyl ether was then evaporated off in a stream of N_2 for 2 min. Thereafter the suspension was neutralized to pH 7.0 before the lipids were extracted as described above (Bligh & Dyer, 1959). The extracted lipids were separated on t.l.c. as described above.

Uptake of fatty acids in intact platelets

The fatty acids were individually bound to BSA: unlabelled and labelled fatty acid were dissolved in acetone. Then the acetone solution was added very slowly to a buffer, pH 7.4, containing 3% BSA, 130 mM-NaCl, 12 mM-Hepes and 2 mM-EDTA, with constant stirring, and under N_2 to evaporate the acetone. Washed human platelets (about 10^9 platelets/ml, suspended in the above buffer without albumin) were incubated for 5 min at 37 °C with the labelled 600 μM fatty acid bound to albumin (molar ratio 4:1). The platelet fatty acid uptake was stopped by adding 1 vol. of buffer to the platelet fatty acid/albumin suspension, followed by immediate centrifugation at 8000 $g_{max.}$ for 1 min in an Eppendorf centrifuge (model 3200). The platelets were washed once, resuspended in buffer, and the fatty acid uptake was measured (nmol/10^9 platelets).

RESULTS

Activity of acyl-CoA: 1-acyl-lysophospholipid acyltransferase in lysates of human platelets

Fig. 1 shows that 1-^{14}C-labelled fatty acids were incorporated into lysoPC by sonicated human platelets in the presence of ATP, CoA and Mg^{2+} and endogenous acyl-CoA synthetase. The reaction rate was linear with incubation time up to 20 min, and with the platelet lysate prepared from (20–120) × 10^8 platelets (results not shown). When lysoPC acylation was assayed under optimal conditions (2 mM-ATP, 3.5 mM-$MgCl_2$, 100 μM-CoA, 200 μM-lysoPC and 150 μM-[^{14}C]fatty acid), EPA and $C_{18:2}$ were incorporated at about the same rate, followed by AA, DHA, $C_{18:1}$ and $C_{16:0}$ (Fig. 1*a*). Fig. 1(*b*) shows that, with lysoPC as the variable substrate and optimal concentrations of [^{14}C]fatty acids, $C_{18:2}$ was incorporated at the highest rate at lysoPC concentrations up to 150 μM, and AA and DHA were incorporated faster than EPA at concentrations of lysoPC up to about 150 and 100 μM respectively.

In the presence of 0.1% Triton X-100, which enhances the acyl-CoA synthetase activity (Vollset & Farstad, 1979; Bakken, 1986), no [^{14}C]PC was formed. This is probably due to the fact that acyl-CoA in the form of mixed acyl-CoA/Triton X-100 micelles (Okuyama *et al.*, 1975; Sun *et al.*, 1979; Berge *et al.*, 1981) is not a substrate for acyl-CoA: 1-acyl-lysoPC acyltransferase. [^{14}C]Acyl-CoA which had not reacted, which remained in the aqueous phase after extraction of [^{14}C]phospholipid and [^{14}C]fatty acids, was found only in trace amounts with up to 90 μM of AA, EPA or $C_{18:2}$ and 500 μM-lysoPE, whereas small but negligible amounts of acyl-CoA were detectable at lower concentrations with $C_{16:0}$, $C_{18:1}$ and DHA. This indicates that [^{14}C]acyl-CoAs were rapidly incorporated into the lysophospholipids in the incubation medium.

When 5 mM-DTT was omitted from the incubation medium, the acylation of lysoPC was decreased by about 30%. During a 10 min incubation period, no radioactive PI, PE or PS was found, and only trace amounts of PC were found when lysophospholipids were omitted. No acylation of lysophospholipids was found when platelet lysates were heat-inactivated at 70 °C for 15 min.

With a constant concentration of lysoPI (200 μM), AA and EPA were incorporated at about the same rate up to a fatty acid concentration of 15 μM (Fig. 2*a*). At higher concentrations, AA was incorporated about twice as fast as EPA. $C_{18:2}$, $C_{18:1}$ and $C_{16:0}$ were incorporated about half as fast as EPA at concentrations under 60 μM. DHA was slightly incorporated into lysoPI. With lysoPI as the variable substrate, the optimal concentration of lysoPI was 80 μM (Fig. 2*b*). Acyl-CoA: 1-acyl-lysoPI acyltransferase had a preference for AA and EPA, although the maximum enzyme activity was only half and one-third, respectively, of that with lysoPC as substrate (Fig. 2).

With the optimal concentration of lysoPE (500 μM), EPA was incorporated twice as fast as AA at 30 μM [^{14}C]fatty acid substrate, with $C_{18:2}$ at a rate in between, and DHA, $C_{18:1}$ and $C_{16:0}$ at lower rates (Fig. 3*a*). At optimal fatty acid concentration AA, EPA and $C_{18:2}$ were incorporated to the same large extent. When lysoPE was the variable substrate, maximal incorporation of [^{14}C]fatty acids occurred at about 600 μM-lysoPE (Fig. 3*b*). The fatty acid incorporation was much the same as with fatty acids as variable substrates at optimal lysoPE concentration, but the acyltransferase had a greater preference for AA than for EPA.

Also, maximal incorporation of [^{14}C]fatty acids into lysoPS took place when lysoPS concentration was high, about 500 μM

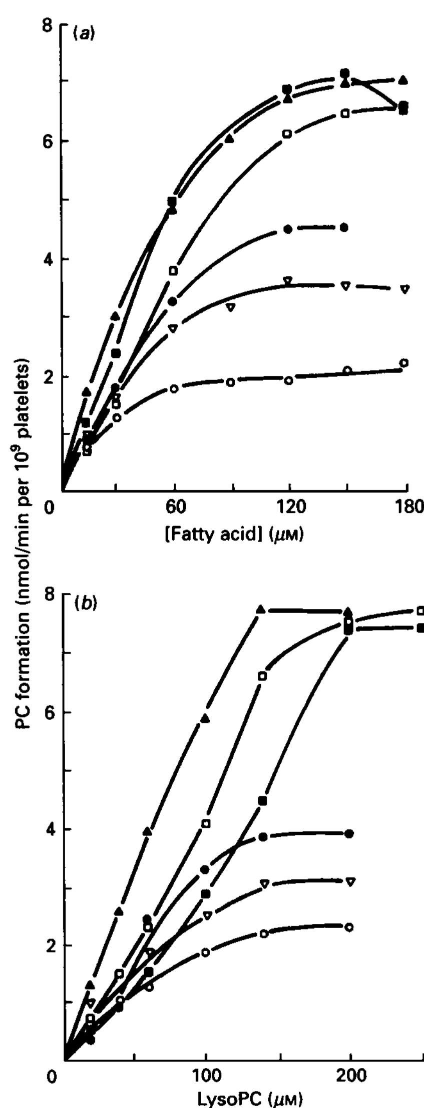

Fig. 1. [^{14}C]PC formation from lysoPC and endogenously formed [^{14}C]acyl-CoA from [^{14}C]fatty acid, ATP and CoA as a function of [^{14}C]fatty acid (*a*) and lysoPC (*b*)

The reaction mixture contained, in a final volume of 250 μl, 50 mM-Hepes, pH 7.4, 3.5 mM-$MgCl_2$, 2 mM-ATP, 5 mM-DTT, 100 μM-CoA, 82 × 10^6 platelets, 200 μM-lysoPC and various amounts of ^{14}C-labelled fatty acids (*a*) or varied concentrations of lysoPC and 150 μM-[^{14}C]fatty acid (*b*). The incubation temperature was 37 °C and the incubation time was 10 min. Fatty acids: □, AA; ■, EPA; ●, DHA; ▲, $C_{18:2}$; ▽, $C_{18:1}$; ○, $C_{16:0}$.

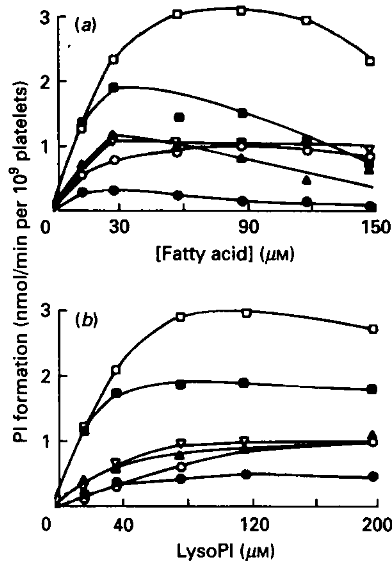

Fig. 2. [^{14}C]PI formation from lysoPI and endogenously formed [^{14}C]acyl-CoA from [^{14}C]fatty acid, ATP and CoA as a function of [^{14}C]fatty acid (*a*) and lysoPI (*b*)

The incorporation of fatty acids into lysoPI was measured, with 200 μM-lysoPI and varied amounts of ^{14}C-labelled fatty acids (*a*) or varied concentrations of lysoPI and 30 μM-[^{14}C]fatty acid (60 μM for AA) (*b*). Otherwise conditions were as in the legend to Fig. 1.

(Fig. 4*b*). At the optimal concentration of lysoPS (600 μM), AA and EPA were incorporated at the same rate at fatty acid concentrations up to 60 μM, followed by $C_{18:2}$ (Fig. 4*a*). When lysoPS was varied, the order of incorporation was AA > EPA > $C_{18:2}$ > $C_{16:0}$. Only traces of $C_{18:1}$ and DHA were incorporated.

To summarize, Figs. 1–4 show that: (1) with lysoPC as acyl acceptor, AA, EPA and $C_{18:2}$ were incorporated at the same rate under optimal conditions, followed by DHA and $C_{18:1}$; (2) with lysoPI as acceptor AA was incorporated fastest, followed by EPA, $C_{18:2}$, $C_{18:1}$ and $C_{16:0}$; DHA incorporation was low; (3) with lysoPE as acceptor, the rate of incorporation was similar to that of lysoPC; and (4) with lysoPS the incorporation resembled that with lysoPI, except that only traces of DHA were incorporated. Figs. 1–4 also show that AA, $C_{18:2}$ and EPA were to some extent preferred for lysoPC and lysoPE. DHA was incorporated 5–20 times as fast into lysoPC and lysoPE as into lysoPI and lysoPS. The incorporation of $C_{18:1}$ was also relatively high, especially with lysoPC as acceptor.

The optimal conditions for maximal incorporation of [^{14}C]fatty acids into lysoPC, lysoPI, lysoPE and lysoPS varied both for the fatty acid substrates and the acyl-acceptor substrates (Figs. 1–4). Thus the pattern of incorporations is very complicated. With lysoPI and lysoPS, the enzyme activity was similar both under conditions that permitted the optimal rate of acylation of the lysophospholids and under conditions giving $V_{max.}/2$ (Figs. 2 and 4).

Degradation of 1-acyl-2-[1-^{14}C]-AA (or -EPA/-DHA/-linoleoyl/-palmitoyl)-PC by PLA$_2$

When [1-^{14}C]AA, [1-^{14}C]EPA, [1-^{14}C]DHA, [1-^{14}C]linoleate or [1-^{14}C]palmitate was incorporated into lysoPC, addition of 15 units of PLA$_2$ to the incubation medium (after adjusting pH to 8.0 and adding 5 mM-$CaCl_2$) resulted in complete exclusion of labelled AA, EPA, DHA and linoleate from the *sn*-2 position of [^{14}C]PC within 1 h of incubation. With labelled palmitate esterified to the *sn*-2 position in PC, less than 5% of the originally labelled PC was detected after incubation with PLA$_2$.

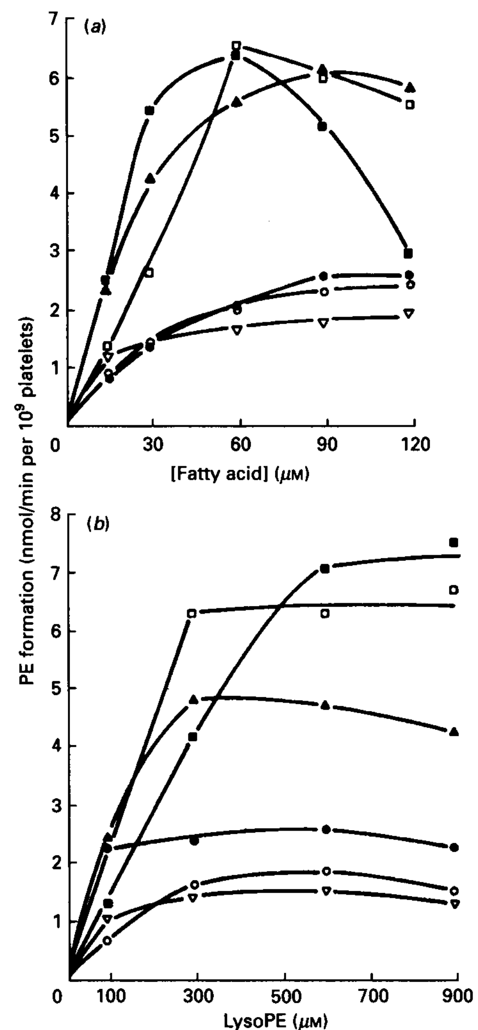

Fig. 3. [^{14}C]PE formation from lysoPE and endogenously formed [^{14}C]acyl-CoA from [^{14}C]fatty acid, ATP and CoA as a function of [^{14}C]fatty acid (*a*) and lysoPE (*b*)

The incorporation of fatty acids into lysoPE was measured, with 500 μM-lysoPE and varied amounts of ^{14}C-labelled fatty acids (*a*) or varied concentrations of lysoPE and 60 μM-[^{14}C]fatty acid (75 μM for AA) (*b*). Otherwise conditions were as in the legend to Fig. 1.

It was observed that the PC spots disappeared from the silica-gel t.l.c. plates. It was also observed that the usually easily seen endogenous PE spots had disappeared, whereas lysoPE spots appeared, on spraying with I_2 in light petroleum.

Degradation of 1-acyl-2-[1-^{14}C]palmitoyl-PC in extract from t.l.c.

After PLA$_2$ treatment of the 1-acyl-2-[1-^{14}C]palmitoyl-PC extract residue, lipids were extracted and separated on silica-gel t.l.c. plates. [^{14}C]Palmitate was detected only in the non-esterified fatty acid spot. No measurable [^{14}C]PC or [^{14}C]lysoPC was found in the position of these phospholipids, showing that palmitate was exclusively incorporated into the *sn*-2 position of the PC formed.

Acyl-CoA:1-acyl-lysoPC acyltransferase activity with 1-[1-^{14}C]palmitoyl-lysoPC as acceptor

By using 200 μM-1-[1-^{14}C]palmitoyl-lysoPC as acceptor and 150 μM-fatty acids and otherwise as stated in the Materials and methods section, the measured activity of acyltransferase was 4–30% higher than with [1-^{14}C]fatty acids and lysoPC. Table 1

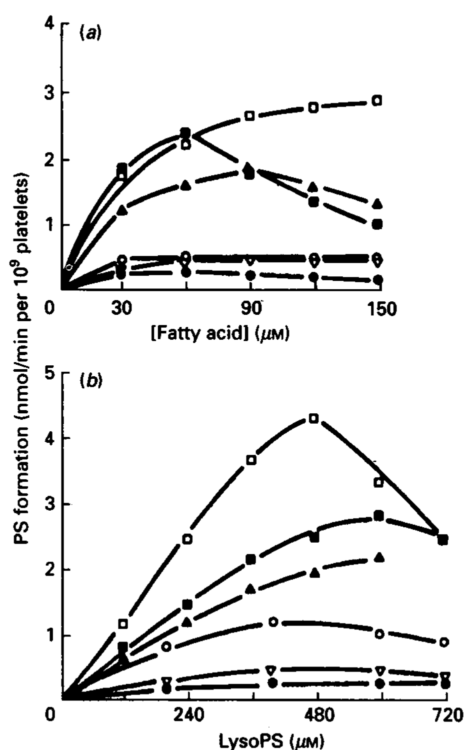

Fig. 4. [^{14}C]PS formation from lysoPS and endogenously formed [^{14}C]acyl-CoA from [^{14}C]fatty acid, ATP and CoA as a function of [^{14}C]fatty acid (*a*) and lysoPS (*b*)

The incorporation of fatty acids into lysoPS was measured, with 600 μM-lysoPS and varied amounts of ^{14}C-labelled fatty acids (*a*) or varied concentrations of lysoPS and 60 μM-[^{14}C]EPA, -DHA, -$C_{18:1}$ and -$C_{16:0}$ or 90 μM-[^{14}C]AA and -$C_{18:2}$ (*b*). Otherwise conditions were as in the legend to Fig. 1.

Table 1. Acyl-CoA:1-palmitoyl-lysoPC acyltransferase activity

Activities of acyl-CoA:1-palmitoyl-lysoPC acyltransferase (nmol/min per 10^9 platelets) were measured in presence of 200 μM-1-[1-^{14}C]palmitoyl-lysoPC and endogenously formed acyl-CoA from 150 μM-fatty acid, CoA, ATP and 82 × 10^6 platelets under optimized conditions, or in the presence of unlabelled lysoPC and [^{14}C]fatty acid. For assay conditions see the Materials and methods section.

Acceptor	Activity					
	20:4	20:5	22:6	18:2	18:1	16:0
[^{14}C]LysoPC	10.0	10.5	5.7	8.3	5.6	3.6
LysoPC	9.1	9.3	5.5	7.1	4.8	2.8

gives the results from experiments with the six fatty acids investigated, as means of three to six single experiments. Labelled palmitate was released from 1-[1-^{14}C]palmitoyl-lysoPC and recovered in the non-esterified fatty acid fraction: 2.0, 2.3, 2.4, 2.4, 2.5 and 3.6% of added [^{14}C]lysoPC with AA, EPA, DHA, $C_{18:2}$, $C_{18:1}$ and $C_{16:0}$ fatty acid respectively.

Transacylation of phospholipids during acylation of 1-acyl-lysophospholipids

Table 2 and Fig. 5 show that phospholipids were formed without addition of the respective lysophospholipid. With lysoPC as acceptor, no other phospholipids were found. With lysoPE as acceptor, some [^{14}C]PC was found with all fatty acids tested. [^{14}C]PC accounted for up to 20% of total [^{14}C]phospholipids with 100 μM-lysoPE as an acceptor. With 500 μM concentration of lysoPE, the formation of [^{14}C]PC fell to about 3–6%. With 20 μM-lysoPI as acceptor both [^{14}C]AA-PE and [^{14}C]EPA-PE were formed; and with 100 μM-lysoPI some [^{14}C]PC was found with all fatty acids tested. When lysoPS was acceptor, both [^{14}C]PC and [^{14}C]PE were formed with all acids except DHA. [^{14}C]PI formation was not detected with any of the acyl acceptors.

With all lysophospholipids tested, the higher the concentration of lysophospholipid added to the incubation medium, the smaller the percentage of endogenously formed [^{14}C]phospholipid of the other species.

Competition between lysoPC and lysoPI or lysoPE for endogenously formed [^{14}C]acyl-CoA

Table 3 shows that at equimolar concentrations of lysoPC + lysoPE as acceptor substrates, all [^{14}C]fatty acids tested (45 μM each) were preferentially incorporated into lysoPC; the formation of [^{14}C]PC was 2–5 times the [^{14}C]PE formation. The amounts of [^{14}C]PC and [^{14}C]PE formed were almost unchanged when the equimolar lysophospholipid concentrations were increased from 50 to 100 μM, indicating that the endogenous [^{14}C]acyl-CoA was incorporated almost quantitatively into the lysophospholipids. Only the rate of incorporation was increased when the concentrations of the acceptors were increased from 20 μM (Table 3*a*) to 50 μM (Table 3*b*) or 100 μM (Table 3*c*). The highest acyl-CoA:1-acyl-lysoPC acyltransferase activity was achieved with $C_{18:2}$ as fatty acid substrate, in agreement with the results in Fig. 1. Similar results (not shown) were found when 30 or 150 μM of each fatty acid was used.

Fig. 5(*a*) shows an experiment where lysoPC and lysoPE were acyl acceptors, incubated either alone (100 μM) or in equimolar concentrations (100 μM-lysoPC and 100 μM-lysoPE) with the indicated [^{14}C]fatty acids at 30 μM. When both lysoPC and lysoPE were present, all fatty acids studied were preferentially incorporated into lysoPC. However, the sum of PC + PE formed did not exceed the PC formation when lysoPC was the only acyl

Table 2. Endogenous [^{14}C]phospholipids formed parallel to that [^{14}C]phospholipid expected from the added lysophospholipid (nmol/min per 10^9 platelets)

Numbers in parentheses indicate % of phospholipid (PL) compared with the PL formed from lysoPL. Abbreviation: n.d., not detected. For assay conditions, see the legends to Figs. 1–4.

Acceptor	PL formed				
	20:4	20:5	22:6	18:2	18:1
LysoPC	n.d.	n.d.	n.d.	n.d.	n.d.
LysoPE (100 μM)	PC 0.18 (10)	PC 0.19 (12)	PC 0.07 (6)	PC 0.31 (15)	PC 0.16 (20)
LysoPI (20 μM)	PE 0.3 (15)	PE 0.1 (7)	n.d.	n.d.	n.d.
(100 μM)	PC 0.13 (6)	PC 0.1 (5)	PC 0.1 (18)	PC 0.23 (20)	PC 0.13 (10)
LysoPS (240 μM)	PC 0.10 (3)	PC 0.1 (6)	n.d.	PC 0.14 (12)	PC 0.1 (30)
	PE 0.25 (10)	PE 0.25 (17)	n.d.	PE 0.21 (12)	n.d.

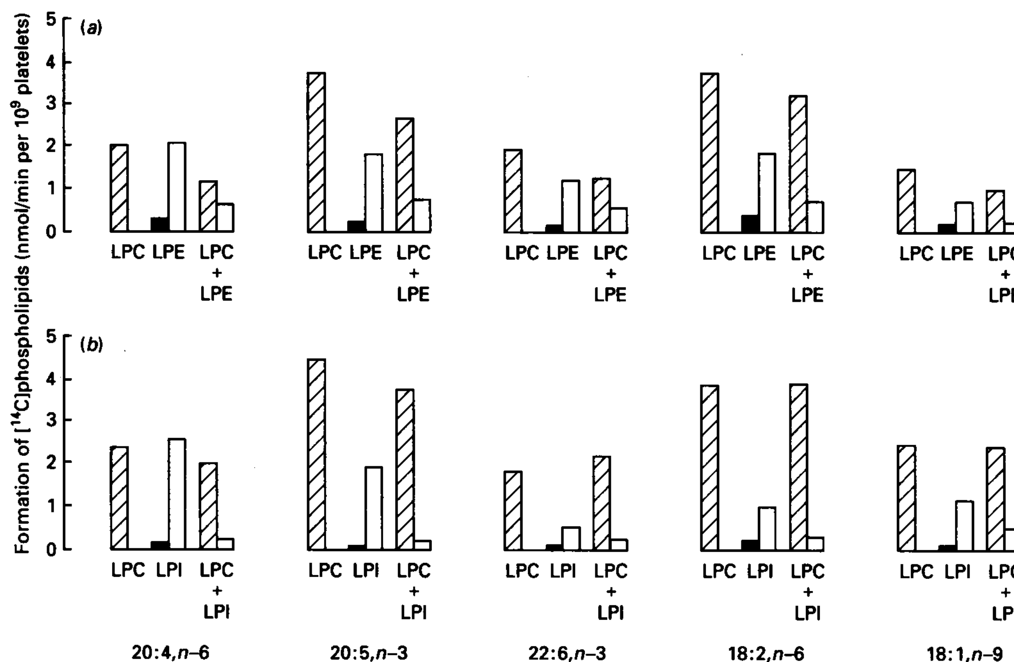

Fig. 5. [14C]PC and [14C]PE formation from lysoPC or lysoPE or both lysophospholipids in equimolar concentrations (*a*), and [14C]PC and [14C]PI formation from lysoPC or lysoPI or both lysophospholipids in equimolar concentrations (*b*), from endogenously formed [14C]acyl-CoA from [14C]fatty acid, ATP and CoA

The reaction mixture contained, in a final volume of 250 μl, 50 mM-Hepes, pH 7.4, 3.5 mM-$MgCl_2$, 2 mM-ATP, 5 mM-DTT, 100 μM-CoA, 30 μM-[14C]fatty acid, 92×10^6 platelets and 100 μM-lysoPC and/or -lysoPE or both in equimolar concentrations (*a*). For (*b*) the conditions were the same, except for lysoPI instead of lysoPE. The incubation temperature was 37 °C and the incubation time was 10 min. The black columns indicate endogenously formed [14C]PC in the absence of lysoPC. The hatched columns indicate formation of [14C]PC from lysoPC, and the white columns [14C]PE from lysoPE in (*a*) and [14C]PI from lysoPI in (*b*).

Table 3. [14C]PC and [14C]PE formation from endogenously formed [14C]acyl-CoA from [14C]fatty acid, ATP, CoA and equimolar concentrations of lysoPC and lysoPE

The incorporation of 45 μM-[14C]fatty acids into equimolar concentrations of lysoPC and lysoPE is given in the Table: 20 μM each (*a*), 50 μM each (*b*) and 100 μM (*c*) each. For other details, see the legend to Fig. 1.

	Acceptor	PC and PE formation (nmol/min per 10^9 platelets)					
		20:4	20:5	22:6	18:2	18:1	16:0
(*a*)	20 μM-LysoPC	1.2	0.9	1.3	1.8	1.0	0.4
	+20 mM-lysoPE	0.4	0.2	0.6	0.5	0.3	0.1
(*b*)	50 μM-LysoPC	2.6	2.7	2.0	3.6	1.2	0.6
	+50 μM-lysoPE	1.0	0.5	0.7	0.8	0.2	0.2
(*c*)	100 μM-LysoPC	2.3	3.1	1.7	3.7	1.5	0.8
	+100 μM-lysoPE	0.7	0.6	0.5	0.5	0.2	0.4

acceptor, indicating that the acyl-CoA formed was quantitatively incorporated into lsyophospholipids.

Fig. 5(*b*) shows a similar experiment where lysoPI was used instead of lysoPE. In this case too, all fatty acids were preferentially incorporated into lysoPC. The incorporation of AA and EPA into lysoPC was slightly decreased by the presence of lysoPI, whereas the PI formation was decreased to only 10 % in presence of lysoPC. With all fatty acids tested some [14C]PC was formed with both lysoPE and lysoPI as acyl acceptor, indicating transacylation (Kramer *et al.*, 1984), or acylation of endogenous lysoPC, or both.

Competition between [14C]EPA and [14C]AA for incorporation into lysoPC

The concentration of the inhibiting fatty acid was chosen from the velocity curves giving $V_{max.}/2$ for the acyl-CoA: 1-acyl-lysoPC acyltransferase(s). Inhibition of [14C]arachidonoyl-PC formation by 45 μM unlabelled EPA, DHA, $C_{18:2}$, $C_{18:1}$ and $C_{16:0}$ was performed with 200 μM-lysoPC and [14C]AA as varied substrate. EPA inhibited the [14C]arachidonate incorporation by about 30 % at 60 μM-[14C]AA. EPA was the most inhibitory fatty acid, whereas DHA inhibited the incorporation least (13 %); the inhibitions by the other fatty acids were in between those of EPA and DHA (results not shown).

With [14C]EPA as variable fatty acid substrate, 200 μM-lysoPC and unlabelled AA, DHA, $C_{18:2}$, $C_{18:1}$ and $C_{16:0}$ at 45 μM, incorporation of [14C]EPA was inhibited by about 30 % by AA and DHA, and only slightly by the other fatty acids.

Incorporation of [14C]DHA was also inhibited by about 25 % by 45 μM-EPA or -$C_{18:1}$, and less by the other fatty acids. This inhibition is similar to the fatty acid inhibition in the acyl-CoA synthetase reaction (Bakken *et al.*, 1991*a*).

Fatty acid uptake in intact platelets

The fatty acid uptake differed only slightly for AA, EPA, DHA, $C_{18:2}$ and $C_{18:1}$: 4.2, 3.7, 5.9, 5.8 and 7.0 nmol/10^9 platelets respectively (means of 2–4 donors, and 2–4 parallel measurements). With $C_{16:0}$ the uptake was 17.9 nmol/10^9 platelets.

DISCUSSION

Human blood platelets derive their fatty acids mostly from the non-esterified fatty acid fraction of plasma (Deykin & Desser, 1968; Spector *et al.*, 1970), although some exchange with fatty

acids of the high-density lipoprotein fraction may take place (Plantavid *et al.*, 1982). Since the plasma fraction of non-esterified fatty acids preferentially comes from hydrolysis of newly synthesized stores, dietary changes will rapidly affect the composition of platelet lipids (Nordøy *et al.*, 1974; Galloway *et al.*, 1985; Bakken *et al.*, 1991*b*). It has also recently been reported that dietary $n-6$ fatty acids inhibit incorporation of $n-3$ fatty acids into human platelet phospholipids (Grønn *et al.*, 1991), independent of the percentage of total energy present as fat. Feeding increasing amounts of linoleic acid with a constant amount of EPA resulted in decreased EPA incorporation into rate platelet lipids (Roshanai & Sanders, 1985). Also, fish oil given to a human population resulted in a greater effect on the AA/EPA ratio in platelet phospholipids when consuming fats from animal sources (saturated) than in those eating vegetable oils (Herold & Kinsella, 1986). The linoleic acid level in rat platelet PC increased rapidly when the diet was changed from sardine oil to corn oil (Ishinaga *et al.*, 1985).

The various fatty acids are unevenly distributed in the platelet phospholipid classes, with AA preferentially in PI and PS, and EPA in PC and PE (Weaver & Holub, 1985; Mahadevappa & Holub, 1987). Transacylation of platelet phospholipids seems to be an important mechanism *in vivo* for distribution of fatty acids in platelets (Kramer *et al.*, 1984). Incorporation of nutritional fatty acids into platelet phospholipids *de novo* has been suggested to be due to specificity of the fatty-acid-activating step (McKeen *et al.*, 1982), and the existence of a specific arachidonoyl-CoA synthetase has been proposed (Wilson *et al.*, 1982; Laposata *et al.*, 1987). Except for some specificity for $C_{20:3,n-6}$, McKeen *et al.* (1986) did not observe any specificity for long-chain acyl-CoA with 1-acyl-lysoPC in human platelets.

Previous studies in our laboratory (Bakken & Farstad, 1989; Bakken *et al.*, 1991*a*) strongly indicate that all long-chain fatty acids (from $C_{12:0}$ up to $C_{22:6}$) are activated by the same enzyme. Some specificity for the incorporation into phospholipids and acyl-CoA formed endogenously must therefore exist. Since human platelets contain an active acyl-CoA hydrolase with a high palmitoyl-CoA-hydrolysing capacity (Berge & Farstad, 1978), it is possible that acyl-CoA hydrolase(s) competes with the acyl-CoA transferases for the different acyl-CoAs. In microsomes from rat heart and liver the acyl-CoA hydrolase is inhibited by lysophospholipids (Sun & MacQuarrie, 1989). The role of the acyl-CoA hydrolase *in vivo* is, however, uncertain, since the activity is inhibited in the presence of ATP concentrations over 200 μM, and by 1 mM-$MgCl_2$ (Berge & Farstad, 1978).

Using endogenous fatty acid activation and 1-acyl-lysophospholipids we have observed the following order of incorporation, assayed both at optimal fatty acid concentration and at fatty acid concentrations giving a velocity of $V_{max.}/2$:

$$\text{LPC } 18:2 \geqslant 20:5 > 20:4 > 22:6 > 18:1 > 16:0$$

$$\text{LPI } 20:4 > 20:5 > 18:2 = 18:1 > 16:0 > 22:6$$

$$\text{LPE } 20:5 \geqslant 18:2 > 20:4 > 22:6 \geqslant 18:1 > 16:0$$

$$\text{LPS } 20:4 > 20:5 > 18:2 > 16:0 \geqslant 18:1 > 22:6$$

In competition experiments all fatty acids tested were preferentially incorporated into PC, and less into PI or PE with equimolar concentrations of lysoPC and the competing lysoPI or lysoPE. It is noteworthy, however, that the kinetics are very complicated, as changing the concentration of either the fatty acid substrate or the concentration of the acceptor substrate (lysophospholipids) changed the optimal rate of incorporation.

The concentration of lysoPC has been reported to be about 4 nmol/mg of platelet protein (McKeen *et al.*, 1981; Hamid *et al.*, 1980) or 3.7 nmol/10^9 platelets (Broekman *et al.*, 1980). This means a concentration of lysoPC of about 500–1500 μM in intact platelets, assuming a homogeneous distribution and a platelet volume about 6 fl. This gives a concentration of about 1.5 μM of endogenous lysoPC in the incubation medium. From Fig. 1(*b*), this accounts for about 0.05 nmol/min per 10^9 platelets with $C_{18:2}$ as fatty acid and less for all the other fatty acids. Thus transacylation must contribute to the results in Fig. 5 and Table 2.

Non-esterified fatty acids have been calculated to be about 1–2% of platelet cholesterol (Marcus *et al.*, 1969), which in rat platelets is about 120–140 nmol/10^9 platelets (Heemskerk *et al.*, 1989) and in human platelets about 200–300 nmol/10^9 platelets (Mendelsohn & Loscalzo, 1988; A. M. Bakken & M. Farstad, unpublished work). This means a concentration of non-esterified fatty acids in human platelets of about 300–700 μM, or close to values in human plasma (Farstad, 1967), or up to 10–100 μM for individual fatty acids.

From these data it is obvious that the present results reflect metabolic events going on *in vivo*.

We conclude that AA, EPA and linoleic acid are the dominating fatty acids incorporated into 1-acyl-lysophospholipids by platelet lysates. DHA is incorporated far less into lysoPC and lysoPE, and negligibly into lysoPI and lysoPS. Thus the availability of fatty acids may to a great extent explain much of the changes observed during various diets with great variations of the composition of fatty acids.

The higher incorporation when 1-[1-^{14}C]palmitoyl-lysoPC was the acceptor is probably due to [1-^{14}C]palmitic acid being split off by endogenous unspecific phospholipase(s) and then re-incorporated into the *sn*-2 position. More non-esterified [1-^{14}C]palmitic acid was found with [^{14}C]lysoPC in the assay and palmitic acid as the fatty acid substrate than with the other fatty acids as substrates. This may explain the relatively higher increase in [^{14}C]PC formation with palmitate than with the other fatty acids.

The effect of incubation with the position-specific PLA_2 demonstrates that the polyunsaturated fatty acids were incorporated into the *sn*-2 position, since the 1-^{14}C-labelled fatty acids were excluded completely from the phospholipid (PC).

From the present results, the content of linoleic acid in phospholipids is not fully understood. Our preliminary experiments with fatty acid uptake showed that linoleic acid was taken up at the same rate as AA and EPA. This fatty acid is very abundant in plasma, and is most rapidly incorporated into lysoPC *in vitro*. But the content in platelet phospholipids is low. Similar results were also found for chick retinal microsomes (Sellner & Philips, 1991). It is therefore likely that some other factor (or factors) is important for its distribution. Such a factor might be the effect of acyl-CoA hydrolase(s) which is very active in platelets, at least *in vitro* (Berge & Farstad, 1978).

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