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Lysocardiolipin formation and reacylation in isolated rat liver mitochondria

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Liver mitochondrial cardiolipin (CL) is distinguished from other phospholipids by the presence of linoleoyl in almost all molecular species, and the biosynthesis of these species is not yet understood. The present study was carried out in order to test the hypothesis that the linoleoyl proportion of CL may be specifically enriched by a deacylation–reacylation cycle. Incorporation of [^{14}C]glycerol 3-phosphate into the metabolites of the CL pathway was accompanied by formation of ^{14}C-labelled monolyso- and dilyso-CL. Labelling of dilyso-CL was increased or decreased by stimulation or inhibition respectively of mitochondrial phospholipase A_2. These data suggest a rapid deacylation of newly formed [^{14}C]CL by phospholipase A_2, whereas endogenous mitochondrial CL was very resistant to hydrolytic degradation. Unlike dilyso-CL, monolyso-CL could be reacylated by [^{14}C]linoleoyl residues. [^{14}C]Linoleoyl incorporation into CL was also observed when exogenous CL was added instead of monolyso-CL, thus indicating the concerted action of de- and re-acylation. Although 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine was a suitable acyl donor under experimental conditions, the reaction was not a transacylation but required splitting of [^{14}C]linoleic acid from phosphatidylcholine and formation of [^{14}C]linoleoyl-CoA as an intermediate. The [^{14}C]linoleoyl was mainly bound to the *sn*-2(2″) position of CL, and a small portion (about 20%) to the *sn*-1(1″) position. It is concluded that a cycle, comprising CL deacylation and monolyso-CL reacylation by linoleoyl-CoA, provides a potential mechanism for the remodelling of molecular species of newly formed CL.

INTRODUCTION

Cardiolipin (CL) is the most specific lipid constituent of the mitochondrial inner membrane. It interacts with several enzymes and carriers in a way that makes it an essential membrane component for respiration and oxidative phosphorylation (Daum, 1985). The structural basis and the functional implications of CL–protein interactions are not completely understood, but magnetic resonance studies have shown that CL has a higher affinity than other phospholipids for binding to mitochondrial membrane proteins such as cytochrome oxidase (Cable & Powell, 1980; Powell *et al.*, 1987) or the ATP/ADP carrier (Beyer & Klingenberg, 1985; Drees & Beyer, 1988).

CL is unique in that it consists of two phosphatidyl residues which are linked by a glycerol bridge, and it therefore carries four acyl chains. Among the acyl chains of heart and liver CL, a high percentage of $C_{18:2}$ has been found (Hostetler, 1982) and separation of the molecular species of their diacylglycerol residues demonstrated a striking predominance of C_{18}–C_{18} double-unsaturated species (Schlame *et al.*, 1990). Likewise, the acyl groups of plant CL essentially consist of $C_{18:1}$, $C_{18:2}$ and $C_{18:3}$ (Bligny & Douce, 1980; Edman & Ericson, 1987). There is now preliminary evidence suggesting that the characteristic acyl composition might be crucial for conferring high binding affinity to proteins (Schlame *et al.*, 1990) as well as enzyme-stimulating activity (Dale & Robinson, 1988) to CL. However, this conclusion awaits further confirmation.

The biosynthetic pathway of CL is entirely confined to mitochondria, starting with the acylation of glycerol 3-phosphate in the outer membrane. The subsequent steps are localized on the inner membrane, involving formation of the intermediates phosphatidyl-CMP, phosphatidylglycerophosphate and phosphatidylglycerol (Hostetler, 1982; Daum, 1985). It seems very unlikely that the characteristic acyl species of CL are already present during formation of the CL precursors (Rüstow *et al.*, 1989). Only the last step of CL formation, i.e. phosphatidyl transfer from phosphatidyl-CMP to phosphatidylglycerol, was reported to prefer $C_{18:1}$–$C_{18:1}$ and $C_{18:2}$–$C_{18:2}$ to long-chain disaturated species (Hostetler *et al.*, 1975; McMurray & Jarvis, 1980), but saturated/unsaturated and short-chain disaturated species were even better substrates (Hostetler *et al.*, 1975). Thus it is tempting to speculate that newly formed CL undergoes an acyl remodelling by deacylation and subsequent reacylation in order to produce the specific diacyl combinations $C_{18:2}$–$C_{18:2}$ and $C_{18:2}$–$C_{18:1}$. However, although acyl-CoA-dependent reacylation of dilyso-CL could be demonstrated in liver microsomes, the process was not specific for $C_{18:2}$-CoA and was almost inactive in mitochondria (Eichberg, 1974). Another obstacle to a CL remodelling might arise from the fact that endogenous CL, unlike mitochondrial phosphatidylcholine and phosphatidylethanolamine, was hardly deacylated upon activation of phospholipase A_2 (PLA_2) (De Winter *et al.*, 1987).

The present paper approaches the problem of lyso-CL formation and reacylation in rat liver mitochondria. It is shown that labelled monolyso- and dilyso-CL appear during biosynthesis of [^{14}C]CL from [^{14}C]glycerol 3-phosphate. Moreover, direct evidence is presented of a deacylation–reacylation cycle by which [^{14}C]linoleoyl is incorporated into exogenous CL added to mitochondria.

Abbreviations used: CL, 1,3-bis-(3-*sn*-phosphatidyl)-*sn*-glycerol (cardiolipin); monolyso-CL, 1-phosphatidyl-3-lysophosphatidyl-*sn*-glycerol or 1-lysophosphatidyl-3-phosphatidyl-*sn*-glycerol; dilyso-CL, 1,3-bis-(lysophosphatidyl)-*sn*-glycerol; PLA_2, phospholipase A_2.

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EXPERIMENTAL

Materials

Rat liver mitochondria were isolated by a standard differential centrifugation procedure (Steinbrecht & Kunz, 1970) in a medium containing sucrose (0.25 mol/l), EDTA (0.2 mmol/l), Tris (0.02 mol/l; pH 7.4) and 2-mercaptoethanol (1 mmol/l). Microsomes were pelleted from the post-15000 *g* supernatant by centrifugation at 100000 *g* for 90 min. ATP, CTP, GTP, AMP, CoA, Hepes, cysteine, glutamate and malate were purchased from Boehringer (Mannheim, Germany), and linoleic acid, linoleoyl-CoA, palmitoylcarnitine, PLA_2 (from *Vipera russeli*; 6 units/mg of protein), succinyl-CoA synthetase (13.5 units/mg of lyophilized powder), Triton X-100 and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO, U.S.A.). Succinic acid and sodium pyrophosphate were from Merck (Darmstadt, Germany). Commercial bovine heart CL was also obtained from Sigma. *sn*-[U-^{14}C]Glycerol 3-phosphate, 1-palmitoyl-2-[1-^{14}C]linoleoyl-phosphatidylcholine and [1-^{14}C]linoleic acid were obtained from Amersham. 1-Acyl-2-[1-^{14}C]linoleoyl-phosphatidylethanolamine was synthesized by acylation of 1-acyl-2-lyso-phosphatidylethanolamine with [1-^{14}C]linoleic acid in the presence of ATP, CoA and rat liver microsomes (Baba *et al.*, 1986). Silica gel 60 plates (20 cm × 20 cm) and silica gel 60 h.p.t.l.c. plates with concentrating zone (10 cm × 10 cm) were purchased from Merck. Silica gel H/florisil (49:1, w/w) layers of 0.5 mm thickness were made on 20 cm × 20 cm defatted glass plates.

Preparation of lyso-CLs

Lyso-CLs were produced by enzymic hydrolysis of bovine heart CL as described by Eichberg (1974), except that PLA_2 from *Vipera russeli* was used (0.2 mg of enzyme/mg of CL). Hydrolysis in ethanol was used to selectively enrich monolyso-CL, while hydrolysis in a two-phase buffer/diethyl ether system was employed to enrich dilyso-CL. However, conversion was not complete and the reaction mixtures were separated by one of the h.p.l.c. methods shown in Fig. 1. The procedure in Fig. 1(*b*) was originally developed by Dale & Robinson (1988), but we were able to replace u.v. detection by a refractive index detector, which allowed the use of less-purified solvents. Monolyso- and dilyso-CL were identified by their chromatographic behaviour in different t.l.c. systems (Wood & Harlow, 1969; Reers & Pfeiffer, 1987). Identity of lyso-CLs was also corroborated by acetylation with acetic anhydride in dry pyridine, which yielded a product running at about the same height as authentic CL on silica gel 60 h.p.t.l.c. plates developed by chloroform/methanol/water (65:25:4, by vol.).

Incorporation of [^{14}C]glycerol 3-phosphate into mitochondrial lipids

Freshly isolated mitochondria, corresponding to 2.3 mg of protein, were incubated at 37 °C in 0.2 ml of medium containing 10 μmol of Hepes (pH 7.8), 200 nmol of EDTA, 2 μmol of cysteine, 33.5 nmol of CoA, 30 μmol of KCl, 2 μmol of $MgCl_2$, 1 μmol of $CoCl_2$, 1 μmol of ATP, 800 nmol of glutamate, 800 nmol of malate, 800 nmol of KH_2PO_4, 500 nmol of CTP and 100 nmol of [^{14}C]glycerol 3-phosphate (specific radioactivity 20 d.p.m./pmol). Incubation was started by addition of mitochondria and stopped by lipid extraction.

Measurement of lyso-CL reacylation

Mitochondria or microsomes, corresponding to 1.6 mg of protein, were added to 0.3 ml of medium containing 10 μmol of Tris (pH 7.4), 4 μmol of 2-mercaptoethanol, 90 nmol of EDTA, 60 nmol of CoA, 1.2 μmol of ATP and 1.4 μmol of $MgCl_2$. 1-Palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine (200000 d.p.m.; specific radioactivity 122320 d.p.m./nmol) was added as acyl donor and CL (12 nmol/mg of protein) or monolyso-CL or dilyso-CL (5 nmol/mg of protein) was added as acyl acceptor. The lipids were dried under a stream of N_2 and sonicated (30 W, 30 s) in the buffer before other additions were made. Incubation, performed at 37 °C, was started by addition of membranes and stopped by lipid extraction.

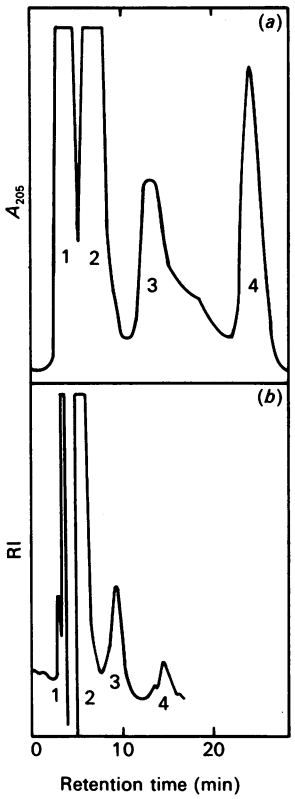

Fig. 1. Two h.p.l.c. methods for separation of CL and lyso-CLs

(*a*) Separation on a Lichrosorb 5 Si 60 column (250 mm × 5 mm) developed by n-hexane/propan-2-ol/water (55:42:3, by vol.) for the first 10 min. From 10 to 30 min a linear gradient was run, changing the solvent mixture to final proportions of 22:15:3 (by vol.). Hewlett–Packard chromatograph 1084 B was operated at a flow rate of 1 ml/min and a u.v. detector was set at 205 nm. (*b*) Isocratic elution on a Polygosil 60 (10 μm) column (250 mm × 5 mm) with cyclohexane/propan-2-ol/H_3PO_4 (0.05 mol/l) (50:50:3.6, by vol.). The flow rate was 0.9 ml/min and a refractive index (RI) detector (ERMA Optical Works, Ltd., Tokyo, Japan) was used at a thermostat temperature of 30 °C. Peak 1, solvent front and non-esterified fatty acids; 2, CL; 3, monolyso-CL; 4, dilyso-CL.

Measurement of mitochondrial PLA_2

PLA_2 activity of mitochondrial membranes was measured at 37 °C as liberation of [^{14}C]linoleic acid from 1-acyl-2-[^{14}C]linoleoyl-phosphatidylethanolamine, essentially as described by De Winter *et al.* (1982). The incubation medium contained 50 μmol of Tris (pH 8.5), 5 μmol of $CaCl_2$, 100 nmol of 1-acyl-2-[^{14}C]linoleoyl-phosphatidylethanolamine (specific radioactivity 1000 d.p.m./nmol), mitochondrial membranes (0.75 mg of protein) and various amounts of CL, lyso-CLs and Triton X-100 in a volume of 0.5 ml.

Lipid analysis

Lipids were extracted according to Bligh & Dyer (1959) using 0.01 M-HCl instead of water in order to facilitate extraction of acidic phospholipids. Lipids were separated by two-dimensional

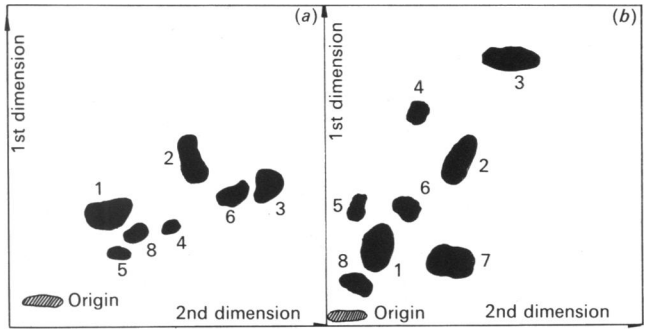

Fig. 2. Two-dimensional t.l.c. separation of mitochondrial phospholipids supplemented by lyso-CLs, phosphatidylglycerol and phosphatidic acid

(*a*) Silica gel 60-precoated plates (20 cm × 20 cm) were developed with chloroform/methanol/water (65:25:4, by vol.) in the first direction and with chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, by vol.) in the second direction. (*b*) Silica gel H/florisil (49:1, w/w) plates were developed as in (*a*), except that the composition of the second solvent was 10:4:2:2:1 (by vol.). The positions of the solvent fronts are indicated by arrows. Lipid spots, stained by iodine vapour, were identified as phosphatidylcholine (1), phosphatidylethanolamine (2), CL (3), monolyso-CL (4), dilyso-CL (5), phosphatidylglycerol (6), phosphatidic acid (7) and phosphatidylinositol (8).

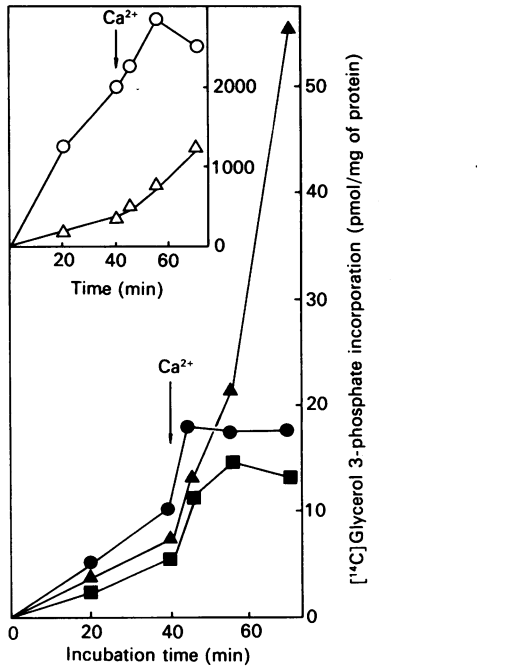

Fig. 3. [^{14}C]Glycerol 3-phosphate incorporation into CL, CL precursors and lyso-CLs

Rat liver mitochondria were incubated in order to incorporate [^{14}C]glycerol 3-phosphate as described in the Experimental section. After 40 min, $CaCl_2$ (20 mmol/l) was added and the incubation was continued for 30 min. The main Figure shows ^{14}C radioactivity incorporation into CL (●), monolyso-CL (■) and dilyso-CL (▲); the inset shows ^{14}C radioactivity incorporation into phosphatidic acid (○) and phosphatidylglycerol (△).

t.l.c. as shown in Fig. 2. These separations proved to be well suited to resolving CL, its lyso compounds, phosphatidylglycerol, phosphatidic acid and the endogenous mitochondrial phospholipids. For studies of [^{14}C]linoleoyl transfer system A was employed, whereas [^{14}C]glycerol 3-phosphate-labelled lipids were analysed using system B. Lipid spots were stained by iodine vapour and radioactivity was determined as described previously (Schlame *et al.*, 1986).

Protein content (Lowry *et al.*, 1951) and phospholipid phosphorus (Hallermayer & Neupert, 1974) were determined according to standard procedures.

RESULTS

Synthesis of lyso-CLs in isolated mitochondria

[^{14}C]Glycerol 3-phosphate may be incorporated into lipids of the CL pathway if mitochondria are supplied with CTP and cofactors of acyl-CoA generation. In four independent experiments, incorporation of [^{14}C]glycerol was in the order phosphatidic acid (40–125 pmol/min per mg of protein) > phosphatidylglycerol (5–25 pmol/min per mg of protein) > CL (0.25–0.55 pmol/min per mg of protein). T.l.c. analysis of mitochondrial lipids was made in the presence of purified monolyso- and dilyso-CL in order to trace labelling of these compounds. After 40 min of incubation, 5–10 pmol of monolyso-CL was synthesized per mg of protein, whereas dilyso-CL was labelled in the range 7–76 pmol of [^{14}C]glycerol/mg. One of these experiments is shown in Fig. 3. Addition of $CaCl_2$ stimulated labelling of dilyso-CL (Fig. 3, Table 1), thereby increasing the [^{14}C]dilyso-CL/[^{14}C]CL ratio by 2.7 ± 1.2-fold ($n = 4$) after 30 min of Ca^{2+} incubation. During parallel incubation in the absence of $CaCl_2$ the labelling rate of all phospholipids remained constant or declined slightly. This stimulation by Ca^{2+} suggests that [^{14}C]dilyso-CL was produced by PLA_2-mediated hydrolysis of newly formed [^{14}C]CL. Under the same incubation conditions, there was no decrease in the endogenous CL content (results not shown), indicating that endogenous CL was not significantly deacylated.

In order to demonstrate the involvement of PLA_2 in [^{14}C]dilyso-CL formation, attempts were made to inhibit this enzyme. Since Reers & Pfeiffer (1987) reported that monolyso-CL is a very specific PLA_2 inhibitor, and since this lysolipid was expected to be non-toxic for other biochemical reactions involved in the formation of dilyso-CL, it seemed to be well suited for this purpose. In fact, applying the same assay conditions as used by Reers & Pfeiffer (1987), we were able to confirm inhibition of mitochondrial PLA_2 at a very low monolyso-CL concentration. The maximal inhibition reached was 70% and half-maximal inhibition required only 1.4 μmol of monolyso-CL/litre, as estimated from the double-reciprocal plot of inhibition versus inhibitor concentration (Fig. 4). Reers & Pfeiffer (1987) found half-maximal inhibition to be at about 0.4 μmol/l, also when using bovine heart monolyso-CL. In Fig. 4 it is shown that CL was also inhibitory to mitochondrial PLA_2, albeit with 10 times lower potency than monolyso-CL. The inhibitory effect of monolyso-CL was then employed to study the role of PLA_2 in dilyso-CL formation. We found that monolyso-CL virtually abolished the Ca^{2+}-stimulated rate of [^{14}C]glycerol 3-phosphate incorporation into dilyso-CL (Table 1).

The above-mentioned experiments on PLA_2 inhibition were performed with a Triton X-100 concentration of 0.1 mg/ml. It should be noted that the effect of monolyso-CL on PLA_2 as well as the PLA_2 activity itself were dependent on the Triton concentration. At a higher Triton concentration (1 mg/ml) both CL and monolyso-CL (25 μmol/l) stimulated rather than inhibited mitochondrial PLA_2 (results not shown). Stimulation of purified mitochondrial PLA_2 by CL has previously been observed (Lenting *et al.*, 1988).

Table 1. Stimulation by Ca^{2+} and inhibition by monolyso-CL of dilyso-[^{14}C]CL formation in isolated liver mitochondria

Rat liver mitochondria were incubated in order to incorporate [^{14}C]glycerol 3-phosphate as described in the Experimental section. After a 30 min preincubation, additions were made as indicated and the incubation was continued for a further 30 min. Incorporation of [^{14}C]glycerol 3-phosphate into dilyso-CL is shown (two independent experiments). Labelling of CL and monolyso-CL remained virtually constant after preincubation.

Experiment no.	Condition	[^{14}C]Glycerol 3-phosphate incorporated into dilyso-CL (pmol/30 min per mg of protein)
1	Preincubation	57
	No addition	32
	+10 mM-$CaCl_2$ +Triton X-100 (0.05 mg/ml)	105
	+10 mM-$CaCl_2$ +0.5 μM-monolyso-CL +Triton X-100 (0.05 mg/ml)	58
2	Preincubation	34
	No addition	19
	+5 mM-$CaCl_2$ +Triton X-100 (0.05 mg/ml)	127
	+5 mM-$CaCl_2$ +4 μM-monolyso-CL +Triton X-100 (0.05 mg/ml)	44

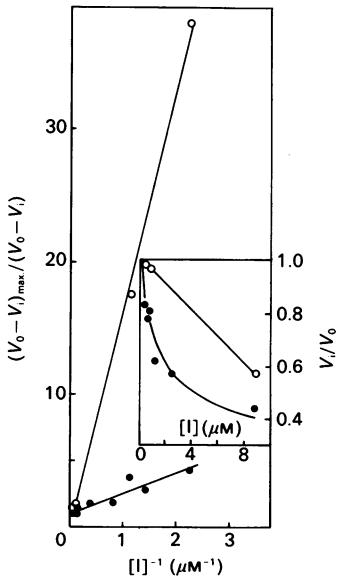

Fig. 4. Inhibition of mitochondrial PLA_2 by CL and monolyso-CL

Mitochondrial PLA_2 activity was measured as described in the Experimental section, using a Triton concentration of 0.1 mg/ml. Either CL (○) or monolyso-CL (●) was added at various concentrations. The main Figure shows a double-reciprocal plot of inhibition (V_0-V_i) versus inhibitor concentration [I]. V_0 is non-inhibited PLA_2 activity; V_i is PLA_2 activity at the respective inhibitor concentration; $(V_0-V_i)_{max.}$ represents maximal inhibition. Linear regression analysis revealed correlation coefficients of 0.999 (○) and 0.945 (●) respectively. The inset is a normal plot of relative activity (V_i/V_0) versus inhibitor concentration [I].

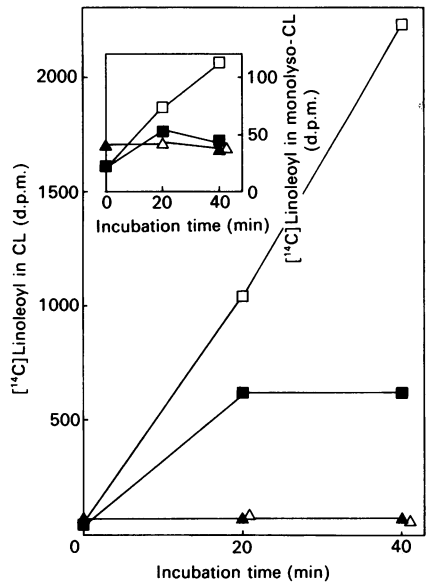

Fig. 5. Reacylation of monolyso-CL in isolated liver mitochondria

Mitochondria were incubated with 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine as acyl donor and either monolyso-CL (□, ■) or dilyso-CL (△, ▲) as acyl acceptor. Incorporation of [^{14}C]linoleoyl into CL (main Figure) or monolyso-CL (inset) is shown. □, △, Incubation in the presence of MgATP and CoA; ■, ▲, incubation without MgATP and CoA. For further details, see the Experimental section.

Deacylation–reacylation cycle of CL

Initial experiments to study lyso-CL reacylation, performed in the presence of [^{14}C]linoleic acid, MgATP and CoA, did not yield reproducible incorporation of radioactivity into CL (results not shown). However, high background levels of radioactivity made evaluation of these experimental data very difficult. When 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine was used as an alternative acyl donor, liver mitochondria catalysed the [^{14}C]linoleoyl acylation of monolyso-CL, which led to a labelling of CL (39±14 d.p.m./min, mean±S.E.M., $n = 2$). This labelling rate was reduced to 25±3% where MgATP and CoA were omitted from the incubation mixture (Fig. 5). Some [^{14}C]linoleoyl was also measured in monolyso-CL (Fig. 5 inset) indicating that a small portion of [^{14}C]CL, formed by the reacylation of monolyso-CL, was again hydrolysed by PLA. In contrast, dilyso-CL was not reacylated under these conditions, since neither of its possible reacylation products, monolyso-CL or CL, was produced (Fig. 5).

The apparent existence of a cycle comprising monolyso-CL acylation and CL deacylation prompted us to study the transfer of [^{14}C]linoleoyl in the presence of exogenous CL instead of lyso-CL (Fig. 6). The transfer of radioactivity from 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine to CL and to monolyso-CL showed that the same cycle may be initiated by CL deacylation followed by monolyso-CL reacylation. Compared with mitochondria, liver microsomes possessed a low activity of [^{14}C]linoleoyl incorporation into CL (Fig. 6).

Further experiments (Table 2) were designed to study the mechanism of [^{14}C]linoleoyl transfer from phosphatidylcholine to CL. CL labelling was markedly reduced in the absence of MgATP and CoA (incubations B and C in Table 2). Although the omission of CoA alone did not result in a decrease of [^{14}C]linoleoyl incorporation (incubation D in Table 2), depletion of the endogenous CoA pool by a CoA-consuming pre-incubation (incubation E in Table 2) significantly suppressed the

CL labelling. This led us to the conclusion that the [^{14}C]linoleoyl transfer from 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine to CL was not a direct transacylation but rather implied splitting of [^{14}C]linoleoyl from phosphatidylcholine and generation of [^{14}C]linoleoyl-CoA. Further evidence was obtained from incubation F (Table 2), showing that product inhibition of acyl-CoA synthesis decreased [^{14}C]linoleoyl incorporation into CL. A decrease in the labelling rate was also brought about by addition of non-labelled linoleoyl-CoA (incubation G) or linoleic acid (incubation H), suggesting that [^{14}C]linoleoyl had to pass through the non-esterified fatty acid and the acyl-CoA pool. Accordingly, there was [^{14}C]linoleoyl labelling of the non-esterified fatty acid fraction during these incubations (results not shown). In the absence of exogenous CL (incubation I) [^{14}C]linoleoyl transfer was reduced by more than 70%, demonstrating that endogenous CL was not a suitable substrate for the deacylation–reacylation cycle.

In order to study the positional distribution of the incorporated [^{14}C]linoleoyl residues, [^{14}C]CL was isolated and subjected to PLA_2 hydrolysis. The results showed that the majority of [^{14}C]linoleoyl was incorporated into the *sn*-2(2″) position, while a minor but significant portion (18%) was esterified to the *sn*-1(1″) position (Table 3). In contrast, the linoleoyl distribution in endogenous mitochondrial CL was characterized by a preference of the *sn*-1(1″) over the *sn*-2(2″) position.

DISCUSSION

Mitochondrial CL of rat liver has a unique pattern of molecular species (Schlame *et al.*, 1990) which is not matched by its precursors in the '*de novo*' pathway (Rüstow *et al.*, 1989). In the

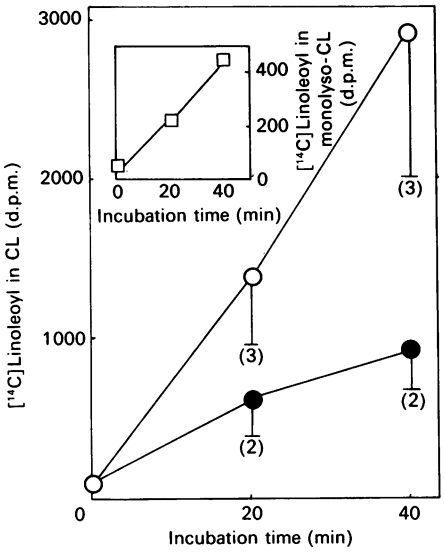

Fig. 6. Linoleoyl transfer from 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine to CL in liver mitochondria and microsomes

Mitochondria (○) or microsomes (●) were incubated with exogenous CL, 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine, CoA and MgATP as described in the Experimental section. [^{14}C]Linoleoyl incorporation into CL is shown as average radioactivity (±S.E.M.) from (*n*) independent experiments. The inset shows incorporation of radioactivity into monolyso-CL during an experiment with mitochondria.

Table 2. Linoleoyl transfer from 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine to CL under various incubation conditions

Rat liver mitochondria were incubated with 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine, CL, MgATP and CoA as described in the Experimental section. The complete assay (A = control) was modified as indicated in the Table. [^{14}C]Linoleoyl incorporation into CL is expressed as a percentage of the control (average values±S.E.M. of *n* experiments). The following concentrations were used for the additional components: AMP and sodium pyrophosphate, 9 mmol/l; linoleoyl-CoA, 85 μmol/l; linoleic acid, 0.15 mmol/l.

Composition of incubation mixture	[^{14}C]Linoleoyl incorporation	*n*
(A) CL+MgATP+CoA (control)	100	3
(B) CL	23±2	3
(C) CL+CoA	44±19	3
(D) CL+MgATP	105±9	3
(E) CL+MgATP/preincubation for CoA depletion*	41±4	2
(F) CL+MgATP+AMP+pyrophosphate	63±5	2
(G) CL+MgATP+linoleoyl-CoA	48±2	2
(H) CL+MgATP+CoA+linoleic acid	56±6	3
(I) MgATP+CoA	24±4	3

* Mitochondria were depleted of endogenous CoA by 15 min preincubation (37 °C) with 0.7 μmol of GTP, 1.8 μmol of succinate and 0.1 unit of succinyl-CoA synthetase per mg of mitochondrial protein.

Table 3. Distribution of [^{14}C]linoleoyl and endogenous linoleoyl among the *sn*-1(1″) and *sn*-2(2″) positions of CL

[^{14}C]CL was isolated after [^{14}C]linoleoyl incorporation (see the legend to Fig. 6) and was subjected to hydrolysis by pig pancreas PLA_2 in a buffer/diethyl ether two-phase system (see the Experimental section). Original [^{14}C]CL and hydrolysed [^{14}C]CL were subject to t.l.c., and radioactivity was counted in the spots of CL, dilyso-CL (representing the *sn*-1 position) and non-esterified fatty acids (representing the *sn*-2 position). The same hydrolysis procedure was applied to non-labelled mitochondrial CL, and separated products were subjected to fatty acid analysis by gas chromatography of their methyl esters as described (Schlame *et al.*, 1986).

T.l.c. fraction	Distribution of [^{14}C]linoleoyl (d.p.m.)		Endogenous fatty acyl composition (%)					
	Original [^{14}C]CL	[^{14}C]CL hydrolysed by PLA_2	$C_{18:2}$	$C_{18:1}$	$C_{16:1}$	$C_{16:0}$	$C_{20:3}$	$C_{18:0}$
CL	3601	50						
Dilyso-CL	36	763	66.9	6.3	8.0	8.9	1.5	2.7
Non-esterified fatty acids	72	3425	30.7	24.2	5.0	13.6	2.6	5.2

present paper it is shown that linoleoyl, the most abundant acyl group of CL, may be incorporated into this lipid by a deacylation–reacylation cycle.

One basic requirement of such a cycle is the formation of lyso-CL mediated by PLA_2. Although there was no measurable deacylation of endogenous mitochondrial phospholipids in our incubation system, we observed the appearance of labelled monolyso- and dilyso-CL during [^{14}C]CL synthesis from [^{14}C]glycerol 3-phosphate (Fig. 3). Lyso-[^{14}C]CLs were most probably produced by deacylation of [^{14}C]CL, since the alternative pathway, namely reaction of lysophosphatidylglycerol with lysophosphatidyl-CMP, could not be confirmed by lysophosphatidyl[^{14}C]glycerol incorporation studies (results not shown). Thus it seems that newly formed CL can be actively deacylated, whereas endogenous mitochondrial CL is very resistant to hydrolytic degradation (see also De Winter *et al.*, 1987).

Stimulation by Ca^{2+} or inhibition by monolyso-CL of mitochondrial PLA_2 resulted in an increase or decrease respectively of [^{14}C]glycerol 3-phosphate incorporation into dilyso-CL (Table 1). Although this supports an involvement of PLA_2 in lyso-CL formation, it does not allow conclusions to be reached about the regulation of this enzyme *in vivo*. Reers & Pfeiffer (1987) were the first to describe monolyso-CL as a specific PLA_2 inhibitor, a finding which was confirmed in the presence of Triton X-100 (0.1 mg/ml) (Fig. 5). The low effective inhibitor concentration seems to exclude the possibility that monolyso-CL changes the physical state of the phospholipid substrate, but rather suggests a direct interaction with the PLA_2. CL, though less potent, was also inhibitory to PLA_2 (Fig. 5), which is perhaps indirectly caused by CL deacylation to monolyso-CL. However, in the presence of Triton X-100 at 1 mg/ml both CL and monolyso-CL stimulated mitochondrial PLA_2. It is not known at present how Triton X-100 affects the susceptibility to lipid effectors of mitochondrial PLA_2, but it may be speculated that the composition and shape of the phospholipid/Triton micelles modulate the enzyme local environment, which might change the exposure of lipid-binding sites. The present study was not aimed at investigating the effect of CL and lyso-CL on PLA_2 under different conditions, and more work is needed to find out whether these phospholipids might possibly be involved in physiological PLA_2 regulation.

The question which arises with regard to the formation of lyso-CLs in mitochondria is whether or not they may be reacylated. Implicit in this is the problem as to whether monolyso- or dilyso-CL, or both, may serve as acyl acceptors, as well as the problem of the suitable acyl donor. Dilyso-CL reacylation has been studied by Eichberg (1974), who found some activity in microsomes but negligible activity in mitochondria from rat liver. Likewise, we did not observe mitochondrial reacylation of dilyso-CL, whereas monolyso-CL was actively reacylated by [^{14}C]linoleoyl (Fig. 5). In these experiments 1-palmitoyl-2-[^{14}C]linoleoyl-phosphatidylcholine was used as acyl donor, since previous experiments revealed difficulties in achieving reproducible incorporation of [^{14}C]linoleic acid in the presence of MgATP and CoA. However, it was clearly established that the [^{14}C]linoleoyl transfer from phosphatidylcholine to CL was not a transacylation, but rather involved a non-esterified fatty acid and an acyl-CoA intermediate (Table 2). At the present stage, it would be premature to assign to phosphatidylcholine the function of physiological linoleoyl donor for CL, although its molecular species contain a high proportion of $C_{16:0}$–$C_{18:2}$ and $C_{18:0}$–$C_{18:2}$ (Schlame *et al.*, 1988). Interestingly, Arthur *et al.* (1987) demonstrated lysophosphatidylcholine reacylation in guinea pig heart mitochondria to be highly specific for linoleoyl. Thus the involvement of phosphatidylcholine in the supply of linoleoyl residues for CL is conceivable, but cannot be proven by the available evidence.

Exogenous CL, added to liver mitochondria, was deacylated to monolyso-CL, which was in turn immediately reacylated to CL (Fig. 6). This is the first demonstration of a CL/monolyso-CL cycle which seems well suited for remodelling of CL molecular species. Assuming that acyl remodelling is the physiological function of this cycle, newly synthesized CL should be its natural substrate. Exogenous CL was used only for the purpose of simulating a metabolically accessible CL pool, since endogenous CL was rather resistant to hydrolysis by mitochondrial PLA_2. The present data (Fig. 3) clearly support the idea that newly synthesized CL may become the subject of PLA_2-catalysed hydrolysis, which is the first step in a potential remodelling process. However, it is not known why [^{14}C]CL, synthesized from [^{14}C]glycerol 3-phosphate *de novo*, was mainly deacylated to dilyso-[^{14}C]CL rather than to monolyso-[^{14}C]CL. It is possible that, under non-physiological incubation conditions, excessive deacylation of [^{14}C]CL prevented the accumulation of monolyso-[^{14}C]CL and, consequently, no reacylation took place. More work is needed to provide evidence for the existence of a CL deacylation–reacylation cycle *in vivo* as well as investigation of its acyl specificity and its physiological significance. The low microsomal activity of [^{14}C]linoleoyl incorporation into CL (Fig. 6) supports the assumption that the CL/monolyso-CL cycle is a specific mitochondrial process.

In contrast with other mammalian phospholipids, which essentially fit the 1-saturated-2-unsaturated configuration, the majority of CL linoleoyl is esterified to the *sn*-1(1″) carbon position and a lesser portion to the *sn*-2(2″) position (Table 3; Wood & Harlow, 1969). Thus a CL remodelling has to account for linoleoyl incorporation into both ester positions. In fact, we found about 20% of [^{14}C]linoleoyl to be linked to the 1(1″)-C and 80% to the 2(2″)-C. Although this finding is consistent with the primary occurrence of A_2-type phospholipase in liver mitochondria (Van den Bosch, 1980), additional action of a PLA_1 or partly positional isomerization of monolyso-CL is required to account for labelling of the *sn*-1 ester position. Although the experiment does not entirely explain the preferred *sn*-1(1″) esterification of linoleoyl in endogenous CL, it demonstrates that the deacylation–reacylation cycle is principally capable of associating linoleoyl with both ester positions.

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REFERENCES

Arthur, G., Page, L. L., Zaborniak, C. L. & Choy, P. C. (1987) Biochem. J. **242**, 171–175
Baba, A., Onoe, H., Ohta, A. & Iwata, H. (1986) Biochim. Biophys. Acta **878**, 25–31
Beyer, K. & Klingenberg, M. (1985) Biochemistry **24**, 3821–3826
Bligh, E. G. & Dyer, W. J. (1959) Can. J. Biochem. **37**, 911–917
Bligny, R. & Douce, R. (1980) Biochim. Biophys. Acta **617**, 254–263
Cable, M. B. & Powell, G. L. (1980) Biochemistry **19**, 5679–5686
Dale, M. P. & Robinson, N. C. (1988) Biochemistry **27**, 8270–8275
Daum, G. (1985) Biochim. Biophys. Acta **822**, 1–42
De Winter, J. M., Vianen, G. M. & Van den Bosch, H. (1982) Biochim. Biophys. Acta **712**, 332–341
De Winter, J. M., Lenting, H. B. M., Neys, F. W. & van den Bosch, H. (1987) Biochim. Biophys. Acta **917**, 169–177
Drees, M. & Beyer, K. (1988) Biochemistry **27**, 8584–8591
Edman, K. & Ericson, I. (1987) Biochem. J. **243**, 575–578
Eichberg, J. (1974) J. Biol. Chem. **249**, 3423–3429
Hallermayer, G. & Neupert, W. (1974) Hoppe-Seyler's Z. Physiol. Chem. **355**, 279–288

Hostetler, K. Y. (1982) in Phospholipids (Hawthorne, J. N. & Ansell, G. B., eds.), pp. 481–492, Elsevier, Amsterdam
Hostetler, K. Y., Galesloot, J. M., Boer, P. & Van den Bosch, H. (1975) Biochim. Biophys. Acta **380**, 382–389
Lenting, H. B. M., Neys, F. W. & Van den Bosch, H. (1988) Biochim. Biophys. Acta **961**, 129–138
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
McMurray, W. C. & Jarvis, E. C. (1980) Can. J. Biochem. **58**, 771–776
Powell, G. L., Knowles, P. F. & Marsh, D. (1987) Biochemistry **26**, 8138–8145
Reers, M. & Pfeiffer, D. R. (1987) Biochemistry **27**, 8038–8041
Rüstow, B., Schlame, M., Rabe, H., Reichmann, G. & Kunze, D. (1989) Biochim. Biophys. Acta **1002**, 261–263
Schlame, M., Rüstow, B., Kunze, D., Rabe, H. & Reichmann, G. (1986) Biochem. J. **240**, 247–252
Schlame, M., Rabe, H., Rüstow, B. & Kunze, D. (1988) Biochim. Biophys. Acta **958**, 493–496
Schlame, M., Horvath, L. & Vigh, L. (1990) Biochem. J. **265**, 79–85
Steinbrecht, I. & Kunz, W. (1970) Acta Biol. Med. Germ. **25**, 731–747
Van den Bosch, H. (1980) Biochim. Biophys. Acta **604**, 191–246
Wood, R. & Harlow, R. D. (1969) Arch. Biochem. Biophys. **135**, 272–281

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