The acylation of lysophosphoradylglycerocholines in guinea-pig heart mitochondria

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The importance of the deacylation–reacylation pathway for attaining the desired fatty acid composition in microsomal phospholipids has been well established. It is not clear, however, whether this mechanism is of equal importance in mitochondria. The absence of acyltransferase activity in mammalian heart mitochondria has been reported in a number of studies. In the present study we report the presence of acyltransferase activities for lysophosphoradylglycerocholines in guinea-pig heart mitochondria. This enzyme showed properties that were considerably different from those of the microsomal enzymes. Of all the acyl-CoAs tested ($C_{18:0}$, $C_{18:1}$, $C_{18:2}$ and $C_{20:4}$) the mitochondrial enzyme utilized only linoleoyl-CoA as fatty acyl donor and utilized both 1-acyl-*sn*-glycero-3-phosphocholine and 1-alkenyl-*sn*-glycero-3-phosphocholine as fatty acyl acceptors. The presence of significant quantities of fatty acids other than linoleate at the C-2 position of mitochondrial acylglycerophosphocholines, coupled with the specificity of the enzyme for linoleoyl-CoA, suggest that, in addition to reacylation, other mechanisms play a significant role in producing the molecular composition of these phospholipids found in the mitochondria.

INTRODUCTION

The acylation of lysophospholipids serves a number of important functions in the cell. It is part of the mechanism for the remodelling of the fatty acyl chains of cellular phospholipids, and it also prevents the accumulation of cellular lysophospholipids, which are potent cytolytic agents. The reaction is catalysed by the action of acyltransferases, which have been well studied over the last two decades. Although acyltransferase activities have been detected in most mammalian tissues, a number of questions remain unresolved. It is not certain whether a specific acyltransferase is required for the acylation of a given lysophospholipid, nor is it known whether the acyl specificities of the activities arise from the broad specificity of one enzyme or a combination of enzymes each responsible for the transfer of a particular acyl group.

The subcellular localization of the acyltransferases has also been the subject of debate. Although the enzyme has been shown to exist in the microsomal fraction of most tissues, its presence in other subcellular fractions has only been suggested. In rat liver, the enzyme activity was initially reported to be exclusively microsomal [1], but subsequent studies placed some activity in the mitochondrial fraction [2]. However mitochondria from rat small-intestinal epithelium did not possess any acyltransferase activity towards 1-acyl-GPC [3]. In guinea-pig and rat cerebral cortex, acyltransferase activity towards 1-acyl-GPC was found solely in the microsomal fraction [4,5] whereas in the same tissue of the rabbit the enzyme activity was present in both the microsomal and nuclear fractions [6]. There are also discrepancies in the subcellular localization of acyltransferases in the mammalian heart. In rabbit heart the enzyme was reported to be exclusively microsomal by one group [7] and

ubiquitously distributed among all subcellular fractions by another [8]. The enzyme was detected in both the microsomal and cytosolic fractions of rat myocytes [9].

In most mammalian hearts, a substantial portion of the major phospholipids exist in plasmalogen form [10]. The presence of acyl-CoA:1-alkenyl-GPC acyltransferase in the heart in addition to acyl-CoA:1-acyl-GPC acyltransferase was recently reported [11]. These two enzyme activities appear to be attributable to separate enzyme proteins. With oleoyl-CoA as the acyl donor, both enzyme activities were detected exclusively in the microsomal fraction of the guinea-pig heart. However, the presence of acyltransferase activities in other subcellular fractions cannot be entirely ruled out, because these enzymes may be highly acyl-specific and may not utilize oleoyl-CoA. Here we report the presence of a distinct acyltransferase in the guinea-pig heart mitochondrial fraction. The enzyme has the ability to acylate both 1-acyl-GPC and 1-alkenyl-GPC and is highly specific for linoleoyl-CoA.

MATERIALS AND METHODS

Materials

Sucrose, Tris/HCl, Tris/succinate, EDTA, EGTA, stearoyl-CoA, oleoyl-CoA, and linoleoyl-CoA were obtained from Sigma Chemical Co. Arachidonyl chloride and stearic acid were purchased from NuChek Prep, Elysian, MN, U.S.A. Santoquin and 5,5'-dithiobis-(2-nitrobenzoic acid) were obtained from ICN Pharmaceuticals. CoA was purchased from Pharmacia. [$1-^{14}C$]Stearoyl-CoA, [$1-^{14}C$]oleoyl-CoA, [$1-^{14}C$]linoleoyl-CoA and [$1-^{14}C$]arachidonyl-CoA were obtained from Amersham International. Lysophosphatidylcholine and phosphatidylcholine (pig liver) were the products of

Abbreviations used: GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine.

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Serdary Laboratories, London, Ontario, Canada. All other chemicals and solvents were of reagent grade and were obtained from Fisher Scientific.

Methods

Preparation of subcellular fractions. Guinea pigs weighing 200–250 g were used throughout the study. The mitochondrial, microsomal and cytosolic fractions were prepared from guinea-pig hearts by differential centrifugation of the heart homogenate and the purity of each subcellular fraction was analysed by marker enzymes as described previously [12]. Protein in the subcellular fractions was determined by the procedure of Lowry *et al.* [13].

Preparation of lysoplasmenylcholine. Plasmenylcholine (1-alkenyl-2-acyl-glycerophosphocholine) was isolated from bovine heart lipid extract [12] and lysoplasmenylcholine was obtained from the hydrolysis of purified plasmenylcholine by the method of Renkonen [14]. The lysoplasmenylcholine formed was purified by silicic acid column chromatography, and the purity of the lipid was assessed by t.l.c. [11]. The alkenyl content of the lipid was determined by iodine uptake as described by Gottfried & Rapport [15]. Lipid phosphorus was measured by the procedure of Bartlett [16].

Preparation of arachidonyl-CoA. Arachidonyl-CoA was synthesized chemically as described previously [11], and the product was found to be 100% active as determined by the procedure of Lands *et al.* [17].

Enzyme assays. Acyl-CoA:1-alkenyl-GPC acyltransferase and acyl-CoA:1-acyl-GPC acyltransferase activities were assayed essentially as described previously [11]. The assay mixture (0.7 ml) contained 52.5 μmol of Tris/HCl, pH 7.0, 0.1 μmol of lysophospholipid substrate, 32.3 nmol of acyl-CoA and 0.05–0.10 mg of enzyme protein. Labelled acyl-CoA was used for the assays. The reaction mixture was incubated at 25 °C for 30 min and the reaction was terminated by the addition of 3 ml of chloroform/methanol (2:1, v/v). Water (0.8 ml) was added to each tube and the contents mixed. The tubes were centrifuged and the aqueous phase removed. Aliquots of the lower phase were subjected to t.l.c. followed by removal of the acylglycerophosphocholine spot. The amount of radioactivity incorporated was determined by scintillation counting.

RESULTS

Guinea-pig heart microsomal and mitochondrial fractions were isolated by differential centrifugation and the purity of the fractions was assessed by marker enzymes [12]. The mitochondrial fractions had about 14% of microsomal material, whereas the microsomes were contaminated with 5% of mitochondrial material. The cytosolic fraction contained less than 1% of the microsomal and mitochondrial fractions. The mitochondrial fractions were assayed for 1-acyl-GPC and 1-alkenyl-GPC acyltransferase activities with arachidonyl- and linoleoyl-CoA. The activity obtained with linoleoyl-CoA was much higher than with arachidonyl-CoA for both acyltransferase reactions. Thus linoleoyl-CoA was used as the acyl donor to characterize the two

Fig. 1. Effect of pH on guinea-pig heart mitochondrial 1-acyl-GPC acyltransferase and 1-alkenyl-GPC acyltransferase activities

1-acyl-GPC acyltransferase (●) and 1-alkenyl-GPC acyltransferase (▲) activities were determined at different pH values. The buffers (75 mM) used were Tris/succinate (pH 5–6) and Tris/HCl (pH 7–10). Enzyme activities were determined with 50 μg of protein for the 1-acyl-GPC acyltransferase and 100 μg of protein for the 1-alkenyl-GPC acyltransferase. Linoleoyl-CoA (46 μM) was used as the acyl donor and incubations were for 30 min at 25 °C. Each point in the Figure is the mean value for three individual sets of experiments, each of which was performed in duplicate. The S.D. of each point is less than 10% of the mean value.

mitochondrial acyltransferase activities with respect to reaction time, protein concentration and pH.

At 25 °C the reaction rate was linear with up to 250 μg of protein and with incubation times of 5–45 min. The pH profile of the 1-acyl-GPC and 1-alkenyl-GPC acyltransferase activities are shown in Fig. 1. Both acyltransferase activities had similar pH profiles and both had a broad optimum at about pH 7. The effect of 1-acyl-GPC and 1-alkenyl-GPC concentrations on their respective acyltransferase activities were determined. From the Lineweaver–Burk plots the apparent K_m of 1-acyl-GPC acyltransferase activity for 1-acyl-GPC as 12.4 μM , whereas the K_m of the 1-alkenyl-GPC acyltransferase for 1-alkenyl-GPC was 92.2 μM . The effect of Ca^{2+} and Mg^{2+} (0–10 mM) on the two mitochondrial acyltransferase activities were studied with linoleoyl-CoA as the acyl donor. Both activities were slightly inhibited by Ca^{2+} , but were unaffected by Mg^{2+} .

The acyl-CoA specificities of the mitochondrial 1-acyl-GPC and 1-alkenyl-GPC acyltransferase activities were examined with stearoyl-CoA, linoleoyl-CoA and arachidonyl-CoA as fatty acyl donors. The results obtained are shown in Figs. 2 and 3 respectively. Although both activities showed identical acyl-CoA specificities, the 1-acyl-GPC acyltransferase activity was much higher than the 1-alkenyl-GPC acyltransferase activity, regardless of the acyl-CoA substrate. Linoleoyl-CoA was the preferred substrate for both acyltransferase activities, followed by arachidonyl-CoA. Stearoyl-CoA proved to be a very poor substrate for both acyltransferases and was hardly utilized by the 1-alkenyl-GPC acyltransferase, even at relatively high concentrations.

Fig. 2. Acyl-CoA specificity of guinea-pig heart mitochondrial 1-acyl-GPC acyltransferase

The activity of acyl CoA:1-acyl-GPC acyltransferase in the presence of various concentrations of acyl-CoA were determined. The assay mixture (0.7 ml) contained 100 nmol of 1-acyl-GPC, 50 μ g of mitochondrial protein and 75 mM-Tris/HCl, pH 7.0. Other assay conditions were as described in Fig. 1. The acyl-CoA species used in the assays were linoleoyl-CoA (●), arachidonyl-CoA (▲) and stearoyl-CoA (■). Each point in the Figure is the mean result for three separate sets of experiments, each of which was performed in duplicate. The s.d. for each point is less than 12% of the mean value.

Because the enzyme activities obtained with arachidonyl-CoA were much less than those obtained with linoleoyl-CoA, the possibility that the former activity was the result of microsomal contamination was explored. 1-Acyl-GPC and 1-alkenyl-GPC acyltransferase activities in microsomal and mitochondrial preparations were assayed at optimal linoleoyl-CoA and arachidonyl-CoA concentrations. With arachidonyl-CoA as substrate, the mitochondrial 1-acyl-GPC and 1-alkenyl-GPC acyltransferases were 12 and 14% of the microsomal activity respectively. The corresponding results obtained with linoleoyl-CoA as substrate were 61 and 51%. Since our assessment of the microsomal contamination of the mitochondria was 14%, both the 1-acyl-GPC and 1-alkenyl-GPC acyltransferase activities obtained with arachidonyl-CoA could indeed be due to microsomal contamination. In order to determine whether there was any intrinsic acyltransferase activity towards arachidonyl-CoA in the mitochondria, the following experiments were carried out. Equal amounts (19 μ g of protein) of mitochondria were added to various amounts of microsomes (0–25 μ g of protein), and for each combination of subcellular fractions the total acyltransferase activity and the quantity of microsomes present (indicated by glucose-6-phosphatase activity) were determined. Arachidonyl-CoA was used as the acyl donor in one set of experiments and oleoyl-CoA was used in another set. The latter was used as a positive control, since the activity was previously shown to be absent from

Fig. 3. Acyl-CoA specificity of guinea-pig heart mitochondrial 1-alkenyl-GPC acyltransferase

The activity of acyl CoA:1-alkenyl-GPC acyltransferase was determined in the presence of various concentrations of acyl-CoA. The assay mixture (0.7 ml) contained 100 nmol of 1-alkenyl-GPC and 100 μ g of mitochondrial protein. Other assay conditions and the symbols for the acyl-CoA species used are as described in Fig. 2. Each point in the Figure is the mean result for three separate sets of experiments, each of which was determined in duplicate. The s.d. for each point is less than 10% of the mean value.

the mitochondrial fraction [11]. The results obtained are displayed in Fig. 4. The sets of points were analysed by linear regression and yielded correlation coefficients of 0.962 and 0.956 for oleoyl-CoA and arachidonyl-CoA respectively (1.0 for perfect correlation). Thus there appears to be no intrinsic 1-acyl-GPC acyltransferase activity in the mitochondria that utilizes oleoyl- or arachidonyl-CoA. Similar conclusions were arrived at for the 1-alkenyl-GPC acyltransferase activity.

Because of the use of intact mitochondria for our experiments, it is possible that acyltransferase activities that utilized acyl-CoA species other than linoleoyl-CoA were present in the organelle in latent form and were not properly exposed to the substrates. To investigate this possibility, 1-acyl-GPC and 1-alkenyl-GPC acyltransferase activities in intact and disrupted (by sonication for 2–3 min) [18] mitochondrial fractions were determined with oleoyl-, linoleoyl- and arachidonyl-CoA. No differences in acyltransferase activities were observed between disrupted and intact mitochondria.

The near-identical properties exhibited by the mitochondrial 1-acyl-GPC and 1-alkenyl-GPC acyltransferase activities (pH profile, effects of cations, specificity for linoleoyl-CoA) led us to investigate whether the two activities might be catalysed by the same enzyme. Thus the effect of heat treatment at 55 °C on 1-alkenyl-GPC and 1-acyl-GPC acyltransferases (using linoleoyl-CoA as acyl donor) was studied. The results obtained are shown in Fig. 5. The percentage loss of activity with heating time was virtually identical for the two mitochondrial acyltransferases; 70% of both acyltransferase activities were lost after 3 min of heat treatment.

Fig. 4. Variation of 1-acyl-GPC acyltransferase activity with glucose-6-phosphatase activity in guinea-pig heart mitochondrial fractions

1-Acyl-GPC acyltransferase activity and glucose-6-phosphatase activities were determined in a mixture containing different amounts of guinea-pig heart microsomal fractions (0–25 μ g of protein) and a fixed amount of mitochondrial fractions (19 μ g of protein). Arachidonyl-CoA (●) and oleoyl-CoA (○) were used as acyl donors. The assay conditions were as described in Fig. 2. The first point on each line is the activity obtained with mitochondria alone. Each point is the mean value obtained from two separate sets of experiments, each of which was performed in duplicate. The slopes and intercepts were obtained by linear-regression analysis.

Fig. 5. Effect of heat treatment on 1-acyl-GPC acyltransferase and 1-alkenyl-GPC acyltransferase activities in guinea-pig heart mitochondria

Guinea-pig heart mitochondria (100 μ g of protein) were incubated at 55 °C for various time periods. The activities of acyl-GPC acyltransferase (●) and 1-alkenyl-GPC acyltransferase (▲) activities were determined after the heat treatment with linoleoyl-CoA (46 μ M) as the acyl donor. The results are expressed as percentages of enzyme activities without heat treatment (100%). Each point is the mean value of two separate sets of experiments, each of which was determined in duplicate. The S.D. of each point is less than 5% of the mean value.

Kinetic studies were next performed to determine the inhibitory effect of 1-alkenyl-GPC on 1-acyl-GPC acyltransferase activity. 1-Acyl-GPC acyltransferase activity was assayed at different concentrations (17.8–107 μ M) of 1-acyl-GPC in the presence of fixed amounts of 1-alkenyl-GPC (0, 35.7 and 71.4 μ M). The

Fig. 6. Lineweaver-Burk plot of acyl CoA:1-acyl-GPC acyltransferase activity and 1-acyl-GPC concentrations in the presence of 1-alkenyl-GPC

Enzyme activity was determined with various concentrations (17.8–107 μ M) of 1-acyl-GPC at 0 μ M (●), 35.7 μ M (■) and 71.4 μ M (▲) 1-alkenyl-GPC. Activities were determined at 25 °C for 30 min with 50 μ g of mitochondrial protein and 46 μ M-linoleoyl-CoA. Enzyme activity is expressed as nmol of product formed/h per mg of protein. Each point is the mean of two separate sets of experiments, each of which was assayed in duplicate. The slopes and intercepts were obtained by linear-regression analysis. Correlation coefficients were 0.99, 0.98 and 0.96 for lines A, B and C respectively.

acylglycerophosphocholines formed from the reaction were subjected to acid hydrolysis (for the removal of plasmenylcholine formed) and the products were separated by t.l.c. Radioactivity in the phosphatidylcholine fraction was determined. The Lineweaver-Burk plots (Fig. 6) indicate that the acylation of 1-acyl-GPC was inhibited by 1-alkenyl-GPC in a competitive manner.

DISCUSSION

We recently reported the presence of 1-alkenyl-GPC: and 1-acyl-GPC: acyl-CoA acyltransferase activities in the guinea-pig heart [11]. With oleoyl-CoA as the acyl donor we demonstrated that these two activities were localized exclusively in the microsomal fraction of the guinea-pig heart. In the present study we utilized linoleoyl- and arachidonyl-CoA to probe for the presence of acyltransferases in the guinea-pig heart mitochondrial fraction. Our results demonstrate quite conclusively that 1-acyl-GPC and 1-alkenyl-GPC acyltransferase activities exist in the mitochondrial fraction of the guinea-pig heart. These activities differ from the microsomal acyltransferases in a number of respects. Whereas the microsomal acyltransferases utilized oleoyl-, linoleoyl- and arachidonyl-CoA [11], the mitochondrial acyltransferases were specific for linoleoyl-CoA and did not have the ability to utilize oleoyl- or arachidonyl-CoA. This specificity was demonstrated with both intact and disrupted mitochondria, thus eliminating the possible existence of latent acyltransferase activities. Both mitochondrial acyltransferase activities were unaffected by Mg^{2+} and slightly inhibited by Ca^{2+} , whereas in the microsomes both cations inhibited 1-acyl-GPC acyltransferase activity and activated the 1-alkenyl-GPC acyltransferase activity [11]. Our results suggest that the

acyltransferase activities in the mitochondria are different from the ones in the microsomes. The existence of a mitochondrial acyltransferases in the guinea-pig heart with a completely different acyl specificity from the microsomal enzymes might explain the discrepancies reported in the subcellular localization of these enzymes and their reported absence in the mitochondria in the heart and other tissues [3-7,9,11]. In most studies only one acyl-CoA was utilized to probe for the activity. Another distinct feature of the mitochondrial enzyme is that the acylation of both 1-acyl-GPC and 1-alkenyl-GPC appears to be catalysed by a single enzyme. This is in contrast with the situation in the microsomal fraction, where the two enzyme activities appear to reside on different proteins.

In guinea-pig heart mitochondria, linoleic acid is the major fatty acid at the C-2 position of both phosphatidylcholine and plasmenylcholine [19]. Thus the deacylation-reacylation mechanisms involving phospholipase A₂ and the linoleoyl-CoA-specific acyltransferase activity might play a major role in achieving the high proportion of linoleoyl acylglycerophosphocholine species. However, the specificity of the enzyme for linoleoyl-CoA poses problems as to how the organelle acquires and maintains the substantial quantities of plasmenylcholine with arachidonate (at the C-2 position) or phosphatidylcholine with oleate. Although the mechanism by which mitochondria obtain their acylglycerophosphocholines remains unclear, it has been assumed that these phospholipids originate in the microsomes [20]. The presence of acyltransferase activity with a high degree of specificity for linoleoyl-CoA suggests that either a selection of molecular species takes place at the level of the transporting mechanism or, alternatively, enzymes such as transacylases might play a major role in providing the desired molecular species of acylglycerophosphocholines in the mitochondria. A CoA-independent transacylase has recently been described in dog heart microsomes that catalyses the transfer of fatty acids from phosphatidylcholine to 1-acyl-GPE and 1-alkenyl-GPE [21]. It is not known if such transacylases exist in cardiac mitochondria or whether the transfer of fatty acids from other phospholipids to 1-alkenyl-GPC and 1-acyl-GPC occurs in the organelle.

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