Acyl-CoA: 2-acetyl-sn-glycero-3-phosphocholine (GPC) acyltransferase is required for the maintenance of the asymmetric distribution of saturated fatty acids at the C-1 position and unsaturated fatty acids at the C-2 position [1,2]. The maintenance of this asymmetric distribution has been postulated to be due to a deacylation-reacylation mechanism involving phospholipase A₁, phospholipase A₂, acyl-CoA:1-acyl-GPC acyltransferase and acyl-CoA:2-acyl-GPC acyltransferase [1,2]. Phosphatidylcholines isolated from cardiac membranes possess this asymmetric distribution of fatty acids [3-5]. Previous studies from this laboratory have demonstrated the presence of acyl-CoA:1-acyl-GPC acyltransferase and acyl-CoA:1-alkenyl-GPC acyltransferases in guinea-pig heart microsomal and mitochondrial fractions [6,7]. Evidence was presented to support the non-identity of the two enzymes in the microsomes, whereas in the mitochondria the same activity appeared to be responsible for the acylation of 1-acyl-GPC and 1-alkenyl-GPC. The preference of these enzyme activities for unsaturated acyl-CoAs suggested their involvement in maintaining the high levels of unsaturated fatty acids at the C-2 position of choline glycerophospholipids in both subcellular fractions.

Studies on the acylation of 2-acyl-GPC have lagged well behind those on 1-acyl-GPC; however, acyl-CoA:2-acyl-GPC acyltransferase has been described in rat liver microsomes [8-11], where a greater selectivity for saturated acyl-CoAs compared with unsaturated acyl-CoAs were reported [8,9,11]. This specificity of the enzyme was taken as an indication of its involvement in the maintenance of the asymmetric distribution of saturated acyl groups in the phospholipid. Since cardiac phosphatidylcholine possesses this asymmetric distribution, it was somewhat surprising that acyl-CoA:2-acyl-GPC acyltransferase activity was reported to be absent in rat, ox and guinea-pig heart microsomes [9], suggesting the absence of this activity in cardiac tissue.

The present study was therefore undertaken to investigate if indeed acyl-CoA: 2-acyl-GPC acyltransferase was absent from cardiac microsomes, using the guineapig heart as our model, and also to assess the involvement of the enzyme, if present, in the maintenance of the asymmetric distribution of saturated fatty acids in cardiac phosphatidylcholine. In addition, because to our knowledge acyl-CoA:2-acyl-GPC acyltransferase has not been described in mitochondria from any mammalian tissue, it was considered worthwhile to investigate the presence of this activity in the mitochondria.

MATERIALS AND METHODS

[5,6,8,9,11,12,14,15-3H]Arachidonic acid and [9,10(n)-3H]palmitic acid were obtained from Amersham International. Lyso phosphatidylcholine (1-acyl-GPC) was the product of Serdary Laboratories, London, Ontario, Canada. Palmitic acid, arachidonic acid, linoleoyl chloride and arachidonoyl chloride were purchased from NuChek Prep, Elysian, MN, U.S.A. 1-Palmitoyl-2-arachidonoyl-GPC, diarachidonoyl-GPC and dipalmitoyl-GPC were obtained from Avanti Polar Lipids (Birmingham, AL, U.S.A.), Santequin and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) were the products of ICN Pharmaceuticals. Phospholipase A₁ [from snake (Crotalus adamanteus) venom], palmitoyl-, stearoyl- and oleoyl-CoA were obtained from Sigma Chemical Co. Guinea pigs (250-300 g) were obtained from Charles River, Ontario, Canada. DEAE-Sepharose and CoA were purchased from Pharmacia. All other chemicals and solvents were of reagent grade and were obtained from Fisher Scientific.

Abbreviations used: GPC, sn-glycero-3-phosphocholine; DTT, dithiothreitol; NEM, N-ethylmaleimide; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).
Subcellular fractionation

Microsomes and mitochondria were prepared from six to eight guinea-pig hearts by differential centrifugation [4]. The protein content was measured by the method of Lowry et al. [12]. The activities of fumarase and succinate dehydrogenase were utilized as mitochondrial enzyme markers and glucose-6-phosphatase and K⁺-stimulated p-nitrophenyl phosphatase as microsomal markers [4].

Synthesis of phosphatidylcholine

The synthesis and purification of 1-acyl-2-[³H]-arachidonoyl- and 1-acyl-2-[³H]-palmitoyl-GPC was carried out as previously described [13] with 1-acyl-GPC and radiolabelled arachidonic and palmitic acids as precursors for the synthesis.

Preparation and characterization of 2-acyl-GPC

The preparation of 2-acyl-GPC was carried out by hydrolysis of the parent phospholipid with guinea-pig pancreas phospholipase A₁ [13]. The specific radioactivities of 2-[³H]arachidonoyl-GPC was 0.17 Ci/mol and that of 2-[³H]palmitoyl-GPC was 0.57 Ci/mol. The substrates were characterized by incubating the purified 2-palmitoyl-GPC and 2-arachidonoyl-GPC with phospholipase A₁ as described by Colard et al. [14]. Control incubations with 1-lyso-3-phosphatidylcholine, 1-[¹⁴C]palmitoyl and 1-acyl-2-[³H]arachidonoyl-GPC were carried out simultaneously. Further characterization involved the incubation of 2-palmitoyl-GPC (214 µM) and 2-arachidonoyl-GPC (214 µM) with 75 mM-Tris/HCl, pH 7, 141 µM-arachidonoyl-CoA, and 100 µg of guinea-pig liver microsomes in a reaction volume of 700 µl. After incubation at 37 °C for 30 min the reaction was stopped by the addition of chloroform/methanol (2:1, v/v) followed by 800 µl of 0.9% KCl. After removal of the upper phase, phosphatidylcholine marker was added to the lower phase and spotted on Sil-G25 precoated t.l.c. plates (Machery–Nagel). The plates were developed in a solvent system of chloroform/methanol/water/acetic acid (35:15:2:1, by vol.). The plates were sprayed with dichlorofluorescein and the phosphatidylcholine fraction eluted by the method of Arvidson [15]. This fraction was then incubated with 50 units of phospholipase A₁ (from Crotalus adamanteus) as previously described [4] for 30 min at 37 °C. The ether was evaporated with nitrogen and the reaction products were separated on Redi plates using a double-development system [16]. The fatty acid and lysophosphatidylcholine bands were revealed with iodine vapour and the radioactivity associated was determined by liquid-scintillation counting.

Preparation of arachidonoyl- and linoleoyl-CoA

Arachidonoyl- and linoleoyl-CoA were synthesized from their respective chlorides by the method described by Reitz & Lands [17].

Acyl-CoA:2-acyl-GPC acyltransferase assay

The incubation mixture contained 75 mM-Tris/HCl, pH 7.0, 150 nmol of 2-acyl-GPC, 50 µg of microsomal protein or 100 µg of mitochondrial protein and acyl-CoA in a total volume of 0.7 ml. The concentration of acyl-CoA used depended on the species, and the values used in each experiment are indicated in the appropriate Figure legends. 2-Acyl-GPC with radiolabelled fatty acids were utilized for the assays. The reaction was started with the addition of the subcellular fraction. Incubations were carried out at 37 °C for 10 min and the reaction was stopped by the addition of 3 ml of chloroform/methanol (2:1, v/v). A 0.8 ml portion of KCl (0.9%) was added and, after mixing, the two phases were clarified by centrifugation. The upper phase was removed by suction, and aliquots of the lower phase were removed for analysis by t.l.c. (Sil-G25) with a solvent system of chloroform/methanol/water/acetic acid (35:15:2:1, by vol.). The radioactivity associated with the phosphatidylcholine band was measured by liquid-scintillation counting. Incubations without acyl-CoA were carried out under identical conditions, and the values were taken as representing the transacylase activity. These values were subtracted from the results obtained in the presence of acyl-CoA to give the true acyl-CoA acyltransferase activities. In experiments carried out with mixed acyl-CoA species (arachidonoyl- and palmitoyl-CoA), the molecular species of phosphatidylcholine were separated on AgNO₃-impregnated plates, revealed by dichlorofluorescein and extracted by the procedures described by Arvidson [15]. Aliquots of the eluted lipids were then taken for liquid-scintillation counting.

RESULTS

The correct interpretation of the data obtained in the present study depends on ensuring that the lysophospholipids utilized were indeed 2-acyl-GPC species. The experiments undertaken to characterize the substrates have been described in the Materials and methods section. When purified 2-palmitoyl-GPC or 2-arachidonoyl-GPC were incubated with guinea-pig pancreas phospholipase A₁, there was no release of labelled fatty acid. In contrast, radiolabelled fatty acid was released from 1-[¹⁴C]palmitoyl-GPC. Incubation of the phospholipase with 1-stearoyl-2-[³H]arachidonoyl-GPC resulted in the formation of labelled lysophosphatidylcholine. When the substrates were acylated to phosphatidylcholine and subsequently incubated with Crotalus adamanteus phospholipase A₂, 98 ± 2% and 97 ± 2% of the label in the reaction products from 2-C₁₆:0-fatty acid-GPC and 2-C₁₄:0-fatty acyl-GPC respectively were associated with the fatty acid fraction. The results are the means of four experiments, each of which was carried out in duplicate. The results clearly demonstrate that neither the synthesis nor the purification of the 2-acyl-GPC resulted in significant acyl migration. The substrates utilized in the studies could therefore be designated as '2-acyl-GPC'.

In contrast with the reported absence of acyl-CoA:2-acyl-GPC acyltransferase activity in guinea-pig heart microsomes [9], our exploratory studies with these microsomes using 2-[³H]arachidonoyl-GPC or 2-[³H]palmitoyl-GPC as acyl acceptors, showed the formation of phosphatidylcholine. When the phosphatidylcholine was isolated and characterized by hydrolysis with Crotalus adamanteus phospholipase A₂ as described above, the results showed that 98% of the label was associated with the fatty acid fraction, indicating that an active acyl-CoA:2-acyl-GPC acyltransferase activity was present in the microsomes. The acylation of 2-arachidonoyl-GPC by guinea-pig heart microsomes was studied with palmitoyl-CoA as the acyl donor. The acylation reaction was characterized with respect to pH,
reaction temperature, time, protein concentration and 2-arachidonoyl-GPC concentration. The subsequent assays were conducted at the optimal conditions obtained. These were pH 7, a 2-arachidonoyl-GPC concentration of 200 μM, 50 μg of protein and incubations for 10 min at 37 °C for the microsomal fraction. The presence of acyl-CoA:2-acyl-GPC acyltransferase activity in guinea-pig heart mitochondria was also investigated. The activities obtained with the mitochondria were considerably lower (7–15 %, depending on the acyl-CoA species) than those obtained for the microsomes. Furthermore the characteristics of the activity were identical with those of the microsomal activity. Our marker-enzyme analysis indicated a 10 % contamination of the mitochondria by microsomes. These observations suggested that activities obtained with the mitochondrial fraction could be due to the contaminating microsomes, and there was either no indigenous mitochondrial activity or an extremely low one indeed.

The acyl-CoA specificity of the microsomal acyl-CoA:2-acyl-GPC acyltransferase was investigated with 2-arachidonoyl-GPC as the acyl acceptor, and the results are displayed in Fig. 1(a). At acyl-CoA concentrations of 24 μM or lower, the order of decreasing enzyme activity was as follows: oleoyl-CoA > stearoyl-CoA > palmitoyl-CoA = linoleoyl-CoA > arachidonoyl-CoA. At concentrations greater than 24 μM, stearoyl- and palmitoyl-CoA inhibited the acylation reaction. The activity with oleoyl-CoA peaked at 50 and 100 μM, whereas activities with arachidonoyl-CoA and linoleoyl-CoA increased with increasing concentrations; at 186 μM the activities obtained with linoleoyl- and arachidonoyl-CoA were 7 and 6 μmol of phosphatidylcholine formed/h per mg of protein respectively.

Because the above observations were quite distinct from those obtained for the acyl-CoA:1-acyl-GPC acyltransferase in the guinea-pig heart microsomes [6], which were assayed with 1-palmitoyl-CoA as the acyl acceptor, it was of interest to determine whether the differences in specificities between the enzymes were due to the differences in the acyl composition of the lysophospholipid rather than the position of the fatty acid. 2-[3H]Palmitoyl-GPC was therefore synthesized and the acyl specificity of the microsomal (Fig. 1b) acyltransferase determined. With the microsomal preparation, the specific activities obtained with 2-palmitoyl-GPC were 2–6 times less than those obtained with 2-arachidonoyl-GPC, depending on the acyl-CoA species. Despite the decreased rates of acylation of 2-palmitoyl-GPC, the acyl-CoA specificities of the enzyme with 2-palmitoyl-GPC as the acyl acceptor were virtually identical with that obtained with 2-arachidonoyl-GPC as acyl acceptor.

Experiments were also conducted with mixtures of arachidonoyl-CoA and palmitoyl-CoA at concentrations of 10 μM and 45 μM of each acyl-CoA and 2-palmitoyl- or 2-arachidonoyl-GPC as acyl acceptors. The above values were selected because the results displayed in Figs. 1(a) and 1(b) indicated that at lower concentrations the enzyme did not show a preference for either CoA species, whereas at higher concentrations a greater utilization of arachidonoyl-CoA was observed. The results obtained with the mixed CoAs are depicted in Tables 1(a) and 1(b). Regardless of the acyl-acceptor utilized, the enzyme displayed the same acyl-CoA selectivity with the mixed CoAs as was observed with the single CoA species (Figs. 1a and 1b). Thus there was a greater utilization of arachidonoyl-CoA only at an acyl-CoA concentration of 45 μM.

The effect of cations and thioc reagents on the acylation of 2-arachidonoyl-GPC by the guinea-pig heart microsomes was investigated. Ca²⁺ (5 mM) did not significantly affect the activity; of the microsomal 2-acyl-GPC acyl-
Table 2. Effect of mixed acyl-CoA species on guinea-pig heart microsomal acyl-CoA:2-acyl-GPC acyltransferase activity

(a) Acyl-CoA:2-acyl-GPC acyltransferase activity in guinea-pig heart microsomes was assayed in a reaction mixture (700 µl) containing 75 mM-Tris/HCl, pH 7, 150 nmol of 2-palmitoyl-GPC, 50 µg of microsomal protein and 10 µM- or 45 µM-arachidonoyl-CoA (C₉₈₆₅), palmitoyl-CoA (C₁₆₆₅) or a mixture of the two acyl-CoAs. Incubations were for 10 min at 37 °C, and the reaction products were extracted into chloroform as described in the Materials and methods section. Dipalmitoyl-GPC and 1-palmitoyl-2-arachidonoyl-GPC were added as phosphatidylcholine (PC) markers and the reaction products were separated by t.l.c. Phosphatidylcholine was isolated as described in the Materials and methods section, and the disaturated and tetraenoic molecular species were separated by t.l.c. as also described in the Materials and methods section. The radioactivity associated with each molecular species was quantified by scintillation counting. The results are expressed as nmol of phosphatidylcholine formed/h per mg of protein and are means for two separate experiments each done in triplicate. (b) Acyl-CoA:2-acyl-GPC acyltransferase activity in guinea-pig heart microsomes was assayed with 150 nmol of 2-arachidonoyl-GPC as the acyl acceptor. All the other reaction conditions were as described in the legend to (a). The phosphatidylcholine markers used for this assay were 1-palmitoyl-2-arachidonoyl-GPC and diarachidonoyl-GPC. The tetraenoic and octaenoic molecular species of phosphatidylcholine in the reaction products were separated by t.l.c. as described in the Materials and methods section. The results are expressed as nmol of phosphatidylcholine formed/h per mg of protein and are means for two separate experiments each done in triplicate.

(a) 

<table>
<thead>
<tr>
<th>[Acyl-CoA]...</th>
<th>10 µM</th>
<th>45 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acyl-CoA species...</td>
<td>C₁₆₆₅</td>
<td>C₂₀₄</td>
</tr>
<tr>
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</tr>
<tr>
<td>Tetraenoic</td>
<td>106</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>93</td>
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(b) 

<table>
<thead>
<tr>
<th>[Acyl-CoA]...</th>
<th>10 µM</th>
<th>45 µM</th>
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<tbody>
<tr>
<td>Acyl-CoA species...</td>
<td>C₁₆₆₅</td>
<td>C₂₀₄</td>
</tr>
<tr>
<td>Tetraenoic</td>
<td>533</td>
<td>−</td>
</tr>
<tr>
<td>Octaenoic</td>
<td>−</td>
<td>503</td>
</tr>
</tbody>
</table>

Table 3. Effect of cations on guinea-pig heart microsomal acyl-CoA:2-acyl-GPC acyltransferase activities

Acyl-CoA:2-acyl-GPC acyltransferase was assayed in a reaction mixture containing 75 mM-Tris/HCl, pH 7, 150 nmol of 2-arachidonoyl-GPC, 50 µg of microsomal protein and 24 µM-palmitoyl-CoA in a reaction volume of 700 µl. Incubations were for 10 min at 37 °C. The reaction products were isolated as described in the Materials and methods section. The results are expressed as a percentage of the activities obtained in the presence of 2 mM-EDTA and -EGTA. The values are means ± s.d. for three separate experiments, each of which was done in duplicate.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Activity (% of control)</th>
</tr>
</thead>
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<tr>
<td>EDTA/EGTA (2 mM)</td>
<td>100</td>
</tr>
<tr>
<td>Ca²⁺ (5 mM)</td>
<td>82 ± 13</td>
</tr>
<tr>
<td>Mg²⁺ (5 mM)</td>
<td>175 ± 6</td>
</tr>
</tbody>
</table>

Experiments with a variety of thiol reagents indicated that GSH and iodoacetate did not affect the activity of the microsomal acyltransferase, whereas DTT, NEM and DTNB were inhibitory to various extents (Table 3).

DISCUSSION

It has been previously reported that acyl-CoA:2-acyl-GPC acyltransferase activity was not present in rat, ox- and guinea-pig heart microsomes [9]. In contrast the present investigation has demonstrated and characterized the presence of a very active acyl-CoA:2-acyl-GPC acyltransferase in guinea-pig heart microsomes. Although the reasons for the discrepancy between the two studies is not clear, the earlier study [9] utilized a spectrophotometric assay to measure the enzyme activity. This involved using 1 mM-DTNB [9], a compound reported here to inhibit the acyltransferase. The inhibition of acyltransferases by DTNB may also not be uniform for all substrates [18]. At non-inhibitory concentrations of the acyl-CoAs, and with 2-arachidonoyl-GPC as the acyl acceptor, the specific activities of the acyl-CoA:2-acyl-GPC acyltransferase were severalfold greater than those reported for acyl-CoA:1-acyl-GPC acyltransferase from the same subcellular fraction [6]. However, with 2-palmitoyl-GPC as the acyl acceptor, the
Table 3. Effect of thiol reagents on guinea-pig heart microsomal acyl-CoA:2-acyl-GPC acyltransferase activity

Acyl-CoA:2-acyl-GPC acyltransferase activity in guinea-pig heart microsomes was assayed in the presence of 0.5 mM thiol reagents. The microsomes were incubated in the reagents for 10 min before the addition of 2-arachidonoyl-GPC (150 nmol) and palmitoyl-CoA (24 μM) to initiate the reaction. The reaction mixture was incubated at 37 °C for 10 min and the reaction products were isolated as described in the Materials and methods section. The results are expressed as a percentage of the activity obtained in the absence of any additions. The values represent means ± S.D. for three separate experiments, each of which was done in duplicate.

<table>
<thead>
<tr>
<th>Addition (0.5 mM)</th>
<th>Activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>DTT</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>GSH</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>Iodoacetate</td>
<td>101 ± 4</td>
</tr>
<tr>
<td>NEM</td>
<td>68 ± 10</td>
</tr>
<tr>
<td>DTNB</td>
<td>76 ± 5</td>
</tr>
</tbody>
</table>

activities of the acyl-CoA:2-acyl-GPC acyltransferase and those reported for the acyl-CoA:1-acyl-GPC acyltransferase [6] were similar. The acyl-CoA specificities for the acylation of 2-acyl-GPC were distinct from those for the acylation of 1-acyl-GPC, regardless of whether 2-arachidonoyl-GPC or 2-palmitoyl-GPC was the acyl acceptor. The most notable differences were: (1) the utilization of saturated fatty acyl-CoA by the acyl-CoA:2-acyl-GPC acyltransferase at rates similar to those obtained with arachidonoyl- or linoleoyl-CoA at low concentrations of the acyl donors; (2) the utilization of oleoyl-CoA (up to concentrations < 48 μM) at rates much greater than for linoleoyl- and arachidonoyl-CoA; (3) the greater rate of utilization of linoleoyl- and arachidonoyl-CoA compared with oleoyl-CoA at concentrations greater than 48 μM. These differences between the two acyltransferases can be attributed to the differences in the position of the fatty acid on the lysophospholipid, since they persisted despite changes in the acyl composition of the 2-acyl-GPC. The distinctive differences between the acyl-CoA:2-acyl-GPC acyltransferases and the acyl-CoA:1-acyl-GPC acyltransferase suggest that these acyltransferases are different enzymes. In rat liver microsomes a similar scenario has been suggested [9,19]. The existence of acyltransferases in particulate fractions that are specific for both the acyl donor and the acyl acceptor has been postulated [1,2]; however, difficulties encountered with the purification of these enzymes have proved to be a stumbling block to obtaining direct proof of their existence. The recent successful purification of an acyltransferase from rat brain microsomes [20] that was specific for both 1-acyl-GPC and arachidonoyl-CoA has provided the strongest evidence yet of their existence. Unfortunately the ability of this enzyme to utilize 2-acyl-GPC was not examined.

Although the present study has identified the existence of acyl-CoA:2-acyl-GPC acyltransferase activity in the guinea-pig heart microsomes the enzymes did not show a distinct preference for saturated acyl-CoAs in experiments with individual acyl donors, as was expected and has been reported for the liver microsomal enzyme [8,9]. When mixtures of palmitoyl- and arachidonoyl-CoA were presented, both substrates were utilized effectively at 10 μM, as would be predicted from Figs. 1 and 2, and at 45 μM arachidonoyl-CoA was the preferred substrate. In studies with the rat liver microsomal acyl-CoA:2-acyl-GPC acyltransferase and mixed linoleoyl- and palmitoyl-CoA, Van den Bosch et al. [11] reported that the enzyme incorporated palmitate at a rate 2.7-fold greater than the incorporation of linoleate into 2-acyl-GPC. However, the authors concluded that the specificity displayed by the enzyme was a lot less than that displayed by the acyl-CoA:1-acyl-GPC acyltransferase. It has also been reported that the rat liver enzyme showed little selectivity between palmitoyl- and stearoyl-CoA [10].

Acyl-CoA:2-acyl-GPC acyltransferases are probably involved in maintaining the asymmetric distribution of saturated fatty acids in phospholipids. The greater utilization of 2-arachidonoyl-GPC relative to 2-palmitoyl-GPC by the guinea-pig heart microsomal acyl-CoA:2-acyl-GPC acyltransferase activity, is consistent with the expectation that unsaturated 2-acyl-GPC will be the natural substrates for these enzymes in vivo. From the properties of the enzyme discerned in the present study, the intracellular concentration and composition of acyl-CoAs may be significant factors in regulating the role of these enzymes in maintaining the asymmetric distribution of saturated fatty acids in guinea-pig heart microsomal phosphatidylcholine. It would therefore appear that acyl-CoA synthetase and acyl-CoA hydrolase may play a greater role in defining the fatty acid composition of phosphatidylcholine than is generally appreciated.

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