Purification, characterization and modulation of a microsomal carboxylesterase in rat liver for the hydrolysis of acyl-CoA

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A carboxylesterase containing long-chain acyl-CoA hydrolase activity was purified to apparent homogeneity from rat liver microsomes. Palmitoyl-CoA was the most preferred substrate, followed by stearoyl-CoA and oleoyl-CoA. Arachidonoyl-CoA, linoleoyl-CoA and acetyl-CoA were not hydrolysed by the enzyme. The purified enzyme had no activity on the hydrolysis of phospholipids and neutral lipids. The molecular mass of the enzyme was found to be 56 kDa by SDS/PAGE and 64 kDa by gel-filtration chromatography. On isoelectric focusing, the

purified enzyme behaved like the ES-4 type, with a pI of 6.15. Determination of the amino acid sequence revealed that its N-terminal sequence is 100% homologous with the only other known N-terminal sequence for a rat carboxylesterase isoenzyme (ES-10). Enzyme activity was inhibited by lysophosphatidic acid and activated by lysophosphatidylcholine. The modulation of enzyme activity by these lysophospholipids might represent a plausible mechanism for the physiological control of acyl-CoA concentrations.

INTRODUCTION

Carboxylesterases are widely distributed in mammalian tissues. High activities of carboxylesterases are found in the microsomal fraction of several tissues, including adipose tissue, liver and kidney [1–7]. Most of the carboxylesterases found in mammalian tissues are designated as ‘non-specific carboxylesterases’ (EC 3.1.1.1), because they have the ability to hydrolyse a variety of ester bonds, including carboxylesters, thioesters and amides [6,8]. Several genetically, electrophoretically and immunologically distinct forms of the enzyme have been purified from mammalian sources [9–12]. The different carboxylesterase isoenzymes in rat liver identified in the last decade have been categorized according to the genetic nomenclature [11] or mobility during isoelectric focusing [6]. Despite the wide distribution of the carboxylesterase, most of the known substrates for the enzyme are compounds not normally found in the body. At present, the exact physiological function of the carboxylesterase has not been defined. The ability of the liver esterase to hydrolyse xenobiotics and other synthetic compounds indicates that the enzyme may be involved in the detoxification of non-physiological metabolites in the liver [13].

Apart from their possible role in drug metabolism, these carboxylesterases may be involved in the catabolism of certain lipids. In the last decade, specific carboxylesterase has been shown to hydrolyse certain lipoidal compounds such as acylcarnitine, palmitoyl-CoA, monoacylglycerol and diacylglycerol [8,14–16]. The ability of carboxylesterases in the rat liver to hydrolyse the acyl groups of phosphatidylcholine and lysophosphatidylcholine at very low rates has been reported [14]. In addition, the ability of some non-specific carboxylesterases in rat liver microsomes to hydrolyse long-chain acyl-CoA has been demonstrated [14,15,17]. In essence, two forms of the enzyme (pI 6.2 and 6.4) within the five major forms of carboxylesterase (pI 5.2, 5.6, 6.0, 6.2 and 6.4) were shown to have the capacity to hydrolyse the long-chain acyl-CoAs. These two carboxylesterases are thought to originate from a single gene product (ES-4) which has undergone post-translational modifications [18]. The hydrolysis of these lipid materials may facilitate the transfer of fatty

acids across the endoplasmic reticulum or may prevent the accumulation of potentially membrane-lysing natural detergents [10]. In view of the importance of acyl-CoA in the biosynthesis of neutral lipids and phospholipids, the ability of this enzyme to regulate the acyl-CoA concentration in the tissues may constitute a simple mechanism for the control of lipid biosynthesis. However, the role of carboxylesterase in the regulation of lipid metabolism is largely unknown. The mechanism for the control of its hydrolytic activity towards acyl-CoA by different metabolites has not been defined.

In mammalian tissues, the presence of other non-specific esterase activities precludes the study on the characterization and modulation of carboxylesterase/acyl-CoA hydrolase activity in the rat liver microsomal fraction without further purification. In this study, we had solubilized, purified and characterized the carboxylesterase/acyl-CoA hydrolase from the rat liver microsomal fraction. The purified enzyme displayed a pI of 6.15 and its activity was found to be modulated by some key phospholipid metabolites.

MATERIALS AND METHODS

Materials

Sprague–Dawley rats of body wt. 250 ± 50 g were obtained from Charles River Canada Inc. (St. Constante, Quebec, Canada). Acetyl-CoA, palmitoyl-CoA, stearoyl-CoA, oleoyl-CoA, octyl glucopyranoside and 4-nitrophenyl acetate were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phosphatidylcholine (pig liver), lysophosphatidylcholine (pig heart), triacylglycerol (pig liver), diacylglycerol (pig liver), mono-olein, phosphatidylethanolamine (pig liver), lysophosphatidylethanolamine (pig liver), phosphatidic acid (egg), lysophosphatidic acid (egg lecithin) and cardiolipin (bovine heart) were products of Serdary Research Laboratory (London, Ontario, Canada). 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB) was obtained from ICN Pharmaceuticals. Ampholine PAGplates (pH 3.5–9.5) and isoelectric-focusing kit (pI 5.2–10.25) were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). [14 C]-

Abbreviation used: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

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Arachidonoyl-CoA was the product of NEN-DuPont. [$1\text{-}^{14}\text{C}$]Oleoyl-CoA, [$1\text{-}^{14}\text{C}$]linoleoyl-CoA, [$1\text{-}^{14}\text{C}$]palmitoyl-CoA, [$1\text{-}^{14}\text{C}$]stearoyl-CoA and [$1,3\text{-}^3\text{H}$]glycerol were products of Amersham Inc. All other chemicals were of analytical grade and obtained locally from Canlab Division of Baxter Co.

Preparation of radiolabelled lipids

^3H -labelled triacylglycerol, diacylglycerol, phosphatidylcholine and phosphatidylethanolamine were prepared from hamster heart by the method of Tardi et al. [19]. In essence, isolated hamster hearts were perfused in the Langendorff mode with Krebs-Henseleit buffer containing 0.4 mM [$1,3\text{-}^3\text{H}$]glycerol ($4\text{ }\mu\text{Ci}/\mu\text{mol}$) for 90 min. After perfusion, hearts were homogenized in chloroform/methanol (1:1, v/v) followed by centrifugation at 1000 *g* for 10 min. Water and chloroform were added to the supernatant to cause phase separation. The phospholipids and neutral lipids in the organic phase were separated by t.l.c. The solvent used for separation of phospholipids was chloroform/methanol/water/acetic acid (35:15:2:1, by vol.), whereas that for separation of neutral lipids was light petroleum (b.p. 37–56 °C)/diethyl ether/acetic acid (60:40:1, by vol.). The lipids on the chromatogram were detected by dichlorofluorescein spray (0.25% dichlorofluorescein in ethanol) and viewing under u.v. light. Fractions containing phosphatidylcholine, phosphatidylethanolamine, triacylglycerol and diacylglycerol on the t.l.c. plates were removed, and the lipids were extracted from the silica gel with 3 × 4 ml of chloroform/methanol/water/acetic acid (50:39:10:1, by vol.). The extracts were combined, and 4 ml of 4 M NH_4OH was added to the pooled extracts to cause phase separation. The upper phase was removed and the lower phase was washed with 2 × 5 ml of methanol/water (1:1, v/v). After washing, the volume of the lower phase was decreased by a stream of N_2 and the concentrated sample was stored at –20 °C. The ^3H -labelled lipids had specific radioactivities of 120 000–180 000 d.p.m./ μmol .

Enzyme assays

Several procedures were used for assay of the carboxylesterase/acyl-CoA hydrolase activities. For routine determination of the carboxylesterase activity, hydrolysis of 4-nitrophenyl acetate was used as the standard assay [20]. The reaction mixture contained 30 mM Tris/HCl buffer (pH 7.8), 1.6 mM 4-nitrophenyl acetate and a suitable amount of the enzyme preparation in a final volume of 1.0 ml. The reaction was monitored spectrophotometrically by measuring the change of A_{405} . The incubation mixture without the enzyme preparation served as a control. The absorption coefficient of $16240\text{ M}^{-1}\cdot\text{cm}^{-1}$ was used to determine the amount of 4-nitrophenol released during the reaction.

Long-chain acyl-CoA hydrolase activity was determined by a spectrophotometric assay and confirmed by the positive identification of the product by a radioisotopic method. The released free thiol reacted with DTNB and the coloured product was monitored spectrophotometrically at 412 nm [17]. The reaction mixture contained 30 mM Tris/HCl buffer (pH 7.8), 40 μM acyl-CoA, 1 mM DTNB and a suitable portion of the enzyme preparation in a total volume of 1.0 ml. The incubation mixture without addition of the enzyme preparation was used as a control. The adsorption coefficient of $13600\text{ M}^{-1}\cdot\text{cm}^{-1}$ was used to determine the amount of CoA released during the reaction [21]. In order to confirm the enzyme activity from the spectrophotometric assay, the hydrolysis of acyl-CoAs was also

determined with [$1\text{-}^{14}\text{C}$]acyl-CoAs as substrates [17]. The assay mixture (0.5 ml) contained the same components at concentrations identical with those in the spectrophotometric assay, except that DTNB was omitted. The reaction was initiated by addition of a suitable sample of enzyme preparation, and the radioactivity in unesterified fatty acid released during the reaction was determined by the procedure of Berge [17].

The ability of the purified carboxylesterase to hydrolyse different lipids was determined with labelled phosphatidylcholine, phosphatidylethanolamine, triacylglycerol and diacylglycerol (emulsified in 10 mM Tris/HCl buffer, pH 7.5, by ultra-sonication). The incubation mixture (1 ml) contained 30 mM Tris/HCl buffer (pH 7.8), 2 mM of the respective ^3H -labelled lipids and 2 μg of the purified carboxylesterase. The assay was carried out for 60 min and the reaction was terminated by addition of 2.1 ml of chloroform/methanol (2:1, v/v). After phase separation, the radioactivity in glycerophosphocholine or glycerophosphoethanolamine was determined in the upper phase. The radioactivity of lysophosphatidylcholine, lysophosphatidylethanolamine, diacylglycerol or monoacylglycerol was determined in the lower phase. Separation of the lysophospholipids in the lower phase was achieved by t.l.c. with the solvent chloroform/methanol/water/acetic acid (35:15:2:1, by vol.). The hydrolysis products of the neutral lipids were separated by t.l.c. with the solvent light petroleum (b.p. 37–56 °C)/diethyl ether/acetic acid (60:40:1, by vol.). Lysophospholipase activity was determined by the procedure of Van den Bosch et al. [22] with 1-[*palmitoyl*- $1\text{-}^{14}\text{C}$]glycerophosphocholine as substrate.

Lineweaver-Burk analyses were performed in the presence and absence of a fixed concentration of the activator or inhibitor with different concentrations of the substrate (palmitoyl-CoA). Enzyme activities versus substrate concentrations were expressed in a double-reciprocal plot. K_m and K_i values were determined from intercepts on the abscissa in the absence or presence of the inhibitor. The binding constant (K_a) of the activator was obtained by the method described by Segel [23]. For non-essential activation, the slope of the double-reciprocal plot of velocity versus palmitoyl-CoA concentration in the presence of the activator was defined as:

$$\text{Slope} = K_s/V_{\text{max}} \cdot \left(\frac{1 + [A]/K_a}{1 + \beta[A]/\alpha K_a} \right)$$

where K_s is the reciprocal of the abscissa intercept in the absence of the activator, and the constants α and β refer to the fold changes in K_m and V_{max} in the presence of saturating concentration of the activator. The value of K_a could be calculated from the above equation.

Molecular-mass determination

The molecular mass of the carboxylesterase was determined by Sephadex G-100 chromatography (1.6 cm × 90 cm column). The column was equilibrated with 10 mM Tris/HCl/100 mM KCl (pH 7.5). The following standard proteins were used for column calibration: aldolase (150 kDa), BSA (67 kDa), ovalbumin (43 kDa) and chymotrypsinogen (25 kDa). The molecular mass of the enzyme subunit was determined by SDS/PAGE with phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa) as standards.

N-terminal amino acid sequence

The amino acid residues at the N-terminus of the purified enzyme was determined by stepwise Edman degradation. The protein

sample was analysed by a gas-phase automated sequencer (Applied Biosystems 470A) coupled to a h.p.l.c. system. The amino acid residues were detected by conversion into phenylthiohydantoin derivatives. The analysis was performed by the peptide sequencing unit, University of Victoria, B.C., Canada.

Other methods

Protein concentrations were determined quantitatively by the method of Lowry et al. [24], with crystalline BSA as the standard. The molar content of lipid P in phospholipids was determined by the procedure of Bartlett [25]. The molar content of glycerol in diacylglycerol or triacylglycerol was determined by the method of Dittmer and Wells [26]. Isoelectric focusing was carried out on an Ampholine PAG plate (pH 3.5–9.5) in a LKB Multiphor IEF chamber at 8 °C. The sample-application filters were removed after 60 min at 40 V/cm and electrofocusing was allowed to continue to another 90 min at 100 V/cm. A calibration kit consisted of β -lactoglobulin A (pI 5.2), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (pI 6.85 and 7.35), lentil lectin (pI 8.15, 8.45, 8.65), trypsinogen (pI 9.3) and cytochrome *c* (pI 10.25) was employed as standards. The gel was fixed in trichloroacetic acid (11.5%, w/v) and sulphosalicylic acid (3.34%, w/v), stained in Coomassie Brilliant Blue R-250 and destained in a solution containing 8% acetic acid and 25% ethanol.

RESULTS

Purification of carboxylesterase

Rat liver microsomes were prepared by differential centrifugation [27], and a portion containing 1.5 g of protein was suspended in 10 mM Tris/HCl (pH 7.5) containing 20% ethylene glycol (buffer A) and 1 M KCl to yield a protein content of 30 mg/ml. Octyl glucoside was added to the suspension to give a final concentration of 30 mM. The solution was stirred at 0 °C for 30 min, and the suspension was centrifuged at 100 000 *g* for 60 min. The supernatant was decanted and applied to a Sepharose 6B column (80 cm $\times$ 3 cm) equilibrated with buffer A. After sample application, the column was washed with buffer A at a flow rate of 20 ml/h, and 10 ml fractions were collected. A considerable portion of the enzyme activity was eluted near the void volume. However, 20–22% of the total activity was eluted in later fractions and distinctly separated from the void volume. The active fractions collected in the second enzyme-activity peak

were pooled and concentrated by ultrafiltration to a final volume of 5–6 ml.

The concentrated sample was dialysed against buffer A (2 $\times$ 1000 ml) for 4–6 h. A portion (1 ml) of the dialysed sample was applied to a Mono Q anion-exchange column (5 cm $\times$ 0.5 cm) equilibrated with buffer A. After sample application, the column was washed with 10 ml of buffer A. A stepwise gradient of 0.5 M KCl in buffer A (buffer B) was used to elute the enzyme activity. The flow rate of the column was maintained at 1 ml/min, and fractions of volume 1 ml were collected. A major enzyme-activity peak (55% of the total activity) was eluted from the column at 6% buffer B. The remaining 45% of activity was found to distribute in two separate peaks, which were eluted at 12% and 50% buffer B respectively. The fractions containing the major activity peak were pooled and concentrated by ultrafiltration. $(\text{NH}_4)_2\text{SO}_4$ was added to this fraction to a final concentration of 1.5 M. The concentrated sample was applied to a Waters Protein Pak Phenyl-5PW column (7.5 cm $\times$ 0.8 cm) equilibrated with buffer A containing 1.5 M $(\text{NH}_4)_2\text{SO}_4$. Enzyme activity was eluted from the column by a negative gradient of $(\text{NH}_4)_2\text{SO}_4$ in buffer A (1.5–0 M in a total volume of 50 ml) at a flow rate of 1 ml/min. The active fractions were pooled and concentrated by ultrafiltration. The sample was applied to a Superose 6 column (30 cm $\times$ 1 cm) equilibrated with buffer A and 0.1 M KCl. The proteins were eluted isocratically from the column at a flow rate of 20 ml/h. The active fractions were pooled and dialysed exhaustively against 10 mM Tris/HCl (pH 6.5) containing 20% ethylene glycol (buffer C). Analysis of the protein sample by SDS/PAGE showed one major and two minor protein bands. The protein sample was applied to a Mono S cation-exchange column (5 cm $\times$ 0.5 cm) equilibrated with buffer C. The column was washed with 10 ml of buffer C at a flow rate of 1 ml/min followed by a 20 ml gradient of 0–0.5 M KCl in buffer C. The active fractions were pooled, concentrated by ultrafiltration and stored at –70 °C. A summary of the purification is given in Table 1.

Both carboxylesterase activity (for hydrolysis of 4-nitrophenyl acetate) and acyl-CoA hydrolase activity (for hydrolysis of palmitoyl-CoA) were monitored throughout the purification. As depicted in Table 1, the same degree of purification was achieved for both activities. The enzyme preparation obtained after Mono-S column chromatography had a specific activity of 66.7 μmol of product formation/min per mg of protein when assayed with 4-nitrophenyl acetate as substrate. When palmitoyl-CoA was used as a substrate, the specific activity was 3.1 μmol of product formation/min per mg of protein.

The enzyme obtained after the Mono S column chromatography was analysed in a 7.5%-polyacrylamide-gel electrophoresis system. The gel after electrophoresis was stained with Coomassie Brilliant Blue G-250. Only one protein band was detected when 1 or 5 μg of protein was used for the analysis. In a separate experiment, the unstained gel obtained under identical conditions was sliced into 0.5 cm sections, and each section was homogenized in 0.5 ml of 50 mM Tris/HCl/0.1 M KCl (pH 7.5). The homogenate was centrifuged to obtain a clear supernatant, which was used to analyse for carboxylesterase and acyl-CoA hydrolase activities. The section that corresponded to the R_f of the protein band of the stained gel contained both enzyme activities, whereas no enzyme activity was detected in the other sections.

Physicochemical properties

Analysis of the enzyme sample by SDS/PAGE under reducing conditions showed only one band, with a molecular mass of

Table 1 Purification of carboxylesterase from rat liver microsomes

Enzyme activities were determined by the ability to hydrolyse 4-nitrophenyl acetate or palmitoyl-CoA (in parentheses) as described in Materials and methods section. One unit of enzyme activity is defined as the formation of 1 μmol of 4-nitrophenol or palmitic acid/min.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Microsomes	1448	3159	2.2 (0.11)	100
Solubilized material	1042	2402	2.3 (0.11)	76
Sepharose 6B	274	465	1.7 (0.08)	15
Mono Q	35	266	7.6 (0.38)	8
Protein Pak Phenyl-5PW	3.2	85	26.6 (1.27)	3
Superose 6	1.5	68	45.5 (2.07)	2
Mono S	0.42	28	66.7 (3.10)	0.9

Figure 1 SDS/PAGE of purified rat liver carboxylesterase

The purified enzyme (5 μ g) was analysed by electrophoresis on a 7.5%-polyacrylamide gel containing 0.1% SDS (lane 1). The protein band(s) was revealed by staining with Coomassie Brilliant Blue R-250. Protein standards in lane 2 were phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa) and carbonic anhydrase (30 kDa).

56 kDa (Figure 1). Alternatively, the protein was eluted from a Sephadex G-100 column in a single symmetrical peak with an average molecular mass of 64 kDa (results not shown). The pI of the enzyme was found to be 6.15, which suggests that it was the ES-4 (4B) carboxylesterase. The N-terminal amino acid sequence of the purified protein was determined by an automated gas-phase sequencer. Although a reproducible assignment for the N-terminal residue could not be obtained, a reproducible sequence for residues 2–19 was obtained as follows: -PSSPPVDDTTK-GKVLGKY-. This is identical with the corresponding N-terminal sequence deduced from the cDNA sequence obtained for the ES-10 carboxylesterase [28].

Substrate specificity

The purified enzyme displayed high activity towards 4-nitrophenyl acetate. However, its hydrolytic activity towards acyl-CoA was considerably lower (20-fold less) than that of 4-nitrophenyl acetate. Its specificity towards different species of acyl-CoA is depicted in Figure 2. The hydrolytic activity of the enzyme was highest with palmitoyl-CoA as substrate, followed by stearoyl-CoA and oleoyl-CoA. However, the enzyme displayed no hydrolytic activity towards both linoleoyl-CoA and arachidonyl-CoA or acetyl-CoA. In addition, the enzyme had no activity towards the hydrolysis of phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, triacylglycerol and diacylglycerol (results not shown).

Modulation of carboxylesterase by phospholipids

The hydrolytic activity of the purified enzyme towards acyl-CoA was found to be modulated by different lipids (Table 2). For the

Figure 2 Hydrolysis of acyl-CoA by rat liver microsomal carboxylesterase

The hydrolysis of acyl-CoA by the enzyme was determined radiochemically at 25 °C as described in the Materials and methods section. Each assay mixture contained 0.8 μ g of purified enzyme and 40 μ M [14 C]palmitoyl-CoA (○), [14 C]stearoyl-CoA (●), [14 C]oleoyl-CoA (△), [14 C]linoleoyl-CoA (□) or [14 C]arachidonyl-CoA (▲). Each value is the mean of three separate experiments.

Table 2 Effects of lipids on the hydrolysis of acyl-CoA by rat liver microsomal carboxylesterase

Each assay mixture (1 ml) contained 0.8 μ g of purified enzyme, 40 μ M acyl-CoA and 60 μ M lipid. The enzyme activity was monitored spectrophotometrically as described in the Materials and methods section. Each value represents the mean of three separate determinations.

Lipid added	Enzyme activity (μ mol/min/mg protein)	
	Palmitoyl-CoA	Oleoyl-CoA
Control (no lipid)	3.10	1.10
Phosphatidic acid	2.39	1.10
Lysophosphatidic acid	0.50	0.36
Cardiolipin	0.70	0.36
Phosphatidylethanolamine	3.49	1.10
Phosphatidylcholine	4.04	1.65
Lysophosphatidylethanolamine	4.04	1.29
Lysophosphatidylcholine	6.30	2.03
Triacylglycerol	3.13	1.48
Diacylglycerol	3.30	1.10

hydrolysis of palmitoyl-CoA, lysophosphatidylcholine was found to be an effective activator of the enzyme, whereas lysophosphatidic acid and cardiolipin were shown to be strong inhibitors of the enzyme. In the presence of lysophosphatidylcholine (60 μ M), a 2-fold increase in enzyme activity was observed, whereas in the presence of lysophosphatidic acid (60 μ M) or cardiolipin (60 μ M) over 75 % of the enzyme activity was inhibited. Phosphatidylcholine, phosphatidylethanolamine and lysophosphatidylethanolamine also displayed an activation effect on the enzyme, but a marginal modulation effect was observed with the neutral lipids. The enzyme activity was modulated in a similar manner when oleoyl-CoA was used as substrate (Table 2).

The modulation of enzyme activity by lysophosphatidylcholine, lysophosphatidic acid and cardiolipin at different concentrations was examined. As depicted in Figure 3, the enzyme activity was activated by 0–30 μ M lysophosphatidylcholine in a dose-dependent manner. Increase in lysophosphatidylcholine concentration beyond 30 μ M did not elicit any further increase in enzyme activity. Addition of 0–80 μ M lysophosphatidic acid or

Figure 3 Effects of lysophosphatidic acid, cardiolipin and lysophosphatidylcholine on the hydrolysis of palmitoyl-CoA by rat liver microsomal carboxylesterase

The hydrolysis of palmitoyl-CoA by the enzyme was determined spectrophotometrically at 25 °C as described in the Materials and methods section. The assay mixture (1 ml) contained 0.6 μg of purified enzyme, 40 μM palmitoyl-CoA and different amounts of lysophosphatidic acid (●), cardiolipin (△) or lysophosphatidylcholine (○). Each value is the mean of three separate experiments.

Figure 4 Double-reciprocal plot of acyl-CoA hydrolytic activity versus substrate concentration in the presence of phosphatidic acid and lysophosphatidylcholine

The rate of substrate hydrolysis versus $[1\text{-}^{14}\text{C}]$ palmitoyl-CoA concentrations in the absence (○) and presence of 12 μM lysophosphatidic acid (△) or lysophosphatidylcholine (●) was determined. The reaction contained 3–20 μM palmitoyl-CoA and 0.2 μg of purified enzyme. Each value is the mean of two separate experiments. The straight line for each set of experiments was obtained by least-square plot.

cardiolipin caused a progressive decrease in enzyme activity. At 80 μM lysophosphatidic acid or cardiolipin, the enzyme activity was decreased to less than 15% of the control value.

Kinetic study

The mechanism for the modulation of carboxylesterase by lysophosphatidylcholine, lysophosphatidic acid and cardiolipin was investigated. The hydrolysis of palmitoyl-CoA was monitored at different substrate concentrations in the presence of the modulators. The results are depicted in a double-reciprocal plot, as shown in Figure 4. The K_m value for palmitoyl-CoA, estimated to be 17 μM, was comparable with the value for carboxylesterase/acyl-CoA hydrolases reported previously [14]. The activation of enzyme activity by lysophosphatidylcholine appears to be a 'mixed' type, with a K_a value of 12 μM. The inhibition of enzyme activity by lysophosphatidic acid appears to be the classical 'competitive' type, which indicates that inhibition

might be at the substrate level. The K_i for lysophosphatidic acid was estimated to be 7.5 μM.

DISCUSSION

Long-chain acyl-CoA esters are intermediates involved in the acylation of lipids and proteins. The intracellular acyl-CoA content and composition have been shown to be important factors in determining acyl groups in phospholipids produced by the acylation process [29–31]. In addition, acyl-CoA esters may act as modulators in the regulation of several enzyme systems [32,33], including the cardiac Na^+ pump [34] and protein kinase C [35]. The ability to utilize acyl-CoA *in vivo* appears to be dependent on its availability, which in turn is regulated by its rate of synthesis via the acyl-CoA synthase system. In the last decade, several enzymes have been found to have the ability to hydrolyse long-chain acyl-CoA. However, none of these enzymes are specific for the hydrolysis of the thioester bond, and it is not clear if these hydrolytic enzymes may play a role in the regulation of long-chain acyl-CoA levels *in vivo*.

In this study, we have purified a carboxylesterase which has the ability to hydrolyse long-chain acyl-CoA esters. On the basis of its substrate specificity, its mobility during isoelectric focusing and its K_m for palmitoyl-CoA, the enzyme appears to be the ES-4 carboxylesterase with the assigned pI of 6.2. Despite the non-specific nature of most carboxylesterases, our enzyme preparation has a remarkable specificity towards the long-chain acyl-CoAs. Its inability to hydrolyse phospholipids and neutral lipids is contrary to the common view of the non-specificity of carboxylesterases. We are the first to show that the activity of a carboxylesterase is modulated by lysophospholipids and cardiolipin. The results of the kinetic studies clearly demonstrate that the mechanisms of enzyme modulation are different with lysophosphatidylcholine and with lysophosphatidic acid. However, the exact physiological significance of enzyme modulation by the lipids remains undefined. Since the vast majority of cardiolipin is found in the mitochondrial membrane, whereas the carboxylesterase is located in the endoplasmic reticulum, it is quite unlikely that cardiolipin may have a regulatory role on the enzyme. Alternatively, lysophosphatidic acid is an intermediate for the formation of phosphatidic acid (a precursor of all glycerolipids), and long-chain acyl-CoA is required for the acylation reaction [36]. The inhibition of acyl-CoA hydrolase by lysophosphatidic acid may be an important positive control for the synthesis of glycerolipids. The enhancement of carboxylesterase activity by lysophosphatidylcholine observed in the present study has not been identified in previous studies [37]. Lysophosphatidylcholine, which is formed from phosphatidylcholine by the action of phospholipase A_2 , is further deacylated or acylated back to the parent compound. The mechanism for the diversion of lysophosphatidylcholine for the deacylation or acylation reaction remains unknown [31]. Since the acylation reaction is dependent on the concentrations of lysophosphatidylcholine and acyl-CoA, the activation of acyl-CoA hydrolase by a high concentration of lysophosphatidylcholine may lower the concentration of acyl-CoA. The ability of lysophosphatidylcholine to regulate the acyl-CoA concentration may be a plausible mechanism to balance the levels of the two substrates for maintaining the rate of the acylation reaction.

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