Cholate extracts of mitochondrial outer membranes increase inhibition by malonyl-CoA of carnitine palmitoyltransferase-I by a mechanism involving phospholipids

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It has been reported that sodium cholate can separate the catalytic component of carnitine palmitoyltransferase-I (CPT-I) from a putative malonyl-CoA-binding regulatory protein capable of conferring sensitivity to malonyl-CoA on CPT-II. We found that cholate preferentially extracted a contaminating malonyl-CoA-sensitive CPT from mitochondrial inner membranes. When cholate extracts of outer membranes were incubated either with cholate extracts of inner membranes or with osmotically swollen mitochondria, inhibition of CPT by malonyl-CoA was increased. Treatment of intact mitochondria with subtilisin abolished the increased inhibition by malonyl-CoA, suggesting that the outer-membrane CPT-I was responsible for the increased inhibition. Incubation of cholate extracts with proteinase K did not prevent

the increased inhibition. Fractionation of the cholate extract indicated the presence of phospholipids. Addition of cardiolipin or phosphatidylglycerol to osmotically swollen mitochondria increased sensitivity of CPT to malonyl-CoA, but several other phospholipids did not. When cardiolipin was added to intact mitochondria from either starved or fed rats, there were large increases in inhibition by malonyl-CoA; sensitivity in mitochondria from starved rats increased to that normally observed with mitochondria from fed rats. These results suggest that phospholipids are responsible for the increased inhibition of CPT by malonyl-CoA with added cholate extracts and that changes in membrane composition may be involved in the physiological regulation of CPT-I.

INTRODUCTION

There are different forms of carnitine palmitoyltransferase (CPT; palmitoyl-CoA:L-carnitine *O*-palmitoyltransferase; EC 2.3.1.21) distributed in mitochondria, peroxisomes and microsomes (for reviews see [1,2]). Hepatic mitochondria contain two forms of the enzyme: a malonyl-CoA-sensitive form located in the mitochondrial outer membrane (CPT-I) [3] and a malonyl-CoA-insensitive, detergent-extractable, form located on the inside of the mitochondrial inner membrane (CPT-II) [4]. CPT-I and CPT-II are distinct proteins, synthesized from separate mRNAs [5,6]. One means of regulating fatty acid oxidation in the liver is through inhibition of CPT-I by malonyl-CoA [7]. Starvation and diabetes increase hepatic CPT-I activity and decrease its sensitivity to inhibition by malonyl-CoA [8–14]; the K_i for malonyl-CoA is increased about 10-fold [11–14]. The peroxisomal [15] and microsomal [16] enzymes are also sensitive to malonyl-CoA, with peroxisomal CPT exhibiting changes in sensitivity to inhibition by malonyl-CoA [17].

Several recent publications suggest that a regulatory component binding malonyl-CoA can be separated from a catalytic component of mitochondrial CPT-I [18–23]. In two studies, Woldegiorgis et al. [19] and Chung et al. [20] solubilized mitochondria with Triton X-100, and then used acyl-CoA affinity chromatography to obtain a fraction enriched with respect to binding malonyl-CoA and devoid of CPT activity. The fraction binding malonyl-CoA was then combined with an octylglucoside extract of liver mitochondria containing either a mixture of CPT-I and CPT-II [19] or purified rat heart CPT [20]. In a third study, Ghadiminejad and Saggerson [21–23] extracted mitochondrial

outer membranes with cholate to obtain a fraction with greater ability to bind malonyl-CoA, but low CPT activity; this fraction increased inhibition by malonyl-CoA when combined with a cholate extract of mitochondrial inner membranes [21–23]. All three systems used a CPT preparation that exhibits minimal inhibition by malonyl-CoA and is responsive to the addition of a malonyl-CoA-binding fraction. These experiments have not unambiguously demonstrated that in detergent extracts a specific protein is responsible for the increased inhibition of CPT by malonyl-CoA. In the reconstitution procedures of Woldegiorgis et al. [19] and Chung et al. [20] a mixture of phospholipids, the CPT fraction and the fraction binding malonyl-CoA were incorporated into liposomes; a sample was subsequently removed for assay of CPT. The incorporation of CPT into phospholipid liposomes resulted in both stimulation of basal CPT activity and inhibition by malonyl-CoA [4,24]. Ghadiminejad and Saggerson [21–23] used a crude cholate extract of isolated mitochondrial outer membranes as a source of the binding-protein fraction; this would contain a mixture of proteins, phospholipids and other constituents of the outer membrane.

Ghadiminejad and Saggerson [21–23] claim to have conferred malonyl-CoA-sensitivity on CPT-II in reconstitution experiments with extracts of inner membranes (i.e. CPT-II). Their CPT assay conditions were, however, originally designed for the measurement of CPT-I, not CPT-II, and their inner membranes were contaminated by marker enzymes for mitochondrial outer membranes, peroxisomes and microsomes [25]. The purpose of the present work was a re-examination of the effect of cholate extracts on CPT-I and CPT-II and a better characterization of the components of such extracts.

Abbreviations used: CPT-I and CPT-II, carnitine palmitoyltransferase of the mitochondrial outer and inner membrane, respectively; PEG, poly(ethylene glycol); PMSF, phenylmethanesulphonyl fluoride.

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EXPERIMENTAL

Mitochondria and membrane preparations

Male Sprague-Dawley rats (150–250 g; Harlan Industries, Indianapolis, IN, U.S.A.) were either fed on Purina Rodent chow (Ralston Purina Co., Richmond, IN, U.S.A.) *ad libitum* or starved for 48 h. Pig livers were obtained from a local abattoir. Hepatic mitochondria were isolated by the method of Johnson and Lardy [26] with modifications previously described [27]. Mitochondrial outer and inner membranes were prepared by the method of Parsons et al. [28], and stored at -70°C in 20 mM potassium phosphate buffer (pH 7.4).

Extraction of membranes and identification of components

Membranes were extracted essentially as described by Ghadiminejad and Saggerson [21]. Rat and pig mitochondrial inner or outer membranes (1–3 mg of protein/ml) were mixed with 15 mM sodium cholate to give a cholate/protein ratio of 150 nmol of cholate/mg of protein, and placed on a slowly rocking mixer at 4°C for 1 h, then centrifuged at 100 000 *g* for 1 h at 4°C . Some cholate extracts were digested for 1 h at 37°C with proteinase K (0.25 mg of proteinase K/ml), and the reaction was stopped by addition of 20 mM phenylmethanesulphonyl fluoride (PMSF) in ethanol (final concentration of 1 mM PMSF), followed by 15 min at 37°C and 15 min in boiling water. Cholate extracts were fractionated further by the method of Bligh and Dyer [29] as modified for cellular preparations [30]. Methanol, chloroform and cholate extracts were mixed (10:10:9, by vol.) and centrifuged to yield a two-phase system with a proteinaceous interface. It is important to understand that the original procedure of Bligh and Dyer [29] contained no detergent and that the presence of cholate may significantly alter the fate of some membrane components. The three fractions were separated, dried under nitrogen and resuspended in distilled water for reconstitution experiments. After finding that the aqueous phase contained the component responsible for the increased inhibition by malonyl-CoA, we added 5 vol. of cold acetone to the aqueous phase, followed by centrifugation at 4°C for 15 min [31] to see if phosphatides would be precipitated. The supernatant was decanted and the precipitate was dried in a vacuum desiccator to yield a waxy solid which was easily solubilized in distilled water for reconstitution experiments.

Proteins in the outer membranes, the cholate extract of outer membranes, the cholate extract after proteinase K digestion and the aqueous phase of the cholate extract after addition of chloroform/methanol were separated by SDS/PAGE (10% gels) [32] followed by silver staining [33].

Malonyl-CoA binding assay

Cholate extracts used in the malonyl-CoA binding experiments were concentrated approx. 10-fold by ultrafiltration in a Centricon-10 Concentrator (Amicon, Beverly, MA, U.S.A.). Malonyl-CoA binding in mitochondrial outer membranes was measured by a method similar to that of Ghadiminejad and Saggerson [25]. Assays were performed in micro-centrifuge tubes at 0°C in a total volume of 1 ml (pH 7.0) containing 70 mM imidazole, 70 mM KCl, 1 mM EGTA and 400 nM [2- ^{14}C]-malonyl-CoA. Specific binding was estimated by subtracting non-specific binding from total binding. Non-specific-binding values were obtained in the presence of a 250-fold molar excess of unlabelled malonyl-CoA. Incubations were initiated by addition of 300–400 μg of outer membranes. After 1 h, 100 μl of γ -globulin (20 mg/ml) and 400 μl of poly(ethylene glycol) (PEG)

6000 (130 mg/ml) were mixed together and added to the tubes. They were vortex-mixed and centrifuged at 10000 *g* for 5 min at 4°C . The supernatant was removed, and any remaining liquid was quickly removed with absorbent paper. The pellets were dissolved in 500 μl of 10% (v/v) Triton X-100, transferred to scintillation vials and mixed with 5 ml of scintillation cocktail. This binding assay resulted in complete precipitation of outer-membrane proteins, but when the PEG/ γ -globulin mixture was added to the 100 000 *g* supernatant of cholate-extracted outer membranes (i.e. cholate extract) there was only partial precipitation of the proteins. Malonyl-CoA binding was measured in the cholate extracts by decreasing the total volume to 0.5 ml and adding only 50 μg of protein while otherwise keeping the binding mixture the same. The binding mixture was incubated in an Ultrafree-CL Polysulfone 30000 NMWT Unit (Millipore Corp., Bedford, MA, U.S.A.) at $0-4^{\circ}\text{C}$. After 1 h the tubes were centrifuged at 30000 *g* for 15 min at 4°C in a swinging-bucket rotor. This resulted in spinning the filter to dryness while retaining 94% of the protein on the filter. The filter was removed from the tube, mixed with scintillation cocktail and counted for radioactivity.

Enzyme assays

A modified procedure of Bremer [9] described earlier [12] was employed to measure CPT activity. Final concentrations in a total volume of 1 ml at 37°C were: 80 mM sucrose, 70 mM KCl, 70 mM imidazole (pH 7.0), 1 mM EGTA, 1 μg of antimycin A, 2 mg of BSA. A 5 min preincubation period was initiated by the addition of 10–200 μM acyl-CoA (if CPT-II activity was measured, the assay also contained 0.04% Triton X-100). The reaction was started with 0.25–2.0 mM L-carnitine (0.4 mCi/mmol L-[methyl- ^3H]carnitine), and stopped after 10 min by adding 4 ml of 1.0 M HClO_4 . If 5 μM 2-bromopalmitoyl-CoA was included as an inhibitor of outer-membrane CPT, the preincubation began with the addition of L-carnitine and the reaction was started with myristoyl-CoA (these conditions produced $97 \pm 1\%$ inhibition of CPT activity in intact mitochondria). Monoamine oxidase and catalase were determined spectrophotometrically as previously described [34,35].

Reconstitution assay

Initial experiments were performed with isolated mitochondrial inner and outer membranes from rats, but pig livers were used in most experiments in order to obtain more membranes. In all reconstitution experiments the cholate concentration was kept constant. Some of the mitochondria were treated with subtilisin as previously described, except that subtilisin treatment was for 20 min [27]. Before the assay, concentrated mitochondria were mixed with a solution of PEG 6000 to give 5 mg of mitochondrial protein/ml and 100 mg of PEG/ml, and the mixture was left on ice for 30 min. Mitochondria (125 μg of mitochondrial protein) were mixed with different amounts of mitochondrial cholate extract of outer membranes or phospholipids, adjusted to 20 vol. of the mitochondrial suspension with water, and allowed to incubate on ice. After 1 h, 250 μl of a reaction cocktail was added as tubes remained on ice. This cocktail contained all the components of the CPT reaction mixture (excluding substrates) at 4 times the final concentrations in the CPT assay listed above. Malonyl-CoA (to give a final concentration of 100 μM) or an equivalent volume of water was added, and a 5 min preincubation period at 37°C was initiated after adding 50 μM myristoyl-CoA. The CPT reaction was started by adding 0.5 mM L-carnitine (0.4 mCi/mmol L-[methyl- ^3H]carnitine), which gave a final

Figure 1 Apparent K_m and $V_{\max}$ values of CPT-I and CPT-II for palmitoyl-CoA

(a) CPT-I activity was measured in intact mitochondria at 0.5 mM L-carnitine, and CPT-II was measured in frozen/thawed mitochondrial inner membranes in the presence of 0.04% Triton X-100 at 2.0 mM L-carnitine. Values are means $\pm$ S.E.M. ($n = 8$, CPT-I; $n = 4$, CPT-II); S.E.M. bars are hidden by the data points. (b) Plot obtained by inserting measured K_m values of CPT-I and CPT-II for L-carnitine and palmitoyl-CoA and the concentrations of L-carnitine and palmitoyl-CoA used by Ghadiminejad and Saggerson [21–23] into the equation: Observed rate = rate of CPT-I + rate of CPT-II. The theoretical ratio of the amount of CPT-I to CPT-II activity was varied and plotted as the CPT-I percentage of the total activity (abscissa) to represent different theoretical amounts of contamination of inner-membrane CPT by outer-membrane CPT. The ordinate gives the calculated percentage of the total activity that would be contributed by outer-membrane CPT if activity were measured at 40 μM palmitoyl-CoA and 0.4 mM L-carnitine.

reaction volume of 1 ml. After 10 min at 37 °C the reaction was stopped by adding 1.0 M HClO_4 , and radiolabelled acyl-carnitine was extracted and measured as described previously [9,12]. When the effects of phospholipids were to be examined, phospholipids were transferred to test tubes and the solvent was evaporated in a vacuum centrifuge. Phospholipids were resuspended (by using a bath sonicator) in either 100 μl of 0.45 mM sodium cholate for addition to osmotically swollen mitochondria, or 250 μl of reaction cocktail (above) for addition to intact mitochondria.

Materials

Ultra-pure (99.9%) sucrose was obtained from ICN Biomedicals (Cleveland, OH, U.S.A.). L-[methyl- ^3H]Carnitine hydrochloride and [2- ^{14}C]malonyl-CoA were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Protein was determined by the BCA Protein Kit (Pierce, Rockford, IL, U.S.A.).

RESULTS AND DISCUSSION

The physiological directions of the reactions catalysed by CPT-I and CPT-II are reflected in their K_m values for palmitoyl-CoA and L-carnitine (Figure 1a). CPT-I (measured in intact rat liver mitochondria) and apparent K_m values of 48 ± 6 μM ($n = 8$) for palmitoyl-CoA and 0.26 ± 0.02 mM ($n = 4$) for L-carnitine, and CPT-II (measured in frozen/thawed mitochondrial inner membranes in the presence of 0.04% Triton X-100 to release CPT-II and inhibit contaminating CPT-I activity [36]) had much higher apparent K_m values of 275 ± 40 μM ($n = 4$) for palmitoyl-CoA and 0.63 ± 0.06 mM ($n = 4$) for L-carnitine. The apparent K_m values for palmitoyl-CoA are useful only for comparison purposes, since they depend on the albumin concentration used. Because albumin binds palmitoyl-CoA, a true K_m cannot be obtained. In their CPT assay, Ghadiminejad and Saggerson [21–23,25] used substrate concentrations of L-carnitine (0.4 mM) and palmitoyl-CoA (40 μM) that were substantially lower than these latter K_m values for CPT-II. They report 13% contamination of their inner membranes by outer-membrane marker

enzymes and considerable contamination by peroxisomal and microsomal marker enzymes [25]. Since the contaminating CPT-I activities have lower K_m values, their measurement would be preferentially favoured. Using the kinetic model that the observed rate of the reaction is equal to the rate of CPT-I plus the rate of CPT-II, and substituting into the rate equation (see [37] for derivation) the K_m values for palmitoyl-CoA and L-carnitine that we obtained and the concentrations of palmitoyl-CoA and L-carnitine used by Ghadiminejad and Saggerson [21–23], we constructed a plot of the percentage contamination by CPT-I versus the actual contribution of CPT-I to total activity (Figure 1b). Assuming also that the $V_{\max}$ values for CPT-I and CPT-II are equal (Figure 1a), a 13% contamination by CPT-I protein would result in an assay in which CPT-I would account for 46% of the total measured activity.

We repeated the reconstitution experiments of Ghadiminejad and Saggerson [21–23] using cholate extracts of rat liver mitochondrial outer and inner membranes, and obtained identical results except for one deviation; we found that malonyl-CoA could inhibit approx. 20% of CPT activity in the extracts of inner membranes (results not shown). We attributed the difference to the fact that the radioisotopic assay for CPT typically yields greater inhibition values than the spectrophotometric assay [21]. Because of the low yield of purified mitochondrial membranes from rat liver, we isolated inner and outer membranes from pig liver and repeated the reconstitution experiments with these membranes. The extraction with cholate of mitochondrial outer membranes from pig liver is shown in Table 1. Only 6% of the total protein and 2% of the CPT activity was solubilized by cholate, but a substantial amount of malonyl-CoA-binding protein was extracted, with a 9-fold increase in the specific binding of malonyl-CoA. Cholate extracted 27% of the total protein from pig mitochondrial inner membranes, including 2% of the total CPT activity (Table 2). Marker enzymes in the inner membranes and cholate extracts of inner membranes suggest that CPT-I of the outer membranes and peroxisomal CPT were present in these preparations. There was more than a 5-fold increase in inhibition of CPT by malonyl-CoA in the cholate extract of inner membranes compared with the intact inner

Table 1 Cholate extraction of isolated hepatic mitochondrial outer membranes

Mitochondrial outer membranes were isolated from pig liver and extracted with sodium cholate as described in the text. CPT activity was determined at 50 μ M myristoyl-CoA and 0.5 mM L-carnitine with or without 50 μ M malonyl-CoA. Malonyl-CoA binding was measured at 400 nM [2^{14} C]malonyl-CoA. Numbers in parentheses are the percentages of enzymic activity or malonyl-CoA binding recovered in the respective fractions relative to outer membranes. Values are means $\pm$ S.E.M. for 3 experiments.

	Protein (%)	CPT activity (nmol/min per mg of protein)	Malonyl-CoA inhibition (%)	Malonyl-CoA binding (pmol/min per mg of protein)
Outer membranes	100	9.1 $\pm$ 0.6	81 $\pm$ 3	24.5 $\pm$ 3.7
Outer membranes + cholate	100	9.4 $\pm$ 0.9 (99%)	83 $\pm$ 3	19.5 $\pm$ 2.1 (77%)
100 000 g pellet	94 $\pm$ 4	9.3 $\pm$ 0.9 (98%)	82 $\pm$ 4	18.0 $\pm$ 1.6 (65%)
100 000 g supernatant	6 $\pm$ 1	3.4 $\pm$ 2.0 (2%)	47 $\pm$ 9	190 $\pm$ 5 (29%)

Table 2 Cholate extraction of isolated hepatic mitochondrial inner membranes

Mitochondrial inner membranes were isolated from pig liver and extracted with sodium cholate as described in the text. CPT activity was determined at 50 μ M myristoyl-CoA and 0.5 mM L-carnitine. Numbers in parentheses are the percentages of enzymic activity recovered in the respective fractions relative to inner membranes. Values are means $\pm$ S.E.M. for 4 experiments.

	Protein (%)	CPT			Monoamine oxidase (nmol/min per mg of protein)	Catalase (μ mol/min per mg of protein)
		(nmol/min per mg of protein)	Malonyl-CoA inhibition (%)	2-Bromopalmitoyl-CoA inhibition (%)		
Inner membranes	100	6.2 $\pm$ 0.3	6 $\pm$ 3	23 $\pm$ 5	34 $\pm$ 4	197 $\pm$ 2
Inner membranes + cholate	100	6.1 $\pm$ 0.1 (98%)	—	—	29 $\pm$ 2 (83%)	192 $\pm$ 1 (95%)
100 000 g pellet	72 $\pm$ 2	8.1 $\pm$ 0.2* (98%)	—	—	29 $\pm$ 2 (78%)	142 $\pm$ 2* (56%)
100 000 g supernatant	27 $\pm$ 2	0.4 $\pm$ 0.1* (2%)	35 $\pm$ 7*	51 $\pm$ 8*	Not detectable	389 $\pm$ 37* (65%)

* Significantly different ($P < 0.05$) compared with inner membranes.

membranes, and there was more than a 2-fold increase in inhibition of CPT activity by 2-bromopalmitoyl-CoA in the cholate extracts of inner membranes compared with intact inner membranes (Table 2). 2-Bromopalmitoyl-CoA is an active-site inhibitor of CPT-I and peroxisomal CPT, but it does not inhibit CPT-II [27]. These results indicate that cholate extracts from mitochondrial inner membranes contain a much larger proportion of CPT activity that is sensitive to inhibition by malonyl-CoA than of CPT activity that is insensitive to malonyl-CoA. After repeating with pig liver the initial reconstitution experiments, with results identical with those obtained with rat liver (results not shown), we examined the cholate extracts in more detail.

When cholate extracts of mitochondrial outer membranes from pig liver were added to osmotically swollen rat liver mitochondria, there was a concentration-dependent increase (with respect to added cholate extract) in inhibition of CPT by malonyl-CoA (Figure 2). These results are important, since they show identical effects of the cholate extracts in a system in which the preponderance of the CPT activity is CPT-I (based on 87% inhibition by 2-bromopalmitoyl-CoA). Treatment of mitochondria with subtilisin before adding extracts of the outer membrane resulted in little loss of CPT activity, but there was a large decrease in inhibition of CPT by malonyl-CoA (Figure 2). The addition of cholate extracts of outer membranes resulted in only a slight increase in inhibition of CPT by malonyl-CoA in the

subtilisin-treated mitochondria. Subtilisin treatment has been shown [4,27] to destroy the portion of the protein on the cytoplasmic side of the mitochondrial outer membrane that is responsible for inhibition of CPT-I by malonyl-CoA, while doing minimal damage to the active CPT-I protein protected within the membrane.

In order to identify the component(s) of the cholate extract of outer membranes which was responsible for the increased inhibition by malonyl-CoA of CPT-I, part of the cholate extract was digested with proteinase K for 1 h, followed by 15 min in a boiling-water bath, and another part of the cholate extract was fractionated with chloroform/methanol [30]. Proteins in these fractions were separated by SDS/PAGE followed by silver staining. Proteinase K treatment substantially decreased the amount of all proteins present in the sample (results not shown). Inhibition of CPT by malonyl-CoA was still increased to the same extent as with the untreated extract (Table 3). Chloroform/methanol fractionation of the cholate extract of outer membranes gave two phases with a white solid proteinaceous interface. Neither the chloroform phase nor the interface was able to stimulate inhibition of CPT by malonyl-CoA, but the aqueous phase increased inhibition (Table 3). An acetone precipitate of the aqueous phase also increased inhibition of CPT by malonyl-CoA (to 92%) when added to mitochondria. Proteins in each of these fractions were separated by SDS/PAGE followed by silver staining (the volume added to each lane was equal to the volume

Figure 2 Addition of mitochondrial cholate extracts of outer membranes to mitochondria

(a) Mitochondria and subtilisin-treated mitochondria were mixed with PEG to give final concentrations of 5 mg of mitochondrial protein/ml and 100 mg of PEG/ml and left on ice for 30 min. A 25 μ l portion of the mitochondria/PEG suspension was mixed with the indicated amounts of mitochondrial cholate extract of outer membranes, then adjusted with distilled water to a volume 20 times that of the original mitochondrial suspension, and allowed to incubate on ice for 1 h before the CPT assay (see the text). $\circ$, $\square$, Control and subtilisin-treated mitochondria respectively. $\bullet$, $\blacksquare$, Control and subtilisin-treated mitochondria respectively, with added malonyl-CoA (100 μ M). (b) Inhibition of CPT activity by malonyl-CoA in control ($\bullet$) and subtilisin-treated ($\blacksquare$) mitochondria. CPT activity was determined at 50 μ M myristoyl-CoA and 0.5 mM L-carnitine. Values are means $\pm$ S.E.M. for 3 experiments. Some S.E.M. bars are hidden by the data points.

Table 3 Addition of proteinase K-digested and chloroform/methanol-fractionated cholate extracts of mitochondrial outer membranes of osmotically swollen mitochondria

Mitochondria were mixed with PEG and left on ice for 30 min. A 25 μ l portion of the mitochondria/PEG suspension was mixed with 100 μ l of either the cholate extract of outer membranes, the extract after treatment with proteinase K or the extract after fractionation. Distilled water was added to adjust the volume to 20 times that of the original mitochondrial suspension, and the mixture was allowed to incubate on ice for 1 h before the CPT assay was begun (see the text). CPT activity was determined at 50 μ M myristoyl-CoA and 0.5 mM L-carnitine with or without 100 μ M malonyl-CoA. Values are means $\pm$ S.E.M. of 3 experiments.

Addition to mitochondria	CPT (nmol/min per mg of protein)		
	Without malonyl-CoA	With 100 μ M malonyl-CoA	Percentage inhibition
None	4.4 $\pm$ 0.4	1.3 $\pm$ 0.06	70 $\pm$ 3
Extract	4.5 $\pm$ 0.1	0.7 $\pm$ 0.09*	83 $\pm$ 2*
Proteinase K-treated extract	4.6 $\pm$ 0.8	0.7 $\pm$ 0.13*	83 $\pm$ 4*
Chloroform phase of extract	4.3 $\pm$ 0.4	1.1 $\pm$ 0.02	74 $\pm$ 2
Extract interface	4.5 $\pm$ 0.3	1.3 $\pm$ 0.02	75 $\pm$ 2
Aqueous phase of extract	4.2 $\pm$ 0.3	0.6 $\pm$ 0.15*	88 $\pm$ 2*

* Significantly different ($P < 0.05$) compared with control.

Table 4 Addition of phospholipids to osmotically swollen mitochondria

Mitochondria were mixed with PEG and left on ice for 30 min. A 25 μ l aliquot of the mitochondria/PEG suspension was mixed with 125 μ g of the indicated phospholipid. Phospholipids were prepared by removing the solvent for the phospholipid by evaporation and then resuspending them in 100 μ l of 0.45 mM sodium cholate. The final volume was adjusted to 20 times that of the mitochondrial suspension with distilled water, and mixtures were allowed to incubate on ice for 1 h before the CPT assay was started (see the text). CPT activity was determined at 50 μ M myristoyl-CoA and 0.5 mM L-carnitine with and without 100 μ M malonyl-CoA. Values are means $\pm$ S.E.M. for 4 experiments.

Addition to mitochondria	CPT (nmol/min per mg of protein)		
	Without malonyl-CoA	With 100 μ M malonyl-CoA	Percentage inhibition
None	3.50 $\pm$ 0.34	0.78 $\pm$ 0.14	78 $\pm$ 3
Phosphatidylcholine	4.20 $\pm$ 0.43	1.01 $\pm$ 0.25	77 $\pm$ 3
Phosphatidylethanolamine	4.25 $\pm$ 0.36*	0.59 $\pm$ 0.19	87 $\pm$ 3*
Phosphatidylinositol	5.11 $\pm$ 0.32*	1.40 $\pm$ 0.25*	72 $\pm$ 6
Phosphatidylserine	4.66 $\pm$ 0.47*	0.63 $\pm$ 0.06	87 $\pm$ 2*
Cardiolipin	4.06 $\pm$ 0.23	0.23 $\pm$ 0.06*	95 $\pm$ 1*
Sphingomyelin	3.69 $\pm$ 0.29	0.97 $\pm$ 0.20	74 $\pm$ 4
Phosphatidic acid	4.88 $\pm$ 0.32*	1.27 $\pm$ 0.48	75 $\pm$ 9
Phosphatidylglycerol	4.07 $\pm$ 0.25	0.32 $\pm$ 0.03*	93 $\pm$ 1*

* Significantly different ($P < 0.05$) compared with control.

added to mitochondria in Table 3). There were no detectable bands in the aqueous phase or the acetone precipitate of the aqueous phase (results not shown). Since acetone precipitation is a common method used to remove phospholipids from solutions [31], we checked the effects of phospholipids in the CPT assay system. The addition of individual phospholipids to osmotically swollen mitochondria is shown in Table 4. Of the phospholipids tested, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidic acid significantly increased CPT activity, and phosphatidylinositol was the only phospholipid to increase CPT activity significantly in the presence of malonyl-CoA. Cardiolipin and phosphatidylglycerol mimicked the effect of the cholate extract of outer membranes by having no significant effect on CPT activity, but stimulating inhibition of CPT by

malonyl-CoA. The fatty acid composition of phosphatidylglycerol was 33% $C_{16:0}$, 13% $C_{18:0}$, 31% $C_{18:1}$ and 15% $C_{18:2}$, and the fatty acid composition of cardiolipin was 5% $C_{18:1}$ and 90% $C_{18:2}$ (fatty acid compositions furnished by the supplier, Sigma Chemical Co.) These are fatty acids common to mammalian cells, but the composition of these two phospholipids was quite different. On the contrary, the fatty acid composition of phosphatidylglycerol was identical with that of phosphatidylcholine. This suggests that the effects observed are the function of similar polar head groups rather than fatty acid compositions.

The average phospholipid composition of mitochondrial outer membranes from four references [38–41] is 49% phosphatidylcholine, 30% phosphatidylethanolamine, 11% phosphatidyl-

Figure 3 Addition of cardiolipin to intact mitochondria from fed and starved rats

Cardiolipin (150 μg) was prepared by evaporation of the solvent in a vacuum centrifuge and resuspended in CPT assay buffer by sonication before placing on ice. Malonyl-CoA (5–60 μM) was added, followed by mitochondria, and an assay for CPT activity was begun within 5 min. ○, □, Fed and starved rats respectively. ●, ■, Fed and starved rats respectively, with added cardiolipin. CPT activity was determined at 50 μM myristoyl-CoA and 0.5 mM L-carnitine. Values are means ± S.E.M. for 3 experiments.

inositol and 7% cardiolipin. At this point, it seemed reasonable to believe that cardiolipin was the component in the cholate extract of the outer membranes and could have caused increased inhibition of CPT by malonyl-CoA. The addition of cardiolipin to intact mitochondria from fed and starved rats is shown in Figure 3. The usual pattern [8–14] of increased CPT activity and diminished inhibition by malonyl-CoA was observed in the starved rats compared with fed rats. Addition of cardiolipin increased basal CPT activity in both fed and starved rats, and a tremendous increase in malonyl-CoA sensitivity was produced. Although inhibition by malonyl-CoA increased in both fed and starved rats, mitochondria from starved rats were more responsive to the added cardiolipin. At 5 μM malonyl-CoA, inhibition was increased by 200% in fed rats and 400% in starved rats by adding cardiolipin. The most striking observation was that cardiolipin increased malonyl-CoA sensitivity in mitochondria from starved rats to the level normally seen only in mitochondria from fed rats. We also added cardiolipin to mitochondria from rat hearts, but we found only a $13 \pm 7\%$ ($n = 3$) increase in inhibition at 1 μM malonyl-CoA (inhibition in controls was $62 \pm 7\%$ for three preparations of heart mitochondria).

The data presented here suggest that the mixing of a cholate extract of outer membranes with a cholate extract of inner membranes was more likely a reconstitution of outer-membrane components resulting in increased inhibition of CPT-I rather than conferring malonyl-CoA sensitivity to CPT-II. The results with subtilisin treatment of mitochondria provide additional evidence that the target of the outer-membrane extract is a protein that is exposed to the cytoplasmic compartment. The recent sequencing of a cDNA for CPT-I suggests that it has a single membrane-spanning region and would be exposed to the cytosol [6], whereas CPT-II would not. These experiments do not solve the issue of the existence of any auxiliary malonyl-CoA binding protein that would interact with CPT-I, but the data clearly indicate that there is no absolute requirement for a protein to be present in the cholate extract to produce the stimulation of malonyl-CoA-sensitivity.

These data raise the question of whether the phospholipid content of hepatic mitochondrial outer membranes may be involved in a physiological mechanism for regulation of CPT-I. Although previous publications [4,20,24,42] have implicated phospholipids as being important in altering CPT-I activity and

its sensitivity to inhibition by malonyl-CoA, data presented in Figure 3 represent the first demonstration that hepatic mitochondria from starved rats, normally relatively unresponsive to inhibition by malonyl-CoA, can be completely restored to the more malonyl-CoA-sensitive mode seen in mitochondria from fed animals. We have previously shown that cholesterol, supplied artificially in culture [43] or naturally in the diet [44], can alter the activity of CPT-I, presumably by some kind of membrane interaction. Other investigators [45,46] have demonstrated that the malonyl-CoA-sensitivity of CPT-I can be altered in response to changes in membrane fluidity. A dramatic increase in malonyl-CoA-sensitivity occurring as a result of lowered assay temperature (and thus decreased fluidity) led some investigators to suggest that changes in sensitivity occurring in different physiological conditions should be examined in relation to changes in the lipid composition of the membrane [45]. Additional work is needed to ascertain whether the phospholipid composition of hepatic mitochondrial outer membranes is altered in physiological and pathophysiological states that alter the regulatory properties of CPT-I.

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