Evidence against direct involvement of phosphorylation in the activation of carnitine palmitoyltransferase by okadaic acid in rat hepatocytes

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1. The mechanism of activation of mitochondrial overt carnitine palmitoyltransferase (CPT I) by treatment of hepatocytes with okadaic acid (OA) was investigated. Activation was observed when cells were permeabilized with digitonin, but not when a total membrane fraction was obtained by sonication. Both cell disruption methods preserved the activation of phosphorylase observed in OA-treated hepatocytes. Activation of CPT I was also observed in crude homogenates of OA-treated hepatocytes, but it was lost upon subsequent isolation of mitochondria from such homogenates. 2. In all experiments, any activation observed did not depend on the presence or absence of fluoride ions in the permeabilization/homogenization media. When hepatocytes were permeabilized in the absence of fluoride and further incubated with exogenous phosphatases 1 and 2A, the OA-induced activation of CPT was not reversed, whereas the activation of glycogen phosphorylase in the same cells was rapidly reversed. 3. Treatment of hepatocytes with OA, followed by permeabilization and incubation before assay of CPT I,

demonstrated that OA had no short-term effect on the sensitivity of CPT I to malonyl-CoA, although the difference in sensitivity between cells isolated from fed and starved rats was fully preserved. 4. Incubation of isolated mitochondria or purified mitochondrial outer membranes with cyclic AMP-dependent or AMP-activated protein kinases, under phosphorylating conditions, did not affect the activity of CPT I or its sensitivity to malonyl-CoA inhibition. Under the same conditions, the use of [³²P]ATP resulted in the labelling of several outer-membrane proteins but, unlike [³H]jetomoxir-labelled CPT I, none of them was specifically removed from membrane extracts by a specific polyclonal antibody to the enzyme. 5. We conclude that the increase in overt CPT activity observed in permeabilized hepatocytes is not due to direct phosphorylation of CPT I, but may involve interactions between the mitochondrial outer membrane and other membranous or soluble cytosolic components of the cell.

INTRODUCTION

The possibility that the activity of the mitochondrial overt carnitine palmitoyltransferase (CPT I) of hepatocytes is modulated by a phosphorylation–dephosphorylation mechanism was first raised in [1]. The validity of those studies was questioned [2] on the basis that the use of an anti-CPT II antibody to immunoprecipitate the phosphorylated protein invalidated the conclusion that CPT I was phosphorylated. In an attempt to obtain a functional measure of the effects of glucagon on CPT I in isolated hepatocytes, we conducted studies [2] in which cells were first exposed to glucagon and subsequently permeabilized (in the presence of fluoride and EDTA) with low concentrations of digitonin. This protocol was designed to retain mitochondrial integrity (essential for minimization of the contribution of CPT II towards total CPT activity) as well as any changes in phosphorylation state of CPT I, which was measured in the resulting (washed) cell ghosts. Those studies showed that there was no significant effect of glucagon either on the activity of CPT I in the absence of malonyl-CoA or on the sensitivity of the enzyme to malonyl-CoA inhibition. However, the differences in kinetic characteristics of CPT I induced by starvation and diabetes [3–6] were entirely preserved, irrespective of the previous treatment of the cells [2]. On the basis of similar experiments, Stephens and Harris [7] concluded that glucagon had a small (25%) stimulatory effect on CPT I activity, but that it had no effect on malonyl-CoA sensitivity.

An alternative method for measuring CPT I activity in permeabilized cells was described in [8–11]. In this method, much

higher concentrations of digitonin are included directly in the assay medium. Under these conditions, permeabilization and enzyme assay occur simultaneously. Several studies using this method showed that various agents have short-term effects on CPT I activity in the direction expected from the parallel changes in the rates of fatty acid oxidation measured in the same cell preparations [8–11]. In particular, it was shown [10,11] that effectors that increase intracellular cyclic AMP (e.g. glucagon, forskolin) and okadaic acid (OA; an inhibitor of protein phosphatases 1 and 2A) were able to increase CPT I activity by up to 50% in parallel with activation (by the same magnitude) of the rate of oxidation of [1-¹⁴C]palmitate by these hepatocytes. It was also found that treatment of hepatocytes with OA apparently decreased the sensitivity of CPT I to malonyl-CoA [11]. It was concluded that inhibition of the phosphatases might have resulted in increased phosphorylation of CPT I, with consequent changes in the kinetic characteristics of the enzyme.

In the present paper we show that OA-induced activation of CPT I in hepatocytes can be observed in crude homogenates and in digitonin-permeabilized cells, but not when intact mitochondria or a total membrane fraction are prepared. When observed, activation is not dependent on the presence of fluoride in the extraction or permeabilization media and is not reversed by phosphatases 1 and 2A. These observations, together with the absence of direct effects of cyclic AMP-dependent and AMP-activated kinases on the kinetic properties of CPT I in intact mitochondria *in vitro*, suggest that any effects of increased cellular phosphorylation on CPT I are likely to be indirect.

MATERIALS AND METHODS

Preparation and incubation of hepatocytes

Isolated hepatocytes were prepared essentially by the method of Seglen [12]. Viability (as judged by Trypan Blue exclusion) was routinely greater than 90%. They were washed once and finally resuspended to a density of $(5.0\text{--}6.0) \times 10^6$ cells/ml in Krebs–Henseleit medium [13] supplemented with 12 mM glucose and 2% (w/v) BSA.

Incubations (1 ml) were performed at 37 °C in 25 ml silicone-treated glass conical flasks with constant shaking (180 oscillations/min) and gassing (with O_2/CO_2 , 19:1). In all experiments the cells were incubated for 10–15 min before making any additions. Flasks received 1 μl of DMSO only (controls) or 1 μl of stock solutions to yield final concentrations of 5 μM tetradecylglycidic acid (TDGA) or 1 μM OA. Other additions were as indicated in the text.

Permeabilization of hepatocytes and preparation of mitochondria

Permeabilization with digitonin was performed by using the same detergent/cell protein ratio as that used in [8], except that the procedure was not performed in assay medium but in the incubation flask. In brief, to incubating cells $[(5.0\text{--}6.0) \times 10^6$ cells in 1 ml] was added 1.0 ml of prewarmed medium containing 0.25 mg of digitonin, 5 mM EDTA, 5 mM EGTA, 5 mM Tris (pH 7.4), 50 mM KF and 100 mM KCl (F^- medium). The flask contents were immediately mixed for 5 s or 10 s, as indicated, and rapidly diluted (cold-quenched) by transfer to tubes containing either 40 ml of ice-cold F^- medium or of medium in which an equivalent amount of KCl replaced KF (Cl^- medium). The permeabilized cells were sedimented by centrifugation at 350 g for 15 s, at 4 °C, as described in [14]. Samples of the supernatant were retained for marker-enzyme activity measurements. Permeabilized cell pellets were resuspended in 1.0 ml of prewarmed Cl^- or F^- medium, as appropriate, and the suspension was transferred to silicone-treated glass conical flasks (25 ml) and incubated, with shaking, at 37 °C in the absence or presence of exogenous protein phosphatases. Samples were taken at the indicated times for assay of CPT activity and for rapid freezing (into liquid N_2) for subsequent assay of phosphorylase activity [14]. Samples of the cell suspensions were also kept for assay of protein content.

When hepatocytes were disrupted by sonication, the cells were cold-quenched and harvested, as above, but without prior permeabilization. The cell pellet was rapidly resuspended in 0.5 ml of F^- medium and sonicated for two 10 s periods (see [14]) while being cooled in ice/water. The total membrane fraction was obtained by centrifugation of 240 μl samples of the sonicated preparation at 160 000 g for 30 min at 4 °C in an Airfuge microcentrifuge (Beckman). Samples of total sonicated preparation and post-membrane supernatants were kept for assays of phosphorylase and/or marker-enzyme activities. The high-speed membrane pellets were resuspended in F^- medium (0.6 ml) and used for assay of CPT activity and protein content.

Mitochondria were isolated from hepatocytes as described in [15]. Briefly, cells were sedimented by centrifugation at 350 g (15 s) and resuspended in either Cl^- or F^- medium, followed by homogenization with a hand-held tight-fitting Potter–Elvehjem homogenizer (15 strokes) held in ice/water. The crude homogenate was centrifuged at 3000 g for 1 min to remove debris and the supernatant was used for the isolation of mitochondria as in [15]. CPT activity was measured in the various fractions obtained (and corrected for values obtained in fractions prepared from cells incubated with TDGA; see part A in the Results section).

Preparation and incubation of intact mitochondria and outer membranes

Mitochondria from intact liver were isolated by standard differential centrifugation from livers of 48 h-starved male rats. They were further purified on self-forming Percoll (30%) gradients as described previously [16]. Outer membranes were prepared and freed from inner-membrane contamination by affinity purification using Sepharose–cytochrome *c* (see [16]).

In phosphorylation experiments, outer membranes (0.2 mg of protein) were incubated (at 30 °C) for 10–30 min in either of two media: (i) 25 mM Tris/HCl (pH 7.5), 25 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, 5% (w/v) glycerol, 0.25 mM AMP, 2.5 mM ATP, 6.25 mM MgCl_2 and 2 m-units of AMP-activated protein kinase in the absence or presence of 50 μM cyclic AMP, or (ii) 25 mM Mops (pH 7.0), 5 mM dithiothreitol, 2.5 mM ATP, 20 mM magnesium acetate and 2 m-units of cyclic AMP-dependent protein kinase. When CPT I activity was to be preserved, the mitochondria were sedimented, resuspended in fresh KF medium (100 mM KCl, 50 mM KF, 5 mM Tris/HCl, pH 7.4) and used for assay of CPT activity (as in [15]). In experiments in which proteins were to be labelled with ^{32}P , the membranes were incubated as in (ii) above, except that 2.5 μCi of [^{32}P]ATP was added and the final concentration of ATP was 0.1 mM. Labelling of CPT I with [^3H]etomoxir-CoA was performed in the presence of 10 μCi of [^3H]etomoxir {2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylate dihydrate} (40 $\mu\text{Ci}/\text{mmol}$), 0.5 mM CoA, 10 mM ATP and 8 mM MgCl_2 in medium containing 350 mM Tris/HCl and 5 mM dithiothreitol (pH 7.4). After incubation at 37 °C for 30 min (^{32}P experiments) or 120 min ([^3H]etomoxir experiments), the membranes were sedimented and solubilized in 0.1% SDS (see [16]). Half of the resulting solubilized protein was treated with agarose beads coupled to anti-CPT I antibody. The other (control) half was incubated for 30 min at 30 °C with agarose only, with constant mixing. The agarose was removed from suspension by light centrifugation, and the supernatant proteins were prepared for SDS/PAGE [15]. After separation of the proteins, autoradiography (^{32}P) or fluorography (^3H ; see [15]) was performed to localize those labelled in control and anti-CPT I antibody-treated extracts.

Other methods

Phosphorylase *a* and *b* activities were measured as described previously [14].

Materials

The sources of chemicals and other materials were as described previously [16]. In addition, [^{32}P]ATP was from Amersham International (Amersham, Bucks., U.K.). Cyclic AMP-dependent protein kinase was from Sigma (Poole, Dorset, U.K.). AMP-activated protein kinase was given by Dr. D. G. Hardie (University of Dundee) and Dr. D. Carling (Hammersmith Hospital, London). [^3H]Etomoxir was given by Dr. T. Holland (Byk Gulden, Kostanz, Federal Republic of Germany).

RESULTS

(A) Effects of okadaic acid on CPT activity

(i) Digitonin permeabilization of hepatocytes

In order to be able to distinguish the direct effects of putative phosphorylation on CPT activity from the effects of cell permeabilization itself, hepatocytes were permeabilized by incubation

Table 1 Effects of OA, dibutyryl cyclic AMP (db cAMP), fructose and ionophore A23187 on CPT I activity in digitonin-permeabilized cells or the high-speed (160 000 g) particulate fraction obtained from sonicated cells

Hepatocytes were incubated with the agents indicated for 10 min before preparation and isolation of digitonin-permeabilized cells (D) or of a total cell membrane fraction by sonication (S). Values for CPT activity are given as a percentage of the respective controls performed concurrently on the same cell preparation. CPT activities were 2.24 ± 0.45 nmol/min per mg of permeabilized-cell protein and 6.24 ± 0.64 nmol/min per mg of total membrane protein, at 37 °C. The CPT activity obtained in TDGA-treated cells is set at 0. The numbers in parentheses indicate the numbers of separate cell preparations used for each determination. Paired values that are significantly different from controls ($P < 0.01$) are indicated by *. Abbreviation: DMSO, dimethyl sulphoxide.

Animals	Additions	Cell disruption	CPT activity (% of control)	Phosphorylase $a/(a+b)$ ratio
Fed (9)	Control (0.1% DMSO)	D	100	0.21 ± 0.05
Fed (9)	OA (1 μ M)	D	$142.0 \pm 4.9^*$	$0.86 \pm 0.05^*$
48 h-starved (4)	OA (1 μ M)	D	$145.0 \pm 4.9^*$	—
Fed (4)	db cAMP (50 μ M)	D	$115.2 \pm 2.7^*$	—
Fed (4)	OA (1 μ M)	S	91.1 ± 4.0	$0.71 \pm 0.05^*$
Fed (4)	db cAMP (50 μ M)	S	93.7 ± 1.6	$0.82 \pm 0.03^*$
Fed (4)	Fructose (12 mM)	S	102.2 ± 7.0	$0.58 \pm 0.05^*$
Fed (4)	A23187 (10 μ M)	S	102.1 ± 7.1	$0.81 \pm 0.02^*$
Fed (4)	TDGA (5 μ M)	S	0	0.17 ± 0.03
Fed (4)	Control (0.1% DMSO)	S	100	0.13 ± 0.01

Table 2 Loss of OA-induced activation of CPT after isolation of mitochondria from treated hepatocytes

Hepatocytes were incubated with OA (1 μ M) and used to prepare intact mitochondria (A) from total cell homogenates (B) and a post-nuclear supernatant (C) (see the Materials and methods section) in the presence or absence of 50 mM KF in the homogenization medium. Cells from the same preparations were concurrently used to prepare digitonin-permeabilized cells in which CPT I activity was measured by the 'direct' assay (D) or after separation and incubation (E). CPT activity obtained from cells/fractions derived from hepatocytes incubated with TDGA (5 μ M) was routinely subtracted to obtain the TDGA-sensitive CPT activity (CPT I). Extractions/permeabilizations were performed either in medium containing Cl^- only or in which 50 mM KF replaced a similar concentration of Cl^- , except for 'direct' assays (n.a.) when cells were added directly to the permeabilization/assay medium. Values are means ($\pm$ S.E.M.) for the numbers of separate hepatocyte preparations shown in parentheses; n.a., not applicable.

Protocol/ cell fraction	OA in incubation	Fluoride in medium	CPT activity (nmol/min per unit of glutamate dehydrogenase)
A (4)	—	+	1.07 ± 0.15
	+	+	1.16 ± 0.17
	—	—	1.12 ± 0.34
	+	—	1.14 ± 0.22
B (4)	—	+	1.99 ± 0.18
	+	+	$3.44 \pm 0.22^*$
	—	—	1.74 ± 0.30
	+	—	$2.82 \pm 0.51^*$
C (3)	—	+	1.73 ± 0.15
	+	+	$3.25 \pm 0.17^*$
	—	—	1.80 ± 0.34
	+	—	$3.49 \pm 0.59^*$
D (5)	—	n.a.	1.43 ± 0.26
	+	n.a.	$2.26 \pm 0.41^*$
E (3)	—	+	1.87 ± 0.81
	+	+	$2.84 \pm 0.45^*$
	—	—	1.60 ± 0.12
	+	—	$2.49 \pm 0.63^*$

with digitonin (0.25 mg/10⁶ cells in a total volume of 2 ml) in F^- -containing medium for 5 s, followed by rapid dilution with 40 ml of ice-cold F^- medium and sedimentation. After resuspension in F^- or Cl^- medium, the cells were incubated at 37 °C for 10 min (or longer, as indicated) in the presence or absence of exogenous

phosphatases. Judging by the phosphorylase $a/(a+b)$ activity ratio (see Table 1 and Figure 1) measured in the same cells used for CPT assays, the procedure preserved the phosphorylation status of phosphorylase attained when cells were incubated in the presence or absence of OA (and other activators of phosphorylase). Routinely, each set of incubations included control flasks to which TDGA was added (5 μ M) to obtain a value for the TDGA-insensitive activity, which was subtracted to obtain CPT I activity as in [11]. Peroxisomal acyltransferase activity has been shown not to be inhibited by the TDGA concentrations used [11]. Routinely, TDGA-insensitive activity accounted for a small proportion (5–10%) of total CPT activity, and no further attempt was made to characterize it in terms of its subcellular distribution.

When hepatocytes were exposed to 1 μ M OA before permeabilization, CPT I activity in the permeabilized cells was increased by 40–50% (see Table 1), in agreement with [11]. The degree of activation was similar whether cells were obtained from fed or 48 h-starved rats (Table 1). The phosphorylase $a/(a+b)$ activity ratio was also increased from 0.15 ± 0.03 to 0.85 ± 0.05 ($n = 8$).

(ii) Alternative methods of cell disruption

(a) Sonication of hepatocytes. Intact hepatocytes were incubated exactly as described above, but, after cold-quenching and sedimentation, the cells were resuspended and sonicated (2×10 s) in ice-cold F^- -containing medium [14]. Samples of the supernatants were centrifuged at 120 000 g for 30 min to obtain a total membrane fraction. CPT I activity was measured in the resuspended membranes after subtraction of TDGA-insensitive activity (see above). Routinely, this accounted for 20–30% of total CPT activity. As expected, this value was higher than in digitonin-permeabilized cells, as, in addition to peroxisomal and microsomal acyltransferase activities, sonication would have produced a greater disruption of the mitochondrial inner membrane, as indicated by the release of 70% of glutamate dehydrogenase activity into the supernatant. (No glutamate dehydrogenase activity was released after digitonin treatment.)

In contrast with digitonin permeabilization, when hepatocytes were disrupted by sonication after OA treatment, we could not observe any difference between the CPT I activity of membranes obtained from control or OA-treated hepatocytes (Table 1). This was the case whether CPT I activity was measured in total

Figure 1 Treatment of permeabilized hepatocytes with a combination of exogenous protein phosphatases 1 and 2A reverses the OA-induced activation of glycogen phosphorylase, but not that of CPT I activity

Hepatocytes were incubated for 10 min in the absence (○) or presence (●) of $1 \mu\text{M}$ OA. They were then permeabilized with digitonin in either the presence (b, e) or absence of F^- . They were rapidly diluted, sedimented and resuspended (zero time) and incubated (at 37°C) in KCl medium with added phosphatases (a, d) or medium containing 50 mM KF (b, e) or medium containing only Cl^- (c, f). At the indicated times, samples were taken and either frozen in liquid N_2 for subsequent assay of glycogen phosphorylase (a–c) or used for assay of CPT activity (d–f). The activity of CPT in cells permeabilized after incubation with TDGA was subtracted for each time point to obtain CPT I activity (see the text). Values are given as percentages of the activities obtained at zero time for each condition, and are means ($\pm$ S.E.M.) for four separate hepatocyte preparations. The CPT I activities for control cells at zero time were 2.14 ± 0.35 and 2.02 ± 0.22 nmol/min per mg of permeabilized-cell protein in (e) and (f) respectively. Comparable values for (d) could not be obtained because of the protein added with the phosphatase preparation.

sonicated preparations or in the total cell membrane fraction. However, the activation of phosphorylase (measured in the total cell extracts or particulate fraction) by OA treatment was of the same order as that observed after permeabilization with digitonin. The latter observation indicated that our failure to observe any OA-induced change in CPT I activity after sonication was unlikely to have been due to artefactual changes in the phosphorylation status during sonication and preparation of the membrane fraction. Moreover, no CPT I activation was observed in sonicated-cell membranes after incubation of cells with dibutyryl cyclic AMP or fructose (which raises cellular AMP concentrations and activates the AMP-activated kinase [14]). Ionophore A23187, which may also activate this kinase [14] and raises intracellular Ca^{2+} under the cell incubation conditions used, was also without effect on CPT I. All these agents fully activated phosphorylase *a* in the same cells (Table 1).

(b) Cell homogenization and preparation of mitochondria. In these experiments, CPT I activity was measured directly in intact mitochondria isolated from cells that had been previously incubated in the presence or absence of okadaic acid or TDGA. As shown in Table 2(A), prior incubation of hepatocytes with okadaic acid did not result in significant changes in CPT I activity, irrespective of whether the mitochondria were isolated in F^- - or Cl^- -containing media. It is noteworthy, however, that assays performed on permeabilized hepatocytes obtained from the same incubations and treated with digitonin, either directly in the assay or followed by separation and resuspension [see (i) above] showed the usual activation of CPT I activity (Table 2, D and E). Moreover, this activation was still apparent in the crude

homogenates from which the mitochondria were isolated (Table 2, B and C). Indeed, OA-induced activation was observed even in cells that were merely frozen (in liquid N_2) and thawed (results not shown). In all instances, the apparent activation was independent of whether the cells were disrupted in F^- -containing medium. It therefore appears that the activation of CPT I induced by OA requires a component from the extramitochondrial compartment of the cell for it to be expressed, but is not dependent on the preservation of protein phosphorylation.

(B) Effects of exogenous phosphatase on permeabilized cells

The isolation of digitonin-permeabilized cells [A(i), above] enabled us to test whether activation of CPT I by OA is reversible by the addition of exogenous phosphatases. We used a partially purified phosphatase preparation [14] that contained a mixture of type 1 and 2A phosphatases in the ratio 3/7 (when their activities were measured by their ability to dephosphorylate and inactivate phosphorylase *a* in the presence of 0.1 or $1 \mu\text{M}$ OA). The rationale behind the use of these phosphatases was that these are the protein phosphatases that are inhibited by $1 \mu\text{M}$ OA in intact cells, and therefore the action of these exogenous phosphatases on permeabilized cells would be expected to reverse any putative OA-induced phosphorylation of CPT I.

After incubation, in the absence or presence of OA, cells were permeabilized with digitonin in either the presence or absence of F^- and incubated at 37°C with shaking in the appropriate medium in either the absence or presence of the phosphatases (2 units; combined activity measured with phosphorylase *a* as

Figure 2 OA treatment does not alter the sensitivity of CPT I to malonyl-CoA in hepatocytes from (a) fed or (b) 48 h-starved rats

Hepatocytes were incubated for 10 min in the presence (●) or absence (○) of 1 μM OA. They were then permeabilized with digitonin dissolved in medium containing 50 mM KF (see the Materials and methods section). After dilution and sedimentation, they were resuspended in KF medium and incubated, with shaking, at 37 °C for 10 min before assay of CPT activity in the presence of the indicated concentrations of malonyl-CoA. The activities obtained for cells previously incubated with TDGA were subtracted to obtain TDGA-sensitive (CPT I) activity. Values are expressed as a percentage of the activity assayed in the absence of malonyl-CoA for four different hepatocyte preparations in each instance. The activities of CPT I were 2.27 ± 0.54 and 2.44 ± 0.32 nmol/min per mg of permeabilized-cell protein for cells from fed and starved rats respectively.

substrate). At the times indicated (Figure 1), portions were sampled and assayed for CPT activity (net of the TDGA-insensitive component) or frozen in liquid N₂ for subsequent assay of phosphorylase *a* and *b* activities. As shown in Figures 1(d)–1(f), CPT I activity increased continuously during the 20 min incubation period, irrespective of whether F[−] was present or absent. This increase in activity is presumed to have been due to the effect of high-ionic-strength media on the enzyme, as described previously [17]. The activation of CPT I due to OA was observed as described in (A) above, but its extent did not alter during these incubations, irrespective of whether the permeabilized cells were incubated in F[−] or Cl[−] medium. Moreover, irrespective of whether the phosphatases were present or absent, the OA-induced activation was preserved throughout the incubation period, contrary to what would have been expected if activation was due to a phosphatase-reversible increase in CPT I phosphorylation.

In these experiments, as in those described under A(i) above, the OA-induced increase in CPT I activity was no smaller when

cells were permeabilized in the absence of F[−]. Indeed, the activation observed for cells permeabilized and incubated in the absence of F[−] tended to be larger, again contrary to what would have been expected if preservation of the phosphorylation status of the enzyme is required for observation of the OA-induced increase in activity.

The effectiveness of the exogenous phosphatase preparation added to the incubations was gauged by monitoring its effect on phosphorylase activity in the same permeabilized-cell preparations. As shown in Figure 1(a), inclusion of the phosphatases resulted in the rapid and complete reversal of the activation of phosphorylase induced by previous incubation of the cells with OA. In the presence of F[−], no significant reversal of phosphorylase activation was observed (Figure 1b). Some reversal of the effect of OA on phosphorylase was also observed when F[−] was omitted from the incubations (Figure 1c), even when no exogenous phosphatase was added. This indicated that the permeabilized cells retained sufficient endogenous active protein phosphatase to effect this slower interconversion between phosphorylase *a* and *b*. In this instance too, CPT I was totally unaffected by this endogenous phosphatase activity (Figure 1e).

(C) Effect of OA on CPT I sensitivity to malonyl-CoA

In these experiments, hepatocytes were digitonin-permeabilized in the presence of 50 mM F[−] and further incubated in the presence of F[−] (at 37 °C for 10 min). CPT activity was then assayed in the presence of the indicated concentrations of malonyl-CoA (Figure 2). This protocol was designed to preserve any changes in sensitivity due to possible phosphorylation of the enzyme, but to eliminate the differential effects on CPT I of changes in intracellular malonyl-CoA concentration due to OA treatment of the cells (see [11]) before assay of CPT I activity.

No effect of OA treatment of hepatocytes was observed on CPT I sensitivity to malonyl-CoA inhibition (Figure 2). This was the case for cells prepared from either fed or 48 h-starved rats. It is noteworthy, however, that a marked difference in sensitivity of the enzyme between cells obtained from fed or starved rats was clearly discernible [compare Figures 2(a) and 2(b)]. This difference corresponds to that observed for the enzyme in mitochondria or purified outer membranes isolated from the livers of fed or starved animals [18,19]. Consequently, we conclude that (i) OA treatment of hepatocytes does not affect the sensitivity of CPT I to malonyl-CoA, and (ii) the difference in sensitivity of CPT I observed in permeabilized cells obtained from fed or starved rats is neither eliminated nor mimicked by OA treatment of hepatocytes. In this respect, it will be recalled that the OA-induced

Table 3 Effect of OA treatment of hepatocytes on the retention of lactate dehydrogenase (LDH) and release of adenylate kinase (AdK) after digitonin permeabilization

The total activities of LDH and AdK were 2.05 ± 0.23 and 0.36 ± 0.06 μmol/min per 10⁶ cells. Asterisks indicate that the value is significantly different ($P < 0.001$; paired *t* test) from the corresponding control value. Values are means ($\pm$ S.E.M.) for three separate cell preparations.

Permeabilization		Percentage of total marker enzyme content			
Digitonin concn. (μg/10 ⁶ cells)	Duration (s)	LDH retained in pellet		AdK released	
		Control	OA	Control	OA
40	5	13.9 $\pm$ 2.6	7.1 $\pm$ 1.7*	21.8 $\pm$ 2.3	22.8 $\pm$ 3.7
40	10	7.4 $\pm$ 1.0	3.5 $\pm$ 0.5*	21.5 $\pm$ 1.5	31.3 $\pm$ 7.8
60	5	11.3 $\pm$ 0.8	5.1 $\pm$ 0.8*	20.0 $\pm$ 3.1	21.7 $\pm$ 3.5
60	10	5.7 $\pm$ 1.0	2.6 $\pm$ 0.4*	25.7 $\pm$ 5.1	28.9 $\pm$ 5.8

Figure 3 Labelling of mitochondrial outer-membrane proteins with ^{32}P (a) or ^3H (b) after treatment with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and cyclic AMP-dependent protein kinase or $[\text{H}]\text{etomoxir}$, respectively

Full details of incubation conditions are given in the Materials and methods section. In both (a) and (b) lanes (i) relate to solubilized proteins treated with agarose beads only, whereas proteins in lanes (ii) have been treated with agarose-coupled anti-CPT I antibody. Lane (iii) in (a) refers to incorporation of ^{32}P into proteins from membranes incubated in the presence of protein kinase, but in the absence of cyclic AMP. The autoradiographs are representative of those obtained from two separate experiments.

activation of CPT I [A(i), above] was also independent of the physiological state of the donor animals. The retention of 'fed' and 'starved' properties of the enzyme in permeabilized cells confirms the observations by Boon and Zammit [2].

(D) Effects of digitonin permeabilization on the release of marker enzymes from hepatocytes

Throughout the above studies, we used the same permeabilization conditions as in [11] and limited the exposure of cells to digitonin to 5 s, as 'direct' CPT assays are linear after this time (see [11]), indicating full permeabilization to the reaction substrates. Routinely, permeabilized cells obtained from hepatocytes that had been incubated with OA were found to have a lower protein content (approx. 10% lower) than controls. This indicated that OA-treated cells lost more protein than control cells during permeabilization. Consequently, it appeared that OA was altering the interaction of digitonin with the hepatocytes. In order to find out which cell compartment was being differentially affected by OA treatment, we monitored the release of lactate dehydrogenase (cytosolic), monoamine oxidase (mitochondrial outer membrane), adenylate kinase (inter-membrane space) and glutamate dehydrogenase (matrix) activities. The experiments were performed with two different digitonin concentrations (40 and 60 $\mu\text{g}/10^6$ cells) and two periods of exposure to the detergent (5 and 10 s). As shown in Table 3, OA treatment of hepatocytes resulted in a decreased retention of lactate dehydrogenase in the permeabilized cell pellet, with a correspondingly greater loss of lactate dehydrogenase. This difference was observed at both digitonin concentrations used and for both periods of exposure.

Release of adenylate kinase from the mitochondrial inter-membrane space was substantial (20–35%), indicating that, even at the lower of the two concentrations of digitonin (routinely used in the present study and previously [11]) there is considerable disruption of outer-membrane structure. However, no release of

monoamine oxidase occurred (results not shown). OA treatment of hepatocytes did not appear to exacerbate the damage to the outer membrane. No release of glutamate dehydrogenase was observed under any of the conditions used.

(E) Effects of cyclic AMP-dependent and AMP-activated protein kinases on isolated mitochondria

Incubation of hepatocytes with glucagon and dibutyryl cyclic AMP mimics the effects of okadaic acid on CPT I when the 'direct' assay is used [11] to permeabilize hepatocytes and to measure CPT I activity simultaneously (see also Table 1). If the effect is due to direct phosphorylation of CPT I, the two protein kinases most likely to be involved are cyclic AMP-dependent protein kinase and AMP-activated protein kinase [20]. In order to test the possibility that either of these kinases could act on CPT I directly, isolated mitochondria or purified mitochondrial outer membranes were incubated with these kinases in the presence of ATP-Mg (see the Materials and methods section). Under phosphorylating conditions (which in parallel incubations using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ resulted in the phosphorylation of substrate peptides for these kinases; results not shown), no effects were observed on the activity of CPT I (at maximal or sub-maximal concentrations of palmitoyl-CoA) or its sensitivity to malonyl-CoA inhibition (results not shown). When incubations were performed in the presence of $[\text{H}]\text{etomoxir}$, several proteins of the outer membrane became phosphorylated (Figure 3a). A major phosphorylated protein migrated at a similar position to that expected for CPT I ($M_r = 88\,000$; see [21]). We do not think that this protein represents CPT I, because of its relatively high abundance and the fact that a polyclonal antibody that removed $[\text{H}]\text{etomoxir}$ -labelled CPT I from membrane extracts (Figure 3b) did not alter the amount of ^{32}P radioactivity associated with the band migrating in the 88000- M_r region relative to other phosphorylated bands (Figure 3a). Similar results were obtained throughout for AMP-activated kinase (not shown).

DISCUSSION

The present work has shown that the activatory effect of OA on CPT I activity in isolated hepatocytes can only be demonstrated under certain conditions of cell disruption. The effect was not observed either when mitochondria were prepared from cell homogenates or when a total membrane fraction was prepared after sonication. As all cell-disruption procedures were conducted under conditions known (and shown directly, with respect to phosphorylase activity) to preserve the phosphorylation status of cellular proteins, these observations suggest that the activation of CPT I is not due to direct phosphorylation of CPT, because, if this were the case, it would be expected to survive in all the protocols used (as occurred for phosphorylase), rather than only in some of them. Evidence was obtained that activation is only retained when mitochondria are still associated with other cellular fractions, e.g. in permeabilized cell ghosts and crude cellular homogenates, before isolation of mitochondria. Because of the retention of the effect in digitonin-permeabilized cells after relatively long periods of incubation and a high degree of dilution, the extra-mitochondrial cell components required for the effect to be demonstrated are presumed to be non-diffusible and possibly membranous. Sonication failed to preserve the activation, either in total sonicated preparations or in the total membrane fractions prepared therefrom. It may be relevant that the degree of sonication used disrupted cell structures much more exhaustively. In this respect, it may be relevant that OA appears to affect the ease with which digitonin permeabilizes cells

to at least one cytosolic enzyme, lactate dehydrogenase. OA-treated cells were more easily permeabilized with respect to loss of lactate dehydrogenase from the cytosol, with the result that cell ghosts contained less cytosolic protein. OA, at the concentrations used in the present study, is known to disrupt the cytoskeleton [22,23], and such effects may facilitate the loss of large cytosolic molecules during permeabilization, although it is not likely that access of the enzyme substrates would be affected. It is also evident that, from the sizeable loss of adenylate kinase observed, digitonin permeabilization of hepatocytes results in considerable damage to the mitochondrial outer membrane.

Strong evidence against the involvement of direct phosphorylation of CPT I by OA treatment was obtained from the inability of a mixture of exogenous, partially purified, protein phosphatases 1 and 2A to reverse the OA-induced activation, even when this was observed. As these phosphatases would be those expected to be inhibited in intact cells incubated with 1 μ M OA, it is unlikely that the OA-induced activation of CPT I is mediated through direct phosphorylation. Similarly, the lack of effect of the presence of F⁻ during permeabilization on the degree of activation observed suggests a mechanism other than the direct involvement of phosphorylation of CPT I in the modulation of its activity or its sensitivity to malonyl-CoA inhibition. With respect to the latter observation, we conclude that the previous description of a desensitization of CPT I towards its inhibitor after OA treatment of hepatocytes [11] was due to the different malonyl-CoA concentration experienced by the enzyme in control and OA-treated cells before permeabilization and concurrent assay of activity (by the 'direct' assay [11]). It has been shown [24,25] that CPT I is reversibly sensitized to malonyl-CoA inhibition by malonyl-CoA itself. Thus, incubation of mitochondria in the absence or presence of malonyl-CoA (at physiological temperatures) renders the enzyme less or more sensitive to inhibition, respectively [24]. Extrapolation of these observations to intracellular conditions suggests that, in control cells, CPT I will be exposed to much higher concentrations of malonyl-CoA than in OA-treated cells [11] (due to increased phosphorylation and inhibition of acetyl-CoA carboxylase). Consequently, the conformational state of CPT I will not be equivalent under the two conditions before permeabilization. If the activity is assayed concurrently with permeabilization (as is done in the 'direct' assay, and in which permeabilization and assay are performed over 45 s [11]), the different degrees of sensitization induced by different concentrations of intracellular malonyl-CoA will be reflected during the assay, as the transition between the two

different sensitivity states is relatively slow even at 37 °C [26]. It would be expected that any set of conditions that results in a decrease in intracellular malonyl-CoA concentration will also result in the apparent decrease in the sensitivity of CPT I to the inhibitor if the enzyme is assayed, for a short period and at the same time as permeabilization, rather than after a period of equalization of conditions as used in the present study. It is particularly noteworthy that, when conditions are equalized before assay, the effect of OA on malonyl-CoA sensitivity is abolished, whereas that induced by the physiological state of the donor rats (e.g. 48 h starvation) is not.

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