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Use of a selectively permeabilized isolated rat hepatocyte preparation to study changes in the properties of overt carnitine palmitoyltransferase activity *in situ*

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1. A permeabilized isolated rat liver cell preparation was developed to achieve selective permeabilization of the cell membrane to metabolites and to allow the assay of mitochondrial overt carnitine palmitoyltransferase (CPT I) activity *in situ*. By performing the digitonin-induced permeabilization in the presence of fluoride and bivalent-metal-cation sequestrants, it was possible to demonstrate that the activity of other enzymes, which are regulated by reversible phosphorylation, was preserved during the procedure and subsequent washing of cells before assay. 2. CPT activity at a sub-optimal palmitoyl-CoA concentration was almost totally (~ 90%) inhibited by malonyl-CoA, indicating that mitochondrial CPT I was largely measured in this preparation. 3. The palmitoyl-CoA-saturation and malonyl-CoA-inhibition curves for CPT activity in permeabilized cells were very similar to those obtained previously for the enzyme in isolated liver mitochondria. Moreover, starvation and diabetes had the same effects on enzyme activity, affinity for palmitoyl-CoA and malonyl-CoA sensitivity of CPT I in isolated cells as found in isolated mitochondria. These physiologically induced changes persisted through the cell preparation and incubation period. 4. Neither incubation of cells with glucagon or insulin nor incubation with pyruvate and lactate before permeabilization resulted in alterations of these parameters of CPT I in isolated cells. 5. The results are discussed in relation to the temporal relationships of changes in the activity and properties of CPT I *in vivo* in relation to the effects of insulin and glucagon on fatty acid metabolism *in vivo*.

INTRODUCTION

The kinetic properties of overt carnitine palmitoyltransferase (CPT I) in rat liver mitochondria are altered in different physiological states [1–3]. The maximal activity of the enzyme increases in ketotic states, and both its sensitivity to malonyl-CoA inhibition and its affinity for acyl-CoA are decreased [4–6]. However, the mechanisms through which these changes in the properties of the enzyme are produced are unknown.

Harano *et al.* [7] have suggested that malonyl-CoA inhibition of CPT I is not an important property of the enzyme with regard to the control of the rate of fatty acid oxidation. Their experiments have indicated that the enzyme becomes more highly phosphorylated in response to glucagon treatment of isolated rat liver cells. Increased phosphorylation was associated with a 2-fold increase in the affinity of CPT for acyl-CoA. Consequently, these authors suggested that it is the increased affinity of CPT I for its acyl-CoA substrate, induced by increased phosphorylation, that results in increased flux through the reaction, rather than de-inhibition produced by lowering of hepatic malonyl-CoA concentration and decreased sensitivity of the enzyme to the inhibitor [7]. However, these data may be open to alternative interpretations, owing to the possibility that, in their experiments, both CPT I and CPT II (latent) activities were measured, owing to the freeze–thawing and solubilization of hepatocytes before assay of CPT activity. It is also not possible to ascertain that the antibody used to immunoprecipitate ^{32}P-labelled CPT protein was raised exclusively against CPT I. Moreover, a very low concentration of fluoride (1 mM) was used in their experiments to inhibit protein phosphatase activity, although the $K_{0.5}$ for fluoride inhibition of phosphatases is in the region of 3–5 mM [8]. However, these studies, as well as others indicating that CPT I may be a substrate for protein kinase C [9], have widened the range of possible mechanisms that may be involved in the acute regulation of CPT I activity. It is particularly important to ascertain whether the enzyme is capable of such acute regulation, because the ability of its activity to be altered acutely appears to differ between physiological states [5,6,10].

Difficulties encountered in obtaining information about acute hormonal effects on CPT I arise partly from the fact that, in order to retain the functional separation between CPT I and CPT II activities, it has been necessary to work on isolated intact mitochondria. This preparation is unsuited for rapidly stopping changes in the properties of the enzyme that may be brought about by reversible covalent modification or ligand binding. Similarly, rapid freezing of intact liver or hepatocyte preparations with liquid nitrogen would be inappropriate, owing to inherent permeabilization of the mitochondrial inner membrane to acyl-CoA on freeze-thawing.

We were interested, therefore, in establishing a method whereby the activity of CPT I could be measured *in situ* in intact mitochondria within permeabilized liver cells.

Abbreviations used: CPT, CPT I, CPT II, carnitine palmitoyltransferase, its overt and latent forms.

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Previous studies had indicated that this was possible for cytoplasmic enzymes after selective permeabilization of hepatocytes to small molecules by using filipin [11] or digitonin [12]. We have coupled the technique of rapid permeabilization with the use of media that inhibit protein kinase and phosphatase activities, thus providing us with the opportunity to determine whether prior hormone treatment of the hepatocytes results in altered kinetic properties of CPT I owing to changes in its phosphorylation state. In addition, we have investigated the effects of the physiological state of the donor animals on the properties of CPT I in permeabilized hepatocytes. When this study was complete, a report of CPT I assays conducted on filipin-treated hepatocytes, that were previously exposed to dibutyryl cyclic AMP, was published [13]; it corroborates our results from studies using glucagon-treated hepatocytes.

MATERIALS AND METHODS

Animals

These were female Wistar rats (A. Tuck and Sons, Rayleigh, Essex, U.K.) weighing 200–230 g. Starved animals had food removed for 48 h before being killed. Diabetes was induced by withdrawal of insulin for 5 days from animals that had previously been injected with 100 mg of streptozotocin/kg body wt. and maintained on protamine zinc insulin for a period of 5–6 days (see [6]). Only rats in which blood glucose concentration was greater than 25 mM were used as diabetic animals.

Preparation, incubation and permeabilization of isolated liver cells

Isolated liver cells were prepared as described by Krebs *et al.* [14] with modifications as described by Seglen [15]. In brief, the liver was first perfused with 300 ml of non-recirculating Ca^{2+}-free Krebs–Henseleit medium [16], followed by recirculating perfusion (flow rate 25 ml/min) with complete Krebs–Henseleit medium containing 1.25 mM-$CaCl_2$, 4 mg of bovine serum albumin/ml and 3 mg of collagenase/ml. Media were maintained at 37 °C, and during recirculation were continuously oxygenated (O_2/CO_2, 19:1) by using a bulb oxygenator. After 25 min the liver was excised, cut coarsely with scissors and incubated with shaking (180 oscillations/min) in the collagenase-containing perfusion medium for a further 15 min. The resulting cell suspension was filtered through nylon gauze, and cells were obtained by centrifugation at 50 *g* for 1 min. They were washed twice with Ca^{2+}-free medium and finally resuspended in complete Krebs–Henseleit medium to the required density. Cell viability, as assessed by exclusion of Trypan Blue, routinely exceeded 90%. Rapid estimation of the amount of cell material was performed by the assay of lactate dehydrogenase activity in samples of the cell suspension lysed with Triton X-100 (0.5%). Routinely, cells were resuspended to a concentration that yielded 30–40 mg dry wt./ml, equivalent to 300–400 units of lactate dehydrogenase/ml at 37 °C. Samples of permeabilized cell pellets (see below) were kept from each experiment for measurement of DNA content.

Cell incubations were performed in a total volume of 5 ml of Krebs–Henseleit medium containing 10 mM-glucose, and, in experiments in which permeability to glycerol 3-phosphate was assessed, 20 mM-glycerol. Incubations were performed in 25 ml conical flasks that were continuously gassed with O_2/CO_2 (19:1) and shaken (180 oscillations/min) in a water bath maintained at 37 °C. Hormones were added at the start of the incubations and, in those extended to 60 min, again after 30 min. At the end of the incubation period, cells were permeabilized by addition of 0.1 ml of a solution containing 0.2% digitonin (freshly dissolved by sonication), 2.5 M-KF, 250 mM-EDTA, 250 mM-EGTA and 10 mM-Tris/HCl (pH 7.4 at 37 °C). After exactly 60 s, permeabilization was terminated in one of two ways. (i) For routine studies on the effects of hormones on CPT I activity, the cell suspension was diluted into a 5-fold excess of ice-cold medium containing 100 mM-KF, 5 mM-EDTA, 5 mM-EGTA and 10 mM-Tris/HCl (pH 7.4 at 0 °C), and immediately centrifuged at 1000 *g* for 2 min in a MSE bench-top centrifuge. The cell pellet was resuspended in 25 ml of the same ice-cold medium, washed and resedimented by centrifugation. The permeabilized cells were finally resuspended in 0.5 ml of the same medium and kept on ice until used (within 30 min). (ii) In experiments in which permeabilization of cells to lactate dehydrogenase and glycerol 3-phosphate was studied, the cells were rapidly sedimented, by rapid centrifugation at 3000 *g* for 60 s after addition of the digitonin-containing medium. Samples of the supernatant were taken and either kept on ice without further treatment (for lactate dehydrogenase assays) or acidified with $HClO_4$ for measurement of glycerol 3-phosphate concentrations (after removal of protein and neutralization). Preliminary experiments indicated that equivalent degrees of permeabilization to either lactate dehydrogenase of glycerol 3-phosphate were obtained by using both methods. However, rapid separation of the cells without dilution resulted in more accurate and easier determinations of glycerol 3-phosphate concentrations in the supernatant.

Analytical techniques

CPT I activity was assayed as described previously [5], except that the assay medium (2 ml) was contained in 25 ml conical flasks that were shaken (180 oscillations/min) in a water bath maintained at 37 °C in order to avoid sedimentation of the cells. Enzyme assays were terminated after 1 min by addition of 0.3 ml of 6 M-HCl, and [^{3}H]carnitine incorporation into acylcarnitine was quantified as described previously [2].

Acetyl-CoA carboxylase activity was measured as described previously [17] in the absence or presence of citrate, as indicated. Assays were terminated by addition of acid after 60 s, and acid-stable radioactivity was measured after evaporation of the samples in liquid-scintillation vials. Lactate dehydrogenase and glutamate dehydrogenase activities were measured as described in [18] and [19] respectively.

Glycerol 3-phosphate [20] and DNA [21] concentrations were measured, as described previously, in supernatants and cell pellets respectively.

The rate of fatty acid oxidation in isolated cells was measured by monitoring the incorporation of ^{14}C radioactivity from [1-^{14}C]palmitate into $^{14}CO_2$ and acid-soluble products. Measurements were performed at 15 min intervals, and rates between 15 and 45 min, which were linear, were taken.

Materials

Digitonin and other biochemicals were obtained from

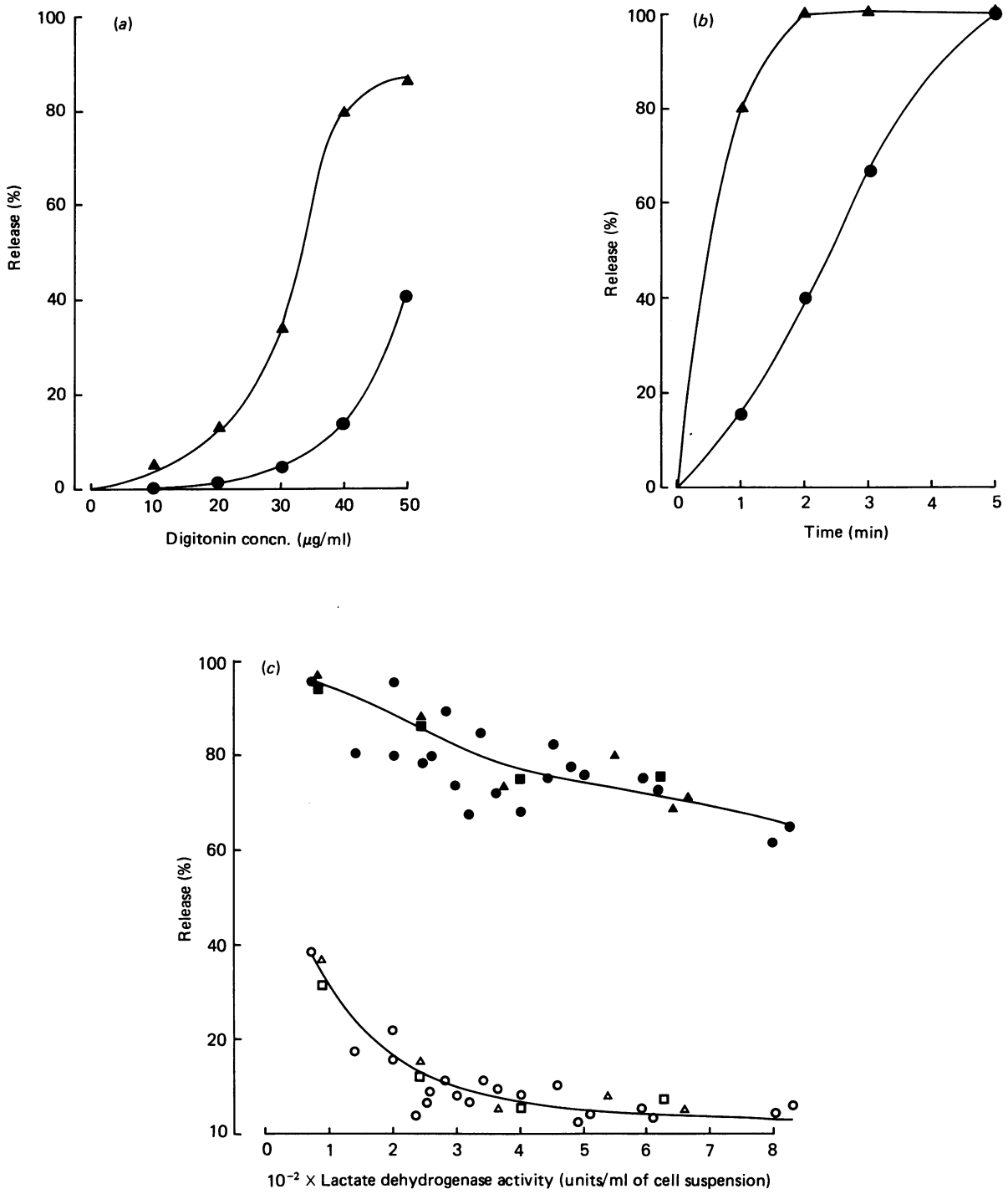

Fig. 1. Effects of increasing (*a*) digitonin concentration, (*b*) time of exposure to digitonin and (*c*) cell concentration on the release of lactate dehydrogenase and glycerol 3-phosphate from isolated rat hepatocytes

Cells were incubated for 15 min in the presence of 10 mM-glucose and 20 mM-glycerol as described in the Materials and methods section. They were then exposed to digitonin at the indicated concentrations (*a*) or to 0.004% digitonin (*b* and *c*). Cells in (*a*) and (*b*) were from fed rats. In (*c*) cells were obtained from fed (●, ○), 48 h-starved (▲, △) or diabetic (■, □) rats. The results in (*a*) and (*b*) are from a single experiment which was repeated twice with similar results: ●, lactate dehydrogenase release; ▲, glycerol 3-phosphate release. Data points in (*c*) represent individual preparations from separate animals. Data are expressed as percentages of the release of total lactate dehydrogenase (▲, ■, ●) or glycerol 3-phosphate (△, □, ○) after treatment of cells with 0.5% Triton X-100 or 3% $HClO_4$ respectively.

Sigma Chemical Co. (Poole, Dorset, U.K.). In addition, collagenase was purchased from BCL Ltd. (Lewes, Sussex, U.K.) and $NaH^{14}CO_3$ and [^{3}H]carnitine were obtained from Amersham International (Amersham, Bucks., U.K.).

RESULTS

Characterization of cell permeabilization

The aim of the permeabilization protocol was to enable access of palmitoyl-CoA to CPT I, but not to CPT II, in hepatocytes, after exposure of the cells to different hormonal treatments. Consequently, a minimal degree of cell membrane damage was required. A method whereby exposure of hepatocytes to very low concentrations of digitonin could be used for selective permeabilization of liver cell plasma membranes to low-M_r compounds was published by Lehninger and co-workers [22]. We tested the suitability of this method by comparing the release of a cytoplasmic enzyme (lactate dehydrogenase, M_r 140000) and an exclusively cytoplasmic metabolite (glycerol 3-phosphate) as described by Tager and co-workers [11]. The effects of increasing digitonin concentrations over a fixed period of time, and of increasing the length of exposure to the same concentration of digitonin at 37 °C are shown in Fig. 1. The cell density used routinely in experiments was chosen so as to result in the release of 80–90% of glycerol 3-phosphate concentration after 60 s exposure to 0.004% digitonin, accompanied by < 20% release of lactate dehydrogenase. These values were assessed by the rapid centrifugation procedure to terminate permeabilization (see the Materials and methods section). Similar release of lactate dehydrogenase was observed by using either protocol for stopping the effect of digitonin (see above). There was no further release of lactate dehydrogenase activity from the permeabilized cells during centrifugation and washing when the dilution protocol was used to stop the effect of digitonin. However, total permeabilization to small molecules always appeared to be achieved by the dilution protocol, after washing the permeabilized cells twice as judged by Trypan Blue staining of the cells. In control cell preparations that were exposed to the same procedure, but with the omission of digitonin, the release of lactate dehydrogenase and of glycerol 3-phosphate did not exceed 10 and 20% of total respectively, and the relative intactness of the preparations was confirmed by the observation that only 10–15% of total CPT I (measured in digitonin-treated cells) was observed in control preparations. No release either of CPT or of glutamate dehydrogenase activity was detected under any of the conditions used. These results with digitonin-permeabilized hepatocytes were very similar to those obtained by using filipin [11]. Minimal release of cytosolic proteins was sought in order to (i) ascertain minimal damage to the cell membrane, (ii) to enable the retention of cytosolic protein kinases and phosphatases that may be involved in mediating effects on CPT I (this should enable other studies on the direct effects of exogenously added second messengers), and (III) to optimize the probability that CPT I would be measured, not only to the exclusion of CPT II, but also *in situ* in its membrane environment. It is known that a portion of CPT I is very easily released by digitonin from isolated mitochondria (concurrently with monoamine oxidase [23]); indeed, it may reside in the cholesterol-rich outer membrane of mitochondria [24]. That most of the CPT activity measured was due to CPT I was suggested by the very high degree of inhibition of this activity by malonyl-CoA, comparable with that found in isolated mitochondria [1] (see also below).

Quantification of the cellular material in permeabilized cell suspensions used for CPT I assays was performed by measurement of DNA content after final resuspension of the 'ghosts'. Consequently, it was possible to express CPT I activity on a total cell basis rather than per mg of mitochondrial protein. This method of quantification enabled us to obtain a more meaningful measure of CPT I activity, in terms of liver function, than is possible from isolated mitochondrial studies.

Characteristics of CPT I in permeabilized hepatocytes

(i) Activity at different concentrations of palmitoyl-CoA. The curves for velocity versus palmitoyl-CoA concentration for CPT I in permeabilized cells obtained from normal fed, 48 h-starved and diabetic rats are shown in Fig. 2. The increase in maximal activity of the enzyme observed after starvation and diabetes with isolated liver mitochondria was also apparent on a whole-cell basis. Similarly, the small (2-fold) increase in the $K_{0.5}$ for palmitoyl-CoA that is observed for CPT I activity in mitochondria isolated from starved and diabetic rats [4–6,14] was also observed for the enzyme in permeabilized cells (Fig. 3). Values for apparent $K_{0.5}$ and $V_{max.}$ could not be derived from double-reciprocal plots, as these were markedly curvilinear (cf. [5] and [6]). These observations suggest that the present preparation preserves aspects of CPT I properties at least as well as in isolated mitochondrial preparations. They also indicate that the changes in these properties of CPT I that occur in mitochondrial preparations may not be artefacts, and

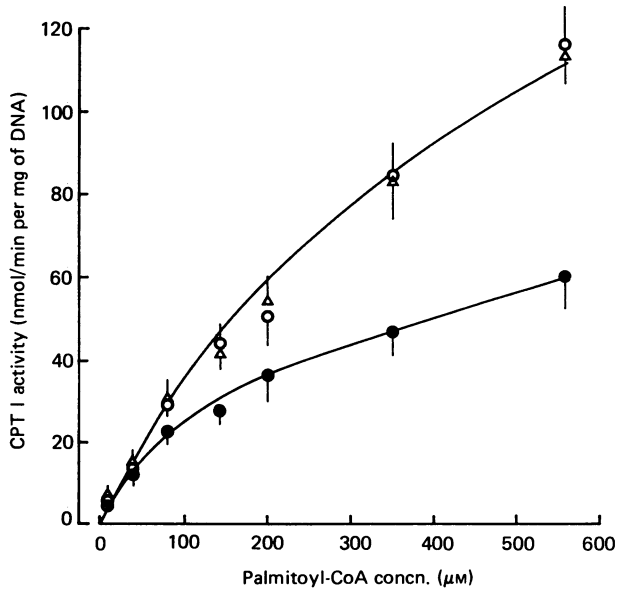

Fig. 2. Activity of CPT I in permeabilized rat liver cells isolated from fed (●), 48 h-starved (○) and diabetic (△) animals

Values are means (±S.E.M.) for three separate determinations on different cell preparations.

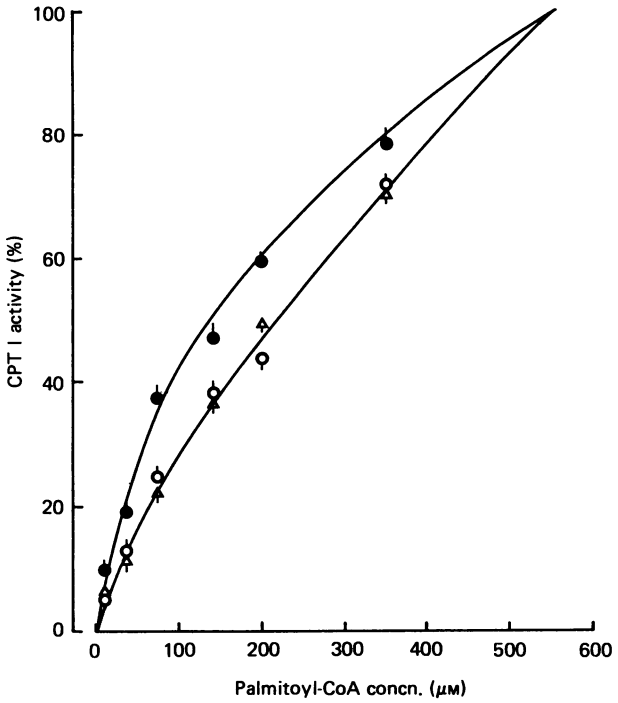

Fig. 3. Affinity of CPT I for palmitoyl-CoA in permeabilized rat liver cells isolated from fed (●), 48 h-starved (○) and diabetic (△) animals

The data from Fig. 2 are plotted as a percentage of the activity, for each individual experiment, obtained at 540 μM-palmitoyl-CoA (in the presence of 10 mg of defatted albumin/ml). Values are means (±S.E.M.) for three separate determinations on different cell preparations.

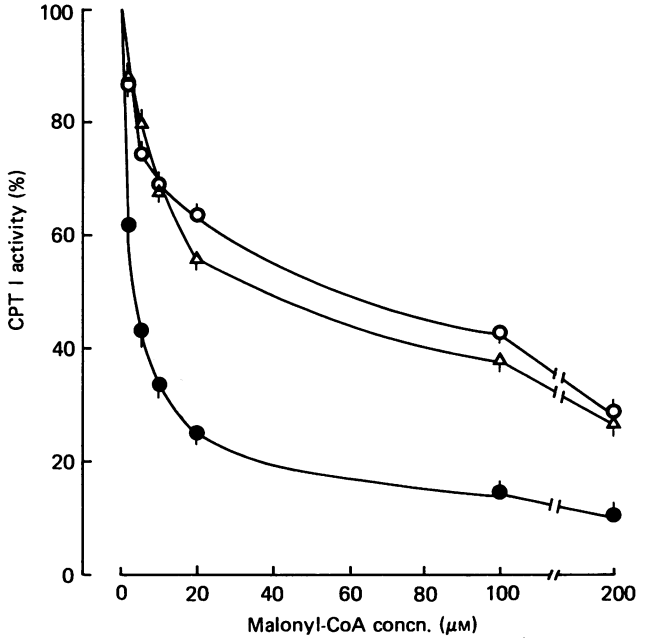

Fig. 4. Sensitivity of CPT I activity to malonyl-CoA inhibition in permeabilized liver cells isolated from fed (●), 48 h-starved (○) and diabetic (△) animals

Data are plotted as a percentage of the activity obtained in the absence of malonyl-CoA. Assays were performed in the presence of 133 μM-palmitoyl-CoA (and 10 mg of defatted albumin/ml). Values are means (±S.E.M.) for three separate determinations on different cell preparations.

that rapid inhibition of protein phosphatase and kinase activity does not alter the relative differences in the kinetics of the enzyme with respect to palmitoyl-CoA.

(ii) Sensitivity of CPT activity to malonyl-CoA inhibition. Several characteristics of the inhibition by malonyl-CoA of CPT activity measured in permeabilized cells indicated that this activity was due to the overt form of the enzyme in the mitochondria. Thus the $I_{0.5}$ values (concn. giving 50% inhibition) observed (6.6±1.2 μM, fed; 39.5±2.6 μM, diabetic; 55.8±4.3 μM starved) were similar to those found under the same conditions for the enzyme in isolated mitochondria [5,6] (Fig. 4). In addition, for cells from fed, starved and diabetic animals maximal inhibition (derived as described in [1]) was 90.9±1.5%, 82.5±2.0% and 81.7±0.6% respectively, which compare favourably with the values obtained in different laboratories, using isolated liver mitochondria [1,4,6]. This was a strong indication that any interference from peroxisomal acyltransferase activity was minimal (< 10%), as this activity is not inhibitable by malonyl-CoA [25]. In addition, it was possible to ascertain whether the activity of CPT assayed in permeabilized cells was in excess of that which would be expected from the activity of CPT I measured in isolated mitochondria, under the same conditions. For these calculations we used values for the appropriate parameters taken from the literature as follows: activity of CPT I, 10.5 nmol/min per mg of mitochondrial protein [5,6]; DNA content of liver, 5 mg/g wet wt. [26]; mitochondrial protein content per g of liver, 27–45.5 mg/g wet wt. [27–30]. From these parameters, a calculated activity in the range 56.5–94.5 nmol/min per mg of DNA is obtained (depending on which value for mitochondrial protein content is taken). Therefore, the actual experimental value of 61.5±0.5 obtained in the present study (see Fig. 2) is not only very close to this range, but towards its lower end. This strengthens the suggestion that peroxisomal activity could not possibly account for more than a small proportion of CPT activity measured in the permeabilized cell preparation used in this study. Had it been a major source of error, then the activity measured would have been expected to be well in excess of the computed value for cell CPT I activity per mg of DNA.

The differences in sensitivity of CPT activity to malonyl-CoA inhibition in cells from fed, starved and diabetic rats (Fig. 4) were very similar to those observed for the CPT I in mitochondria isolated from animals in the same respective patho-physiological states. As in the case of changes in $K_{0.5}$ for palmitoyl-CoA, these observations suggest that these changes are intrinsic to the mitochondria, whether *in situ* or isolated, and are not dependent on the preparation used *in vitro* to study them. They also suggest that the changes induced by starvation and diabetes are not easily (or, at least, not totally) reversible during perfusion of the liver, dispersion and washing of the cells, and a subsequent period of incubation in the absence of hormones. This agrees with

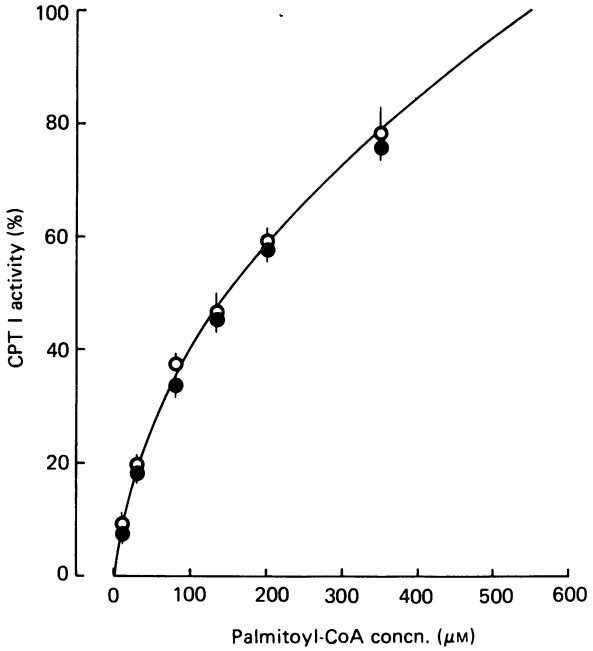

Fig. 5. Lack of effect of glucagon treatment of isolated rat liver cells on the affinity of CPT I for palmitoyl-CoA

Cells isolated from fed animals were incubated for 30 min in the presence of 10 mM-glucose either in the absence (○) or in the presence (●) of 10 μM-glucagon. They were subsequently permeabilized, washed twice, resuspended and assayed for CPT I activity at different concentrations of palmitoyl-CoA. Values are means (±S.E.M.) for three separate determinations on different cell preparations.

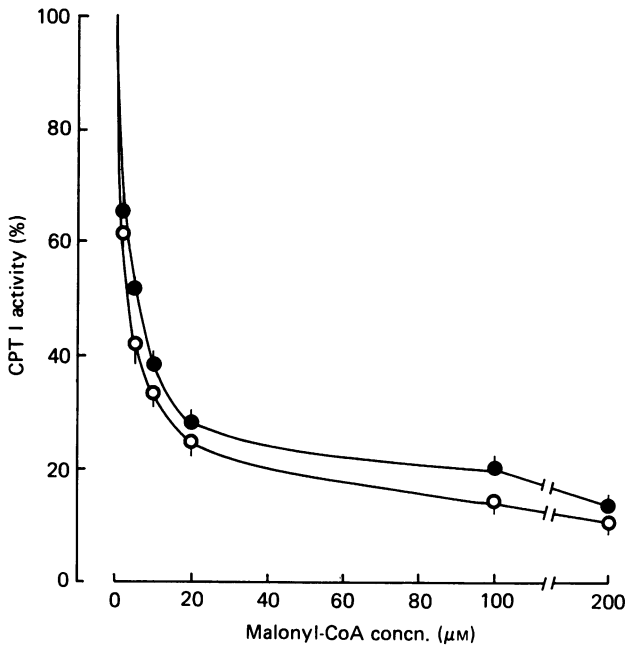

Fig. 6. Lack of effect of glucagon on the sensitivity of CPT I to malonyl-CoA inhibition in rat liver cells

See legend to Fig. 5 for experimental and notation details. CPT I assays were performed in the presence of 133 μM-palmitoyl-CoA and the indicated concentrations of malonyl-CoA. Values are means (±S.E.M.) for three separate determinations on different cell preparations.

the suggestion that at least one component of these changes is stable [31–33] and not acutely dependent on hormone withdrawal.

(iii) Effect of insulin and glucagon. In order to test whether CPT I activity in isolated hepatocytes responded acutely to hormonal stimuli, we exposed cells isolated from rats in different physiological conditions to the two hormones. The length of exposure of the cells to the hormones was chosen so as to resemble as closely as possible the conditions that, in other studies, had been suggested to result in acute changes in the properties of CPT I. Thus cells were exposed to glucagon for 30 min (as performed in [7]) and to insulin for 60 min, since the results from another laboratory [10,34] had suggested that insulin treatment of diabetic rats for 1 h resulted in a partial reversal of the kinetic properties of CPT I, towards those found for the enzyme in mitochondria from normal animals.

Contrary to the observations by Harano *et al.* [7], we did not detect any effects of glucagon on either the dependence of CPT I activity on palmitoyl-CoA concentration (Fig. 5) or the sensitivity of CPT I to malonyl-CoA inhibition (Fig. 6). In addition, we did not observe any effects of glucagon on the absolute activity of CPT I, when this was expressed as units per mg of DNA. Stephens & Harris [13] reported an increase in total CPT I activity in filipin-permeabilized cells after exposure to cyclic AMP. Whether the several differences in the protocols used in the two studies can account for this apparent discrepancy between the effects of glucagon and cyclic AMP is not known. Glucagon was ineffective in mediating any changes in CPT I activity or kinetic characteristics in cells isolated from fed or starved rats incubated with 10 mM-glucose or 9 mM-lactate and 1 mM-pyruvate for 30 or 60 min (Fig. 5). However, the cell preparations used were sensitive to glucagon, as judged by the 1.5-fold stimulation by the hormone of the rate of [1-^{14}C]palmitate oxidation, by cells isolated from fed rats, to $^{14}CO_2$ plus acid-soluble radioactivity (from 5.6±0.4 to 8.6±0.8 nmol/h per mg dry wt. of cells), although the rate of $^{14}CO_2$ production (which accounted for approx. 20% of the total) was only marginally stimulated. This stimulation of oxidation was evident throughout a 60 min period of incubation, suggesting that, even if glucagon had much more rapid and shorter-lasting effects on the properties of CPT I than could be detected by the present techniques, these were not likely to be involved in the mechanism of stimulation of oxidation by the hormone.

Similarly, we did not observe any effects of incubation in the presence of insulin on the properties of CPT I (results not shown). Nevertheless, this treatment with insulin of cells from fed animals resulted in an activation of the 'expressed' activity of acetyl-CoA carboxylase (Table 1). Total acetyl-CoA carboxylase activity was considerably (~ 70%) lower in cells from starved and diabetic animals, making accurate measurement of carboxylase activity more difficult to measure. However, it was evident that insulin elicited a smaller fractional response in acetyl-CoA carboxylase 'expressed' activity in starved-rat cells, and an inconsistent response in

Table 1. Effect of insulin treatment of isolated rat hepatocytes on the activity of acetyl-CoA carboxylase measured in permeabilized cells

Cells were incubated for 30 min in the presence of 10 mM-glucose and either in the presence of 10 μM-insulin or in the absence of the hormone. After permeabilization and washing, the cells were resuspended in 1.0 ml of wash medium (see the Materials and methods section), and samples were used for the assay of acetyl-CoA carboxylase either in the absence of citrate (E) or in the presence of 20 mM-citrate without prior incubation (C) or after preincubation at 30 °C with 20 mM-citrate (T). The + and − signs refer to the presence and absence of insulin, respectively, in the cell incubation medium.

Expt. no.	Animal	Insulin	Acetyl-CoA carboxylase activity (nmol/min per mg of DNA at 30 °C) E	C	T	$(E/T)_+/(E/T)_-$	$(C/T)_+/(C/T)_-$
1	Fed	−	4.25	7.81	38.0	1.03	3.02
		+	4.08	22.09	35.6		
2	Fed	−	3.86	5.35	17.5	1.58	1.45
		+	6.68	8.47	19.2		
3	Fed	−	5.33	9.39	38.3	1.58	1.53
		+	8.43	14.36	38.3		
4	Starved	−	0.68	1.27	7.92	1.36	1.09
		+	1.08	1.61	9.19		
5	Starved	−	0.48	1.73	11.6	1.18	0.97
		+	0.60	1.75	12.2		
6	Diabetic	−	1.27	2.07	10.62	0.97	0.93
		+	1.36	2.13	11.75		
7	Diabetic	−	1.42	1.99	12.11	1.22	0.94
		+	1.83	1.97	12.78		

diabetic-rat cells (Table 1). Other workers have also observed that cells from diabetic rats may fail to be acutely affected by insulin [35].

DISCUSSION

The several similarities found between characteristics of CPT I activity assayed in intact isolated mitochondria and in permeabilized liver cells suggest that minimal permeabilization of isolated liver cells with digitonin is a suitable technique for the study of CPT I activity *in situ*. The use of fluoride and EDTA- and EGTA-containing media (both during and after permeabilization) makes the method suitable for the study of changes in the kinetics of enzymes that are regulated by reversible phosphorylation. As described in the Results section, changes in 'expressed' acetyl-CoA carboxylase activity, induced by physiological state and/or hormone treatment *in vitro* of hepatocytes, appear to be preserved (cf. [36]). We have also shown that the same applies to activities of glycogen phosphorylase and 3-hydroxy-3-methylglutaryl-CoA reductase (P. R. Clarke, V. A. Zammit & P. C. Cohen, unpublished work). The method is particularly useful for the assay of the activities of enzymes that occur in the extramitochondrial compartment (e.g. hydroxymethylglutaryl-CoA reductase; see [37]) or on the cytoplasmic aspect of the mitochondria (e.g. CPT I) and whose assay *in vitro* is liable to interference from intramitochondrial enzymes (e.g. hydroxymethylglutaryl-CoA lyase and CPT II respectively). Because it obviates the requirement for the preparation of mitochondria from isolated cells, which are more resistant to disruption, it also optimizes the 'recovery' of intact mitochondria and enables absolute enzyme activities to be expressed per unit of DNA (cell).

Two sets of observations suggest that the properties of CPT I are not likely to be acutely modulated either by the procedures involved during the preparation and incubation of hepatocytes or by hormone treatment *in vitro* of cells before permeabilization. First, CPT I activity in cells isolated from animals in different physiological states showed the same characteristics as those of the enzyme in isolated mitochondria. Second, we did not detect any effects of incubation of the cells with insulin or glucagon on CPT I characteristics, in spite of the demonstration that these hormones were capable of affecting other aspects of the metabolism of the cell preparations used. Similarly, we failed to observe any effects of incubation of cells with different substrates (e.g. pyruvate and lactate) that raise intracellular malonyl-CoA concentrations [38]. These observations contrast with those of Harano *et al.* [7] on the effects of glucagon on the affinity of total CPT activity for palmitoyl-CoA, although, as indicated in the Introduction, the two studies were probably sufficiently different in design to account for this difference in results. The present results, however, agree with the recent observation [13] that cyclic AMP treatment of hepatocytes does not alter the malonyl-CoA sensitivity of CPT I in filipin-permeabilized cells. Our studies were also designed to test the possibility that acute changes in intracellular malonyl-CoA concentrations may directly affect CPT I sensitivity to the inhibitor [31]. The absence of any such effect after incubation of the cells with pyruvate and lactate provides no evidence for such a direct effect. It is possible, however, that during permeabilization rapid efflux of malonyl-CoA occurred, accompanied by reversal of this putative effect which, at 37 °C, is very rapid [31]. Thus the work of Stephens & Harris [13] on intact liver cells has suggested that the component due to direct malonyl-CoA effects on CPT I may be sizeable, although it is to be appreciated that effects on the sensitivity of the whole pathway of fatty acid oxidation may not always necessarily reflect exclusively those on CPT I. The

present results also emphasize that the 'inherent' component of changes in CPT I properties [31–33] induced by different physiological states is preserved in isolated cells.

The lack of acute response of CPT I to insulin or glucagon treatment may be responsible for the slow response of hepatic ketogenic capacity (relative to changes in other aspects of fatty acid metabolism) to changes in physiological state *in vivo*. Thus, during the first 6 h after commencement of anti-insulin-serum treatment of rats, the concentration of plasma non-esterified fatty acids increases to a maximum [39], suggesting that peripheral lipolysis is fully activated. In contrast, blood ketone-body concentrations are unaffected [39]. During this period, the intrinsic capacity of perfused liver to oxidise a fixed load of fatty acid delivery increased only very gradually [39]. We [6] and others [10] have observed that, when insulin is withdrawn from streptozotocin-diabetic animals [6], or after streptozotocin treatment [10], there is a very gradual change, over the next several days, in the activity and properties of CPT I. The slowness of this transition occurs in spite of the fact that plasma non-esterified fatty acid concentrations are maximal at only 24 h after insulin withdrawal [6, 40]. Similarly, insulin treatment of diabetic rats results in the rapid (< 2 h) depression of plasma non-esterified fatty acid and blood ketone-body concentrations, without any decrease in the intrinsic capacity of the perfused liver to oxidize a fixed load of fatty acids to ketones [39]. This observation agrees with the conclusions drawn from our studies on the effects of insulin treatment of chronically diabetic rats on the properties of CPT I, which required up to 24 h to return to those characteristic of the normal animals [6]. Whether under these conditions the rate of fatty acid supply to the liver becomes the major determinant of the rate of oxidation [6,39], or whether mechanisms that operate distally to CPT I become involved in the control of the oxidation of hepatic fatty acids (see [41]), still has to be determined.

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