Involvement of Ca^{2+} /calmodulin-dependent protein kinase II in the activation of carnitine palmitoyltransferase I by okadaic acid in rat hepatocytes

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The present work was undertaken to study the mechanism by which okadaic acid (OA), an inhibitor of protein phosphatases 1 and 2A, stimulates carnitine palmitoyltransferase I (CPT-I) in isolated rat hepatocytes [Guzmán, Kolodziej, Caldwell, Costorhine and Zammit (1994) *Biochem. J.* **300**, 693–699]. The OA-induced stimulation of CPT-I was abolished by the general protein kinase inhibitor K-252a as well as by KN-62, a specific inhibitor of Ca^{2+} /calmodulin-dependent protein kinase II (Ca^{2+} /CM-PKII). However, neither the protein kinase C-specific inhibitor bisindolylmaleimide nor the protein kinase A/protein kinase C inhibitor H-7 was able to prevent the OA-induced stimulation of CPT-I. Hepatocyte-shrinkage-induced stimulation

of CPT-I as well as OA-induced hepatocyte shrinkage was prevented by KN-62. KN-62 also antagonized the OA-enhanced release of lactate dehydrogenase from digitonin-permeabilized hepatocytes. Exposure of ^{32}P -labelled hepatocytes to OA increased the degree of phosphorylation of Ca^{2+} /CM-PKII, as immunoprecipitated by a monoclonal antibody raised against the α -subunit of rat brain kinase. This effect of OA was also antagonized by KN-62. The results thus indicate that the OA-dependent stimulation of CPT-I may be mediated (at least in part) by increased phosphorylation and subsequent activation of Ca^{2+} /CM-PKII.

INTRODUCTION

Carnitine palmitoyltransferase I (CPT-I) catalyses the pace-setting step of long-chain fatty acid translocation into the mitochondrial matrix [1–3]. It is well established that long-term changes in rat liver CPT-I activity occur in response to alterations in the nutritional and hormonal status of the animal [1–3]. In addition, several studies using permeabilized hepatocytes have shown that various agents exert short-term effects on CPT-I activity in parallel with changes in the rate of long-chain fatty acid oxidation measured in the same cell preparations (reviewed in [3]). In particular, okadaic acid (OA), a potent inhibitor of protein phosphatases 1 and 2A [4], is able to stimulate by up to 50% hepatic CPT-I and palmitate oxidation [5,6], indicating that inhibition of the phosphatases might have resulted in increased phosphorylation of CPT-I, with consequent increase in enzyme activity [5,6]. However, when hepatocytes were treated with OA and then permeabilized with digitonin, addition of purified protein phosphatases 1 and 2A to the permeabilized cell ghosts did not reverse the OA-induced stimulation of CPT-I [7]. In addition, although the effects of OA could be observed in permeabilized hepatocytes, they were lost upon isolation of mitochondria from the same cells. This and other observations showed that (i) the increase in CPT-I activity observed in OA-treated hepatocytes is not due to direct phosphorylation of the CPT-I enzyme, and (ii) diffusible cell component(s) lost on permeabilization of the hepatocyte plasma membrane with digitonin and distinct from protein phosphatases 1 and 2A are essential for the stimulatory effect of OA to be demonstrated [7].

The present study was thus conducted to identify intermediate proteins the phosphorylation (= activation) of which could be triggered by OA, resulting in subsequent activation of CPT-I. OA may influence the phosphorylation state of a particular target protein either by the direct inhibition of the protein phosphatases involved in the dephosphorylation of such protein or by the indirect activation of the protein kinases involved in its activation. Regarding the latter possibility, Ca^{2+} /calmodulin-dependent protein kinase II (Ca^{2+} /CM-PKII) is able to become constitutively activated when autophosphorylated in key serine or threonine residues on the autonomy site of the enzyme [8,9]. Autophosphorylation is sufficient to disrupt the autoinhibitory domain of Ca^{2+} /CM-PKII, leading to a deactivation of the kinase [8]. Hence permanent activation of Ca^{2+} /CM-PKII should be achieved by inhibition of the phosphatases involved in the dephosphorylation (= deactivation) of Ca^{2+} /CM-PKII. In fact, several responses of intact hepatocytes to OA, namely disruption of the cytoskeleton [10], inhibition of autophagy [10,11] and activation of phenylalanine hydroxylase [12], appear to be mediated by the activation of Ca^{2+} /CM-PKII. Likewise, in the present report we present data indicating that Ca^{2+} /CM-PKII is involved in the activation of CPT-I by OA in rat hepatocytes.

EXPERIMENTAL

Materials

L-[methyl- ^{14}C]Carnitine and carrier-free [^{32}P]P_i were from Amersham International (Amersham, Bucks., U.K.). Phosphate-free

Abbreviations used: Ca^{2+} /CM-PKII, Ca^{2+} /calmodulin-dependent protein kinase II; CPT-I, carnitine palmitoyltransferase I; LDH, lactate dehydrogenase; OA, okadaic acid.

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Dulbecco's modified Eagle's medium was from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.). Tetradecylglycidate was donated by Dr. J. M. Lowenstein (Brandeis University, Waltham, MA, U.S.A.). OA, KN-62, K-252a, bisindolylmaleimide and H-7 were from Calbiochem (San Diego, CA, U.S.A.). Anti- Ca^{2+} /CM-PKII monoclonal antibody (raised against the α -subunit of the rat brain enzyme) was from Boehringer-Mannheim (Indianapolis, IN, U.S.A.).

Isolation and incubation of hepatocytes

Male Wistar rats (250–300 g) which had free access to food and water were used throughout the study. Hepatocytes were isolated by the collagenase perfusion method described in [13]. They were incubated in Krebs–Henseleit bicarbonate buffer, pH 7.4, supplemented with 10 mM glucose and 1% (w/v) defatted and dialysed BSA. Incubations (4–6 mg of cellular protein/ml) were carried out at 37 °C in a metabolic gyratory shaker (85 oscillations/min), under an atmosphere of O_2/CO_2 (19:1, v/v).

In some experiments, the osmolality of the medium (305 mOsm in the normal iso-osmotic Krebs–Henseleit bicarbonate buffer) was increased to 385 mOsm (hyperosmotic medium) by changing the concentration of NaCl. This was achieved by adding 10 μl of 4 M NaCl per ml of cell incubation mixture.

CPT-I assay

After incubation of the hepatocytes with the additions indicated in each case, CPT-I activity was routinely determined in digitonin-permeabilized hepatocytes by a 'one-step' assay as the tetradecylglycidate-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine exactly as described previously [6]. In such a procedure, cell permeabilization and assay of enzyme activity are simultaneously performed [6]. In some experiments, however, CPT-I activity was determined by a more complex procedure. In this 'two-step' assay, cells are permeabilized with digitonin, then extensively washed and incubated for 5 min at 37 °C before determination of CPT-I activity. This was achieved exactly as described previously [7].

Immunoprecipitation of ^{32}P -labelled Ca^{2+} /CM-PKII

The protocol for immunoprecipitation of Ca^{2+} /CM-PKII is based on that designed for immunoprecipitation of acetyl-CoA carboxylase as previously described [14]. After isolation, hepatocytes were washed twice in phosphate-free Dulbecco's modified Eagle's medium supplemented with 1% (w/v) defatted and dialysed BSA. Hepatocytes (6–8 mg of cellular protein in 1.5 ml of the aforementioned medium) were subsequently incubated at 37 °C in a metabolic gyratory shaker (85 oscillations/min), under an atmosphere of O_2/CO_2 (19:1, v/v). Hepatocytes were labelled with 100 μCi of [^{32}P]P_i for 1 h and subsequently exposed to the additions indicated. Cells were then permeabilized by exposure for 10 s to 200 μl of a solution containing 1.5 mg of digitonin, 10 mM Tris/HCl, pH 7.4, 5 mM EDTA, 5 mM EGTA, 50 mM KF and 100 mM KCl. The cell ghosts were rapidly sedimented (15 s at 12000 g) and 500 μl of the supernatant was treated with 1% (v/v) Triton X-100 for 1 min. After another centrifugation step, 200 μl of the resulting supernatant was taken for immunoprecipitation after mixing with 50 μl of the following proteinase inhibitor mixture: PMSF (0.5 mM), Tos-Phe-CH₂Cl (15 $\mu\text{g}/\text{ml}$), Tos-Lys-CH₂Cl (18 $\mu\text{g}/\text{ml}$), tosyl-L-arginine methyl ester (18 $\mu\text{g}/\text{ml}$), pepstatin (5 $\mu\text{g}/\text{ml}$), trypsin inhibitor (50 $\mu\text{g}/\text{ml}$), leupeptin (5 $\mu\text{g}/\text{ml}$), benzamidine (0.8 mg/ml), aprotinin (0.4 kIU/ml) and 2-mercaptoethanol (5 $\mu\text{l}/\text{ml}$) in TTBS buffer (20 mM Tris/HCl, pH 7.5, 0.5 M NaCl and 0.05% Tween 20).

Preswollen Protein A–Sepharose (10 μl) was rotated end-over-end with 15 μl (15 μg) of anti- Ca^{2+} /CM-PKII monoclonal antibody (raised against the α -subunit of rat brain kinase) and 25 μl of TTBS for at least 2 h at room temperature. The antibody bound to Protein A–Sepharose was extensively washed with TTBS and then rotated end-over-end overnight at 4 °C with the 250 μl of cell extract supplemented with proteinase inhibitor cocktail (see above). The immune complexes were extensively washed with a medium containing 50 mM Tris/HCl, pH 7.5, 0.15 M NaCl, 0.5% (v/v) Nonidet P40, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS, and subsequently extracted from the Protein A–Sepharose gels by boiling for 5 min in Laemmli disintegration buffer. Samples were subjected to SDS/PAGE as described by Laemmli [15] using 1.0 mm-thick 12% polyacrylamide gels. The pH values of the stacking and resolving buffers were adjusted to 6.8 and 8.8 respectively. After fixing and drying of the gels, ^{32}P -labelled bands were visualized by autoradiography and the intensity of the bands in the autoradiograms was quantified by densitometry.

Other methods

Hepatocyte volume was estimated from the wet to dry cell weight ratio essentially as described in [16]. Briefly, 150–175 mg of fresh hepatocytes were rapidly centrifuged (2000 g, 20 s) in preweighed tubes and the wet weight of the cells as well as the dry weight (overnight at 105 °C) was measured. Lactate dehydrogenase (LDH) activity was determined in permeabilized cells as described previously [7]. Briefly, hepatocytes were permeabilized with 40 μg of digitonin/mg of protein, and the percentage of LDH retained by the permeabilized cells was determined.

Statistical analysis

Results shown represent the means $\pm$ S.D. for the number of hepatocyte preparations indicated in each case. Cell incubations and enzyme assays were always carried out in triplicate. Statistical analysis was performed by Student's *t* test.

RESULTS

Effect of K-252a and KN-62 on the OA-induced stimulation of hepatic CPT-I

The effects of OA on cell metabolism are exerted by the inhibition of protein phosphatases 1 and 2A [4]. Nevertheless, increased phosphorylation of a particular protein in cells treated with OA can result from inhibition of the protein phosphatase(s) and/or by activation of the protein kinase(s) acting on the protein. Table 1 shows the protein kinase-dependent effect of OA on hepatocyte CPT-I activity. The OA-induced stimulation of CPT-I was antagonized by K-252a, a general protein kinase inhibitor which has been shown to inhibit protein kinase A, protein kinase C, protein kinase G, Ca^{2+} /CM-PKII, myosin light-chain kinase and the tyrosine kinase activity of nerve growth factor receptor [17,18]. The effect of K-252a was dose-dependent and evident at micromolar concentrations (Figure 1). Since this observation indicates that activation of a protein kinase may mediate the effect of OA on hepatocyte CPT-I activity, we next set out to dissect the effect of this general protein kinase inhibitor K-252a using more specific protein kinase inhibitors. Alternatively, OA may inhibit dephosphorylation of the unknown target protein downstream of the putative protein kinase.

KN-62, a specific inhibitor of Ca^{2+} /CM-PKII [19], also antagonized the OA-induced activation of hepatic CPT-I (Table 1). The effect of KN-62 was dose-dependent and was evident at micromolar concentrations (Figure 2). In contrast, neither the

Table 1 Effect of protein kinase inhibitors on the OA-induced stimulation of hepatic CPT-I

Hepatocytes were preincubated for 15 min in the presence of the additions indicated, followed by 15 additional min with or without 0.5 μ M OA. CPT-I activity was then determined in digitonin-permeabilized cells either by the standard one-step assay or by the two-step assay. Values correspond to the number of hepatocyte preparations indicated and are expressed on the basis of total cellular protein (one-step assay) or ghost protein (two-step assay). The percentage effect as compared with incubations with no additions is shown in parentheses. * $P < 0.01$ compared with incubations with no additions of the corresponding type of assay. n.d., not determined.

Additions	Presence of 0.5 μ M OA	CPT-I (nmol/min per mg of protein)	
		One-step assay	Two-step assay
None	—	1.47 $\pm$ 0.21 ($n = 11$) (100)	2.27 $\pm$ 0.25 ($n = 4$) (100)
	+	2.15 $\pm$ 0.34* ($n = 11$) (146)	3.54 $\pm$ 0.43* ($n = 4$) (156)
10 μ M K-252a	—	1.54 $\pm$ 0.15 ($n = 6$) (105)	2.21 $\pm$ 0.35 ($n = 3$) (97)
	+	1.50 $\pm$ 0.13 ($n = 6$) (102)	2.34 $\pm$ 0.17 ($n = 3$) (103)
30 μ M KN-62	—	1.53 $\pm$ 0.10 ($n = 11$) (104)	2.28 $\pm$ 0.37 ($n = 4$) (100)
	+	1.50 $\pm$ 0.08 ($n = 11$) (102)	2.41 $\pm$ 0.26 ($n = 4$) (106)
2 μ M bisindolylmaleimide	—	1.44 $\pm$ 0.16 ($n = 4$) (98)	n.d.
	+	2.16 $\pm$ 0.18* ($n = 4$) (147)	n.d.
100 μ M H-7	—	1.51 $\pm$ 0.13 ($n = 5$) (103)	n.d.
	+	2.18 $\pm$ 0.33* ($n = 5$) (148)	n.d.

protein kinase C-specific inhibitor bisindolylmaleimide [20] nor the protein kinase A/protein kinase C/protein kinase G inhibitor H-7 [21] were able to prevent the OA-induced stimulation of CPT-I (Table 1). Hence activation of Ca^{2+} /CM-PKII seems to be involved in the OA-induced stimulation of hepatic CPT-I.

We routinely determine CPT-I activity in digitonin-permeabilized hepatocytes by a rapid one-step assay [6]. In such a procedure, cell permeabilization and assay of enzyme activity are simultaneously performed [6]. The versatility of this type of assay has been repeatedly demonstrated (reviewed in [3]). However, this assay may also reflect the degree of sensitization of CPT-I to malonyl-CoA induced by the different intracellular concentrations of malonyl-CoA at which the enzyme was exposed before cell permeabilization (see ref. [7]). Hence, we also determined CPT-I activity by a more complex procedure which circumvents this potential pitfall. In this two-step assay [7], cells are permeabilized with digitonin, followed by rapid and extensive washing of permeabilized cells and subsequent incubation of the permeabilized cells for 5 min at 37 °C before determination of CPT-I activity. This eliminates any possible interference of malonyl-CoA sensitization in the assay of enzyme activity [7]. As shown in Table 1, the OA-induced stimulation of CPT-I as well as the antagonistic effect of K-252a and KN-62 could be readily demonstrated by using the two-step assay as well.

Effect of KN-62 on hepatocyte-shrinkage-induced stimulation of CPT-I and OA-induced hepatocyte shrinkage

The possible link between changes in hepatocyte volume and the effects of OA and KN-62 on CPT-I activity was studied. Data are

Figure 1 Effect of K-252a on OA-induced stimulation of CPT-I

(A) Hepatocytes were preincubated for 15 min in the absence (○) or presence (●) of 10 μ M K-252a, followed by 15 additional min with various concentrations of OA. (B) Hepatocytes were preincubated for 15 min with various concentrations of K-252a, followed by 15 additional min in the absence (○) or presence (●) of 0.5 μ M OA. CPT-I activity was determined by the one-step assay. In both cases values correspond to three separate hepatocyte preparations.

shown in Table 2. In agreement with a previous report [22], a slight but statistically significant increase in CPT-I activity ensued when hepatocytes were incubated in 385 mOsm medium. Interestingly, this stimulation of CPT-I induced by hepatocyte shrinkage was prevented by KN-62. When the wet to dry cell weight ratio was determined, OA was observed to decrease hepatocyte volume by 6.7%, a magnitude similar to that observed after hepatocyte treatment with glucagon or dibutyryl-cAMP [23]. In addition, KN-62 prevented the OA-induced shrinkage of hepatocytes. As expected, when hepatocytes were incubated in a hyperosmotic (385 mOsm) medium, a marked 11.5% decrease in cell volume was observed. However, KN-62 was unable to antagonize the decrease in hepatocyte volume induced by exposure to hyperosmotic medium.

Effect of KN-62 on OA-induced release of LDH from permeabilized hepatocytes

One of the most remarkable effects elicited by OA in hepatocytes and other cell lines is hyperphosphorylation and subsequent disruption of the cytoskeleton, thereby increasing cell fragility [10,24,25]. Ca^{2+} /CM-PKII is one of the most important protein kinases involved in the control of cytoskeletal integrity by phosphorylation [8,26]. In the context of the present study, it has

Figure 2 Effect of KN-62 on OA-induced stimulation of CPT-I

(A) Hepatocytes were preincubated for 15 min in the absence (○) or presence (●) of 30 μM KN-62, followed by 15 additional min with various concentrations of OA. (B) Hepatocytes were preincubated for 15 min with various concentrations of KN-62, followed by 15 additional min in the absence (○) or presence (●) of 0.5 μM OA. CPT-I activity was determined by the one-step assay. Values correspond to three separate hepatocyte preparations.

been suggested that the increase in CPT-I activity observed in OA-treated hepatocytes may involve interactions between the outer mitochondrial membrane and non-diffusible extra-mitochondrial cell components, probably localized in the cytoskeleton [7]. Thus, in an attempt to quantify hepatocyte fragility, we determined the percentage of LDH retained in the cell ghosts after permeabilization of the plasma membrane with digitonin (see ref. [7]). As shown in Table 2, treatment of hepatocytes with OA resulted in decreased retention of LDH in the permeabilized cells, with a correspondingly greater loss of the cytoplasmic marker enzyme. Once again, KN-62 prevented this effect of OA (Table 2).

Phosphorylation of Ca^{2+} /CM-PKII as affected by OA and KN-62

To gather indications for a link between Ca^{2+} /CM-PKII and the OA-induced stimulation of CPT-I, experiments for measuring Ca^{2+} /CM-PKII activity were performed. It turned out that these measurements could not be conducted on crude cell extracts. Assays on partially purified preparations carry the risk of post-homogenizing modification of the enzyme protein. Therefore we decided to obtain more direct evidence for the involvement of Ca^{2+} /CM-PKII phosphorylation in the OA-induced stimulation of hepatocyte CPT-I. For this purpose, experiments on

Table 2 Effect of KN-62, OA and hyperosmotic medium on CPT-I activity, hepatocyte volume and LDH release from permeabilized hepatocytes

Hepatocytes were preincubated for 15 min in the absence or presence of 30 μM KN-62, followed by either 15 additional min with or without 0.5 μM OA or 30 additional min in hyperosmotic or iso-osmotic medium. Then cells were used for three different purposes: determination of CPT-I activity by the standard one-step assay, determination of the wet to dry cell weight ratio and determination of LDH retained by hepatocytes after permeabilization with digitonin (see the Experimental section). Values correspond to six (CPT-I activity) or four (wet weight/dry weight, LDH activity) separate hepatocyte preparations. The percentage effect as compared with incubations with no additions is shown in parentheses. * $P < 0.01$ or ** $P < 0.05$ compared with the corresponding incubations with no additions in iso-osmotic medium.

Presence of KN-62	Presence of OA	Presence of 385 mOsm medium	CPT-I activity (nmol/min per mg of protein)	Wet weight/dry weight	LDH retained in cell ghosts (%)
—	—	—	1.34 ± 0.25 (100)	3.82 ± 0.14 (100)	6.3 ± 1.4
—	+	—	2.00 ± 0.41* (149)	3.56 ± 0.06* (93.3)	2.3 ± 1.0*
+	—	—	1.27 ± 0.19 (95)	3.83 ± 0.03 (100.2)	6.5 ± 2.0
+	+	—	1.39 ± 0.46 (104)	3.79 ± 0.08 (99.3)	6.6 ± 0.6
—	—	+	1.59 ± 0.11** (119)	3.38 ± 0.17* (88.5)	5.9 ± 1.3
+	—	+	1.36 ± 0.15 (102)	3.49 ± 0.20* (91.4)	5.7 ± 0.9

Figure 3 Immunodetection of hepatocyte Ca^{2+} /CM-PKII

Hepatocytes were permeabilized with digitonin and the supernatant was used for immunoprecipitation with the anti- Ca^{2+} /CM-PKII antibody as described in the Experimental section except for ^{32}P labelling. The gel was fixed and subsequently stained with Coomassie Blue. A 12000 g supernatant of rat brain was used as a control. Molecular-mass markers (kDa) are shown in the left-hand and right-hand lanes. The amount of cell extract (mg of protein) incubated for immunoprecipitation was 0.08 (lane a, brain), 0.40 (lane b, brain), 0.16 (lane c, hepatocytes) and 0.80 (lane d, hepatocytes).

Ca^{2+} /CM-PKII immunoprecipitation were conducted. First we tested whether the antibody raised against the α -subunit of rat brain kinase was able to immunoprecipitate Ca^{2+} /CM-PKII from rat hepatocytes. A rat brain 12000 g supernatant was used as a control. As expected, the antibody was able to precipitate the α -subunit of Ca^{2+} /CM-PKII from rat brain. Gels showed a unique band of molecular mass 54 kDa (Figure 3). This agrees with the size previously reported for the α -subunit (50–54 kDa) [8]. Although several tissue isoforms of Ca^{2+} /CM-PKII have been described to date [8], the molecular mass reported for the subunits of rat liver kinase (50–53 kDa) [27,28] is similar to that of the α -isoform [8]. Thus a unique band of 54 kDa was observed in the gels after immunoprecipitation of rat hepatocyte Ca^{2+} /CM-PKII (Figure 3), indicating that the immunoprecipitation procedure employed is valid for our hepatocyte system.

Experiments in which Ca^{2+} /CM-PKII was immunoprecipitated from ^{32}P -labelled hepatocytes were subsequently conducted.

Figure 4 Effect of KN-62 on OA-induced phosphorylation of Ca^{2+} /CM-PKII

Hepatocytes were labelled with ^{32}P for 1 h and then treated for 15 min with the following: no additions (lane a), 0.5 μM OA (lane b), 30 μM KN-62 (lane c) and 30 μM KN-62 plus 0.5 μM OA (lane d). Immunoprecipitation of Ca^{2+} /CM-PKII was performed as described in the Experimental section. The arrow indicates the 54 kDa band.

As shown in Figure 4, a unique ^{32}P -labelled band appeared in the autoradiograms, the molecular mass of which coincided with that of immunoprecipitated Ca^{2+} /CM-PKII, i.e. 54 kDa (Figure 3). OA produced a marked increase in the phosphorylation of Ca^{2+} /CM-PKII. KN-62 prevented the OA-induced phosphorylation of hepatocyte Ca^{2+} /CM-PKII (Figure 4). Densitometry of the 54 kDa band in the autoradiograms corresponding to three different hepatocyte preparations gave the following relative values of intensity: no additions, 1.00 ± 0.18 ; 0.5 μM OA, 3.61 ± 0.59 ($P < 0.01$ compared with incubations with no additions); 30 μM KN-62, 0.72 ± 0.20 ; 30 μM KN-62 plus 0.5 μM OA, 0.83 ± 0.25 .

DISCUSSION

Several studies performed by our group using digitonin-permeabilized hepatocytes led to the suggestion that phosphorylation-dephosphorylation might be involved in the short-term control of hepatocyte CPT-I activity (reviewed in [3]). However, further research showed that the increase in CPT-I activity observed in OA-treated hepatocytes was not due to the direct phosphorylation of the CPT-I enzyme [7]. In addition, non-diffusible cell component(s) that are retained within permeabilized cells (e.g. cytoskeletal elements) but which are lost on preparation of mitochondria appeared to be essential for the stimulatory effect of OA to be demonstrated [7]. KN-62 has been repeatedly used as a specific cell-permeable inhibitor of Ca^{2+} /CM-PKII autophosphorylation (see refs. [8,29]). Hence, on the basis of the antagonism exerted by KN-62 on the effects of OA, data presented in this report point to the involvement of Ca^{2+} /CM-PKII in the OA-induced stimulation of hepatocyte CPT-I. Thus the OA-dependent stimulation of CPT-I must be dependent on the permanent activation of Ca^{2+} /CM-PKII by autophosphorylation, i.e. OA must inhibit the phosphatases involved in the dephosphorylation (= deactivation) of autophosphorylated Ca^{2+} /CM-PKII. Likewise, activation of Ca^{2+} /CM-PKII appears to be involved in other effects of OA on hepatocytes, namely disruption of the cytoskeleton [10], suppression of autophagy [10,11] and stimulation of phenylalanine hydroxylase [12]. These observations also indicate that protein phosphatases of type 1 and/or 2A are involved in the dephosphorylation of Ca^{2+} /CM-PKII in intact rat hepatocytes, in agreement with reports on rat brain Ca^{2+} /CM-PKII [30]. OA-insensitive protein phosphatase 2C has also been shown to dephosphorylate Ca^{2+} /CM-PKII purified from rat brain [31]. However, the possible role of this phosphatase in the dephosphorylation of hepatic Ca^{2+} /CM-PKII is as yet unknown.

KN-62 was able to antagonize (i) the stimulation of CPT-I induced by OA and hepatocyte shrinkage and (ii) the OA-induced hepatocyte shrinkage. Therefore a link may exist not only between the OA-triggered phosphorylation of Ca^{2+} /CM-PKII and the OA-induced stimulation of CPT-I, but also between the effect of OA and cell shrinkage on CPT-I activity. In line with these data, Häussinger and co-workers [23,32] have shown that Ca^{2+} -mobilizing agents such as vasopressin and extracellular ATP decrease hepatocyte volume. Nevertheless, we are aware that the magnitude of the activation of CPT-I induced by 0.5 μM OA is much greater than that elicited by hyperosmotic medium, although the latter reduces hepatocyte volume much more markedly than the former (see the Results section). In addition, KN-62 was unable to antagonize the decrease in hepatocyte volume induced by incubation of cells in hyperosmotic medium. Hence, although the stimulation of CPT-I by OA might be partially dependent on Ca^{2+} /CM-PKII and in turn on hepatocyte shrinkage, the possible connection between these parameters requires further investigation.

The mechanism by which Ca^{2+} /CM-PKII activates hepatic CPT-I is still not known. Evidence has been presented showing that the stimulation of hepatocyte CPT-I by OA does not involve the direct phosphorylation of CPT-I [7]. We have recently obtained data indicating that cytoskeletal components may be involved in the stimulation of hepatocyte CPT-I by agents such as protein phosphatase inhibitors (including OA), vanadate and cAMP analogues [32a]. All these compounds are potent disrupters of the cytoskeleton of hepatocytes and strong inhibitors of hepatocyte autophagy [10,11,33,34]. Interestingly, these effects of protein phosphatase inhibitors (vanadate and cAMP analogues) have been shown to be antagonized by KN-62 [10,11,33,34]. In fact, Ca^{2+} /CM-PKII is one of the most important protein kinases involved in the control of cytoskeletal integrity by phosphorylation [8]. It binds to both microtubules and intermediate filaments with high affinity, phosphorylating proteins such as vimentin, plectin, microtubule-associated protein-2 and *tau* [8]. The possible connection between activation of Ca^{2+} /CM-PKII, disruption of the cytoskeleton and activation of CPT-I is currently under study in our laboratories.

We are aware that a discrepancy exists between the effects of OA and agents that increase cytosolic free Ca^{2+} concentration on CPT-I activity and Ca^{2+} /CM-PKII phosphorylation [35]. For example, A-23187, like OA, induces a 3–4-fold increase in the phosphorylation extent of rat hepatocyte Ca^{2+} /CM-PKII (G. Velasco, M. Guzmán, V. A. Zammit and M. J. H. Geelen, unpublished work), but it has no effect on CPT-I activity [7,35]. The reason for the lack of stimulatory effect of compounds that increase cytosolic free Ca^{2+} concentration on hepatic CPT-I [35] is not obvious. Most of the previous work on the mechanism of Ca^{2+} /CM-PKII autophosphorylation has been performed with brain kinase. It is well established that neural Ca^{2+} /CM-PKII is first rapidly/transiently activated by Ca^{2+} /calmodulin, and then this activated form of the enzyme undergoes permanent activation by autophosphorylation [8]. However, this mechanism may not be exactly identical in extraneural tissues. For example, smooth-muscle Ca^{2+} /CM-PKII shows a different pattern of Ca^{2+} /calmodulin-dependent autophosphorylation from that of the brain enzyme, e.g. the latter becomes autophosphorylated much more rapidly than the former and the amino acid residues autophosphorylated in the two enzymes are very different [9]. As far as we know, no study has been performed to date on the mechanism of hepatic Ca^{2+} /CM-PKII autophosphorylation. It would thus be interesting to determine whether OA and A23187 induce the phosphorylation of different amino acid residues in hepatocyte Ca^{2+} /CM-PKII, and whether other proteins whose

phosphorylation is triggered by OA (but not by A23187) are required for the stimulation of CPT-I.

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