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REGULACION A CORTO PLAZO DE LA CARNITINA PALMITOILTRANSFERASA I DE HIGADO DE RATA

TESIS DOCTORAL

por

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ABREVIATURAS

ACBP:	Proteína ligante de ésteres de coenzima A
ACC:	Acetil-CoA carboxilasa
AICAR:	5-aminoimidazol-4-carboxiamida ribonucleósido
AMPK:	Proteína quinasa activada por 5'-AMP
Ca^{2+}/CMPKII:	Proteína quinasa dependiente de Ca^{2+} y calmodulina de tipo II
cAMP:	AMP cíclico
CAT:	Carnitina acetiltransferasa
COT:	Carnitina octanoiltransferasa
CPT-I:	Carnitina palmitoiltransferasa I
CPT-II:	Carnitina palmitoiltransferasa II
db-cAMP:	Dibutiril-AMP cíclico
FABP:	Proteína ligante de ácidos grasos
FAS:	Acido graso sintasa
HDL:	Lipoproteínas de alta densidad
IDPN:	3,3'-iminodipropionitrilo
L-FABP:	Proteína ligante de ácidos grasos (isoforma hepática)
MFABP:	Proteína de membrana ligante de ácidos grasos
PKA:	Proteína quinasa dependiente de AMP cíclico
PKC:	Proteína quinasa C
PMA:	4β-forbol 12β-miristato 13α-acetato (=TPA)
OA:	Acido okadaico
VLDL:	Lipoproteínas de muy baja densidad

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ii) *AMPK*

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1. Introducción

ASPECTOS GENERALES DEL METABOLISMO HEPÁTICO

El hígado juega un papel esencial en los mecanismos homeostáticos y en la distribución de sustratos a los distintos tejidos del organismo. Así, el hígado contiene las enzimas necesarias para llevar a cabo tanto la biosíntesis como la degradación de los lípidos y los hidratos de carbono, es el órgano que lleva acabo mayoritariamente el proceso de gluconeogénesis y, en muchas especies, entre las que se encuentra el hombre, es un órgano lipogénico de extraordinaria importancia. Desde el punto de vista anatómico, el hígado se encuentra situado en una posición privilegiada para controlar la homeostasis de la concentración de glucosa en la sangre debido a que la vena porta, antes de bañar el hígado, abarca la mayor parte de la superficie de absorción del intestino delgado. De esta forma el hígado recibe rápida información acerca de la cantidad de glucosa que se ha obtenido a partir de la ingesta, con lo que la maquinaria metabólica hepática retirará glucosa de la sangre o la liberará al torrente circulatorio para mantener constante la glucemia, proceso crucial para el correcto funcionamiento del sistema nervioso central. Sin embargo, aunque se conocen las principales rutas implicadas en el metabolismo los hidratos de carbono y los lípidos, el destino exacto de la glucosa absorbida es aún tema de debate. Así, durante muchos años se mantuvo la idea de que la glucosa pasaría directamente desde el intestino a la vena porta, de donde el hígado la capturaría, siendo entonces fosforilada y utilizada como precursor directo en los procesos de biosíntesis de glucógeno y lípidos. Sin embargo, durante la última década se ha venido acumulando una abundante evidencia experimental que apoya la existencia de una ruta indirecta, que supone la transformación de la glucosa en fragmentos C_3 (especialmente lactato) antes de ser convertida en glucógeno y lípidos. La detección de esta ruta indirecta, aparentemente ineficiente y muy costosa desde el punto de vista energético, ha dado origen al concepto de la *paradoja de la glucosa.* La contribución relativa de las rutas directa e indirecta en los procesos de biosíntesis de glucógeno y lípidos en el hígado está aún por establecer (McGarry *et al.*, 1987; Watford, 1988; Cline y Schulman, 1991; Schulman y Landau, 1992).

Durante periodos de ayuno, en los cuales el cociente insulina/glucagón en la sangre se encuentra disminuido, se movilizan las reservas tanto de glucógeno del hígado como de triacilgliceroles del tejido adiposo. Estas últimas dan lugar a glicerol, que sirve como sustrato para la gluconeogénesis, y a ácidos grasos, que, transportados vía circulatoria

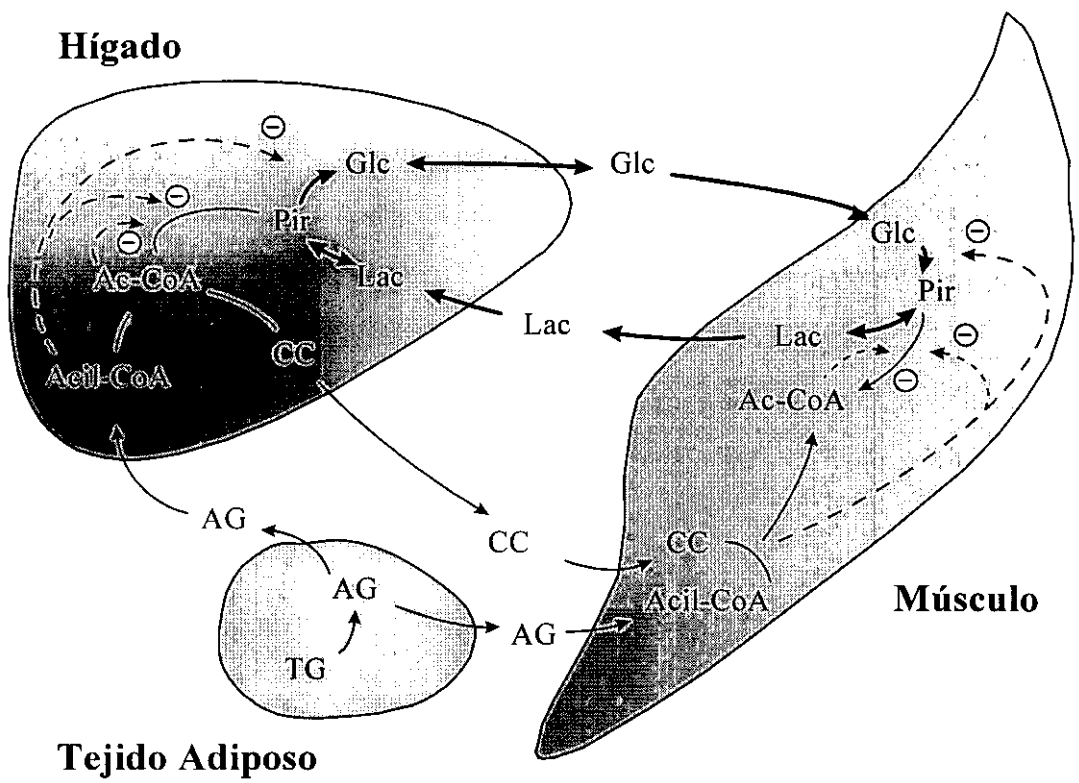

Figura 1. Ciclo de la glucosa y los ácidos grasos y su relación con el ciclo de Cori. Las flechas discontinuas indican las interacciones metabólicas entre los lípidos y los hidratos de carbono, mientras que las flechas gruesas muestran el ciclo de Cori. Abreviauras: Ac-CoA, acetil-CoA; AG, ácidos grasos; CC, cuerpos cetónicos; Glc, glucosa; Pir, piruvato; Lac, lactato; TG, triglicéridos

en forma de complejos con la albúmina, son oxidados por el hígado y los tejidos extrahepáticos (Sugden *et al.*, 1989; Beylot, 1996). En situaciones como ésta, en las que la oxidación de ácidos grasos se encuentra estimulada, parecen una serie de mecanismos adaptativos que conducen a la supresión de la utilización y oxidación de la glucosa (el ciclo glucosa/ácidos grasos), por lo que los requerimientos gluconeogénicos para el mantenimiento de la glucemia disminuyen (Fig.1). Así, la oxidación de ácidos grasos inhibe la fosforilación de la glucosa, el flujo glicolítico a nivel de la 6-fosfofructo-1-quinasa, la enzima reguladora de la primera fase de la glicolisis y la oxidación de piruvato por el complejo de la piruvato deshidrogenasa (Hue *et al*, 1988). Este último efecto permite que se reciclen los esqueletos de carbono entre la glicolisis y la gluconeogénesis (ciclo de Cori, Fig. 1). La oxidación de ácidos grasos estimula además la gluconeogénesis hepática (Sugden *et al.*, 1989). En suma, la oxidación de las reservas lipídicas juega un papel regulador de gran importancia tanto en la conservación como en la biosíntesis de la glucosa.

En situaciones de ingesta correcta, una parte de los ácidos grasos hepáticos se desvía hacia la ruta de esterificación en lugar de ser oxidados (Fig. 2). El control del equilibrio esterificación/oxidación de los acil-CoA de cadena larga es ejercido por factores tales como el cociente insulina/glucagón en el plasma y la disponibillidad relativa de lípidos e hidratos de carbono (Zammit, 1984) (Fig.2). Durante los periodos de alimentación correcta, la baja actividad de la carnitina palmitoiltransferasa I (CPT-I) impide que la oxidación mitocondrial de ácidos grasos se verifique a velocidades elevadas. Sin embargo, esta restricción desaparece en el estado de ayuno, de manera que se desvía hacia la β-oxidación una mayor proporción de acil-CoA de cadena larga (Zammit, 1994). Las demandas energéticas del hígado, son así cubiertas mayoritariamente por la β-oxidación, contribuyendo de forma minoritaria la glicolisis y la oxidación de piruvato. Los ácidos grasos oxidados por el hígado son convertidos principalmente en

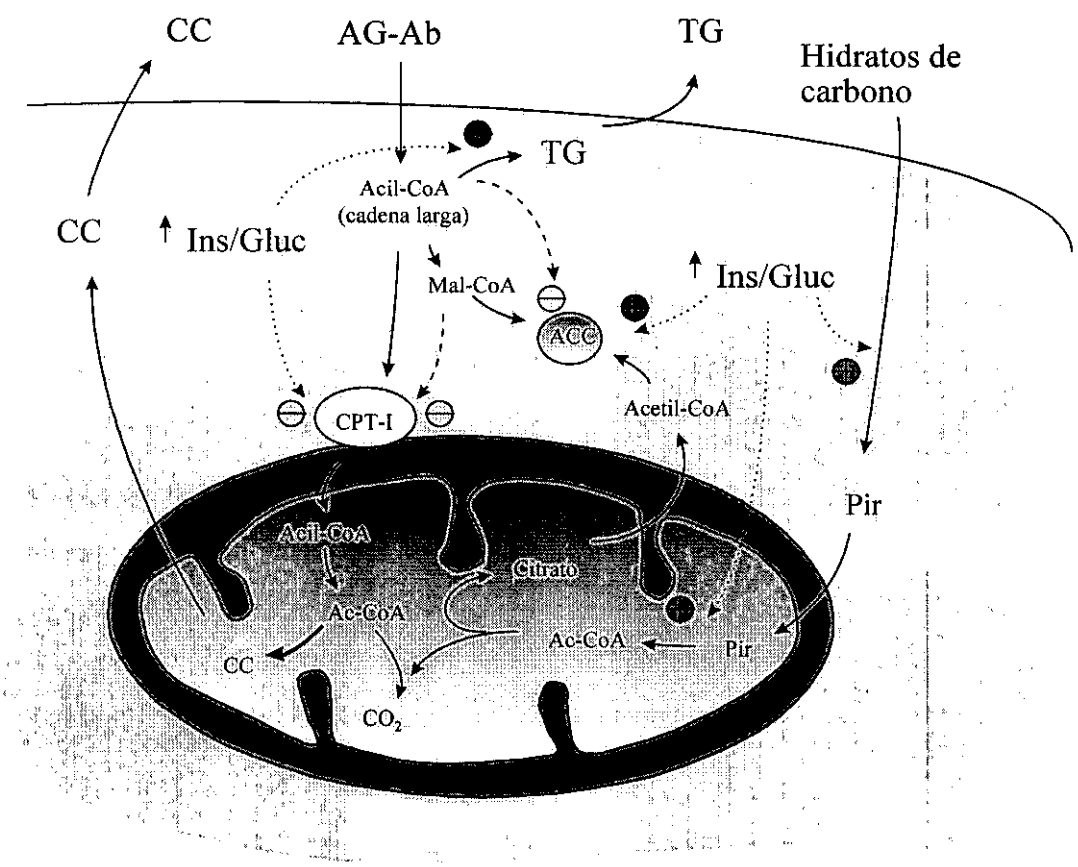

Figura 2. Interrelaciones entre la síntesis y la oxidación de ácidos grasos en el hígado. Las flechas discontinuas indican la inhibición que diversos intermediarios del metabolismo de ácidos grasos ejercen sobre la ACC y la CPT-I. Las flechas punteadas indican los lugares donde el cociente insulina/glucagón ejerce sus efectos. Abreviaturas: Ac-CoA, acetil-CoA; ACC, acetil-CoA carboxilasa; AG-Ab, complejos ácido graso-albúmina; CC, cuerpos cetónicos; CPT-I, carnitina palmitoiltransferasa I; Gluc, glucagón; Ins, insulina; Mal-CoA, malonil-CoA; Pir, piruvato; TG, triglicéridos.

cuerpos cetónicos, que pueden ser utilizados como sustratos alternativos a la glucosa por el hígado y los tejidos periféricos, incluido el cerebro (Zammit, 1984; 1994).

En contraste con su elevada homogeneidad morfológica, una de las características más sorprendentes de las células que constituyen el parénquima hepático (los hepatocitos) es su alta heterogeneidad en lo que a ultraestructura, características metabólicas y equipamiento enzimático se refiere (Gumucio, 1989; Jungermann, 1992; Jungemann y Kietzmann, 1996). Dentro de lo que se considera como la unidad estructural y funcional del hígado, esto es, el acino hepático, los hepatocitos pueden ser subdivididos en dos subpoblaciones celulares, los hepatocitos periportales (situados alrededor de las ramificaciones terminales de la vena porta, es decir en la zona aferente del hígado) y los hepatocitos perivenosos (situados alrededor de las ramificaciones terminales de la vena hepática, es decir en la zona eferente del hígado) (Gumucio, 1989; Lamers *et al.*, 1989ab; Jungermann, 1992; Jungermann y Kietzmann, 1996).

Una primera consecuencia que surge del concepto de heterogeneidad zonal del hígado es que las distintas regiones del hígado poseen una diferente disponibilidad de nutrientes, O_2 y hormonas, ya que la sangre entra a cada acino siguiendo el sentido porta → cava, de forma que la composición de la sangre va siendo modificada a medida que atraviesa el acino desde la "entrada" (zona periportal) hasta la "salida" de éste (zona perivenosa) (Lamers *et al.*, 1989ab; Jungermann, 1992). Cada una de las dos subpoblaciones de hepatocitos posee asimismo una inervación particular (Moorman *et al.*, 1989; Gardemann *et al.*, 1992). Además, tanto los hepatocitos periportales como los perivenosos poseen características fenotípicas propias, ya que aún aislados por separado a partir del hígado conservan sus peculiaridades metabólicas y enzimáticas (Chen y Katz, 1988; Tosh *et al.*, 1988; Jungermann, 1992; Jungermann y Kietzmann, 1996). Así, los hepatocitos perivenosos parecen estar mejor capacitados para llevar a cabo la captura de aminoácidos (Burger *et al.*, 1989) y de 2-oxoglutarato (Stoll y Häussinger, 1989), la biosíntesis de glutamina (Lindros *et al.*, 1989) y quizá la glicolisis (Chen y Katz, 1988; Wals *et al.*, 1988; Moorman *et al.*, 1991; Jungermann, 1992) y la lipogénesis (Guzmán y Castro, 1989; Evans *et al.*, 1989, 1990), mientras que los hepatocitos periportales verifican con mayor eficiencia los procesos de síntesis de glucógeno (Chen y Katz, 1988; Agius *et al.*, 1990), colesterol (Li *et al.*,

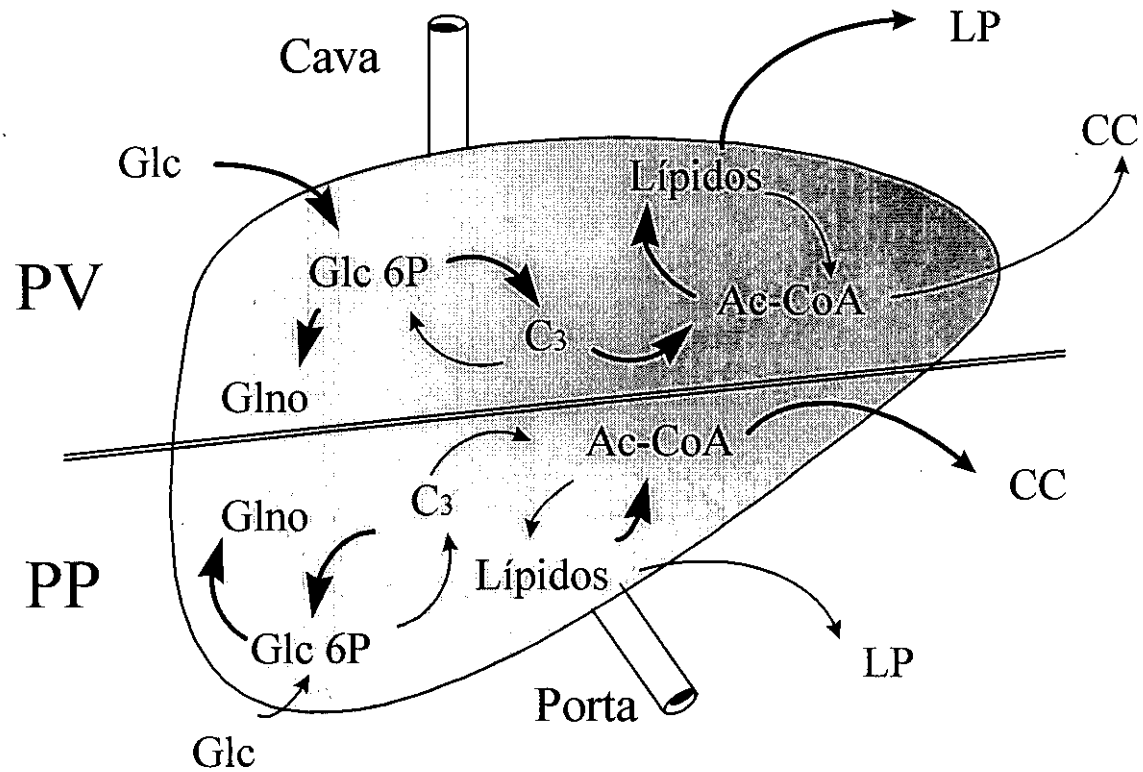

Figura 3. Zonación metabólica del hígado. Abreviaturas: Ac-CoA, acetil-CoA; CC, cuerpos cet\nicos; Glc, glucosa; Glc 6P, glucosa 6 fosfato; Glno, glucógeno; LP, lipoproteínas; PP, zona periportal; PV, zona perivenosa.

1988) y sales biliares (Verhoven y Jansen, 1989; Ugele *et al.*, 1991), así como la gluconeogénesis (Chen y Katz, 1988; Tosh *et al.*, 1988; Jungernmann, 1992), la oxidación de ácidos grasos (Tosh *et al.*, 1988; Guzmán y Castro, 1989a) y la síntesis de urea (Häussinger, 1990; Takei, 1990) (Fig. 3). Desde el punto de *vista de la paradoja de la glucosa*, esta heterogeneidad zonal del metabolismo de hidratos de carbono ha sido explicada de forma que los hepatocitos perivenosos capturarían y fosforilarían la glucosa, pasando seguidamente a la ruta glicolítica hasta generar fragmentos C_3, sobre todo lactato. A continuación, éste pasaría a la circulación sistémica hasta ser capturado por los hepatocitos periportales, que utilizarían el lactato como precursor para la síntesis de glucosa, glucógeno y lípidos (McGarry *et al.*, 1987; Watford, 1988). Sin embargo, los datos de los que se dispone en la actualidad sólo apoyan parcialmente esta hipótesis (Watford, 1988; Youn, 1989; Cline y Schulman, 1991).

Otra cuestión importante es el papel que podría jugar en el control del metabolismo hepático el hinchamiento celular. Aunque los mecanismos bioquímicos a través de los cuales este control sería ejercido no se conocen con precisión, excepto en el caso de la enzima glucógeno sintasa (Meijert *et al.*, 1992), el hinchamiento celular comienza a ser considerado como un elemento importante en la regulación metabólica de diferentes tejidos y especialmente del hígado (Agius *et al.*, 1991; Häussinger *et al.*, 1994; Häussinger, 1996), donde diferentes rutas podrían verse afectadas por este proceso.

REGULACIÓN DEL METABOLISMO HEPÁTICO DE ÁCIDOS GRASOS

El hígado utiliza ácidos grasos de procedencia tanto exógena, mayoritariamente de los complejos ácido graso albúmina presentes en el plasma) cono endógena (sintetizados *de novo* a partir de acetil-CoA u obtenidos a partir de los depósitos intracelulares de triacilgliceroles). Estos ácidos grasos podrán entonces ser, bien oxidados (completamente a CO_2 o parcialmente a cuerpos cetónicos) bien esterificados a lípidos complejos (principalmente triacilgliceroles, fosfatidilcolinas y fosfatidiletanolaminas), bien secretados en forma esterificada como glicerolípidos unidos a proteínas de muy baja densidad (VLDL) o a lipoproteínas de alta densidad (HDL). Todas estas rutas son reguladas de manera coordinada por diferentes factores, de forma que la mayor o menor actividad de alguna de ellas repercute en la actividad de otras. A continuación se repasarán los puntos de control más importantes del metabolismo de ácidos grasos.

Captura

Los ácidos grasos son capturados muy eficazmente por el hígado. Tradicionalmente se ha asumido que los ácidos grasos atraviesan la membrana plasmática siguiendo un proceso no saturable de difusión simple (Spector *et al.*, 1965; De Grelle y Light, 1980). Esta visión clásica de captura de ácidos grasos está asimismo apoyada por experimentos más recientes (Cooper *et al.*, 1989; Ferraresi-Filho *et al.*, 1992). Alternativamente, durante los últimos años se ha ido acumulando una cierta evidencia que apoya la existencia de un sistema saturable de transporte facilitado de ácidos grasos (Stremmel, 1989; Stremmel *et al.*, 1992; Trotter y Voelker, 1994; Glatz y van der Vusse, 1996). En concreto, el grupo de Stremmel ha identificado una proteína de membrana ligante de ácidos grasos de 40 KDa (MFABP). Aunque esta proteína se propuso que pudiera funcionar como intercambiador Na^+/ácidos grasos de cadena larga (Stremel, 1989; Diede *et al.*, 1992; Trotter y Voelker, 1994) parece que su funcionamiento podría ser más bien dependiente de la existencia de un gradiente de protones que podría generar el propio transporte de ácidos grasos al interior del hepatocito (Elsing *et al.*, 1996). Se han aislado además otras proteínas similares a partir de otros tejidos que capturan ácidos grasos con gran actividad, tales como el tejido adiposo (Schwieterman *et al.*, 1988; Abumrad *et al.*, 1994; Schaffer y Lodish, 1994), la mucosa intestinal (Stremmel, 1988) y el corazón (Sorrentino *et al.*, 1988). Estas proteínas podrían constituir un primer punto de control de la entrada de ácidos grasos al interior de la célula. La función de las diversas proteínas ligantes de ácidos grasos en la membrana plasmática podría ser la de actuar como transportadores o quizás, simplemente como aceptores de ácidos grasos que facilitasen la posterior difusión a través de la membrana (van Nieuwenhoven *et al.*, 1996).

Otro punto importante de control lo constituiría la lipasa sensible a hormonas (HSL) del tejido adiposo: los depósitos de triglicéridos del tejido adiposo son, cuantitativamente, los más importantes reservorios de energía del organismo y, de esta manera, los niveles de ácidos grasos libres en plasma vienen principalmente determinados por la actividad de esta enzima, cuya eficacia catalítica está sometida a regulación por fosforilación-desfosforilación.

Tráfico intracelular

El tráfico intracelular de ácidos grasos de cadena larga está mediado por proteínas ligantes de ácidos grasos (FABP). Hasta el momento se han identificado 9 isoformas distintas en diferentes tejidos (van Nieuwenhoven *et al.*, 1996), entre

ellas la isoforma hepática (conocida como L-FABP), cuya expresión aumenta en presencia de diferentes ácidos grasos (Meunier-Durmont *et al.*, 1996). Por otra parte también se ha descubierto la existencia de unas proteínas ligantes de acil-CoA (ACBP) (Gossett *et al.*, 1996) que podrían estar implicadas en la regulación de las enzimas que utilizan acil-CoA, como sustrato o como modulador, a través de la mayor o menor disponibilidad de acil-CoA libre (Faergeman y Knudsen, 1997).

Activación

La activación de ácidos grasos a sus tioésteres de CoA es un requisito previo a su utilización por la célula. Esta reacción es catalizada por una familia de acil-CoA sintetasas que difieren en su especificidad por la longitud de cadena del ácido graso y en su localización subcelular (Zammit, 1984; Schulz, 1991; Waku, 1992). De esta manera el hígado contiene acil-CoA sintetasas específicas para acil-CoA de cadena corta, cadena media, cadena larga y de cadena muy larga. Aunque la acil-CoA sintetasa de cadena larga es inducible por la dieta (Suzuki *et al.*, 1990; Schoojans *et al.*, 1993), y las acil-CoA sintetasas de cadena corta y media son reguladas *in vitro* por el cociente ATP/ADP (Zammit, 1994).

Síntesis *de novo*

La síntesis *de novo* de ácidos grasos se encuentra regulada en primer lugar por la disponibilidad de sustrato. Así, el flujo a través de la glicolisis, el suministro de lactato y la conversión de piruvato en acetil-CoA por el complejo de la piruvato deshidrogenasa son factores de gran importancia que intervienen en el control de la velocidad de síntesis de ácidos grasos (Geelen *et al.*, 1980; Wakil *et al.*, 1983; Hillgartner *et al.*, 1995). Además, las dos enzimas implicadas en la ruta de biosíntesis *de novo* de ácidos grasos, esto es, la acetil-CoA-carboxilasa (ACC) y la ácido graso sintasa (FAS), se encuentran sometidas a un estrecho control por diferentes tipos de mecanismos (Wakil *et al*, 1983; Hardie, 1992; Hardie y Carling, 1997). Esto es especialmente cierto para la ACC, enzima que cataliza la etapa limitante del proceso biosintético de los ácidos grasos (Bijleveld y Geelen, 1987; Hardie, 1992). La ACC está sometida a diferentes mecanismos de regulación: alostérica, con el precursor biosintético citrato como principal activador (Thampy y Wakil, 1988) y el producto malonil-CoA como retroinhibidor (Geelen *et al.*, 1980; Powell *et al.*, 1985); los acil-CoA de cadena larga son asimismo potentes inhibidores alostéricos de la ACC (Wakil *et al.*, 1983). La ACC se regula además por cambios en su estado de agregación (Geelen *et al.*, 1980) y por mecanismos de fosforilación-desfosforilación. En general la activación de la ACC resulta de su desfosforilación (Swenson y Porter, 1985; Bijleveld y Geelen, 1987; Thampy y Wakil, 1988). La proteína quinasa dependiente de AMP (AMPK) parece ser la responsable principal del control de la actividad de la enzima hepática (Carling *et al.*, 1989; Moore *et al.*, 1991; Hardie y Carling, 1997). Se han descrito dos especies de ACC en hígado (Thampy, 1989; Bianchi *et al.*, 1990). Se ha sugerido que la especie de 280 KDa está probablemente implicada en la sintesis de malonil-CoA para el control de la actividad CPT-I (Bianchi *et al.*, 1990; Ha *et al.*, 1996), aunque este aspecto no está aún clarificado (Winz *et al.*, 1994; Guzmán *et al.*, 1995). La isoenzima de 265 KDa parece tener dos isoformas diferentes que difieren es su capacidad para ser fosforiladas por la proteína quinasa dependiente de cAMP (PKA) (Kong *et al.*, 1990; Winz *et al.*, 1994). El hígado contiene principalmente la isoenzima no fosforilablepor PKA (Kong *et al.*,1990), lo que es coherente con la idea de que la fosforilación de la ACC hepática *in vivo* es llevada a cabo principalmente por la AMPK (Hardie y Carling, 1997).

Esterificación

La esterificación de ácidos grasos se encuentra regulada en el hígado de forma coordinada con su oxidación (Zammit, 1994). Así, las variaciones en el estado nutricional y hormonal del animal pueden estimular en el hígado la esterificación y disminuir la oxidación de los ácidos grasos (como son los casos de la realimentación tras el ayuno o el hipotiroidismo) o viceversa (como ocurre en el ayuno, la diabetes o el hipertiroidismo). La esterificación de ácidos grasos a triacilgliceroles se encuentra regulada a nivel de distintas etapas enzimáticas, destacando la glicerol 3-fosfato-acil transferasa (regulada por fosforilación-desfosforilación), la fosfatidato fosfohidrolasa (regulada por fosforilación - desfosforilación, por traslocación citoplasma-retículo endoplásmico y por ácidos grasos) y la diacilglicerol acil transferasa (posiblemente regulada por fosforilación-desfosforilación) (Tijburg *et al*, 1989).

Oxidación

En el hígado, los ácidos grasos de procedencia exógena o endógena son convertidos en sus correspondientes ésteres de CoA por las distintas acil-CoA sintetasas (Zammit, 1984; 1994). En caso de no ser esterificados a lípidos complejos, los acil-CoA pueden ser oxidados en las mitocondrias, aunque también pueden hacerlo en los peroxisomas (Osmundsen *et al.*, 1991). Sin embargo, la membrana mitocondrial interna es impermeable a los acil-CoA de cadena larga, por lo que existe un

sistema de transporte dependiente de carnitina que transloca los acil-CoA a la matriz mitocondrial y cuyo papel, crucial en la regulación del proceso de oxidación de ácidos grasos, se estudia en detalle en un apartado posterior.

Una vez en el interior de la mitocondria, los acil-CoA son escindidos en fragmentos de acetil-CoA a través de la ruta de la β-oxidación. Estos restos C_2 pueden ser completamente oxidados a CO_2 y H_2O a través de la acción subsecuente del ciclo de los ácidos tricarboxílicos y la cadena respiratoria transportadora de electrones o bien ser convertidos en cuerpos cetónicos. En general se puede considerar que la capacidad de oxidación de ácidos grasos por el hígado (u otros tejidos) en una situación fisiopatológica determinada está condicionada por dos factores principales (Edstrom y Grimby, 1986; Zammit, 1994): la disponibilidad de sustrato y la capacidad del tejido en cuestión para oxidar ácidos grasos. En lo que al primer factor se refiere, la velocidad de oxidación de ácidos grasos por el hígado aumenta de forma casi lineal con la concentración de ácidos grasos en el medio, al menos en el rango fisiológico de concentraciones de estos sustratos. Así, suele considerarse que las concentraciones de ácidos grasos que se consiguen *in vivo* no son suficientes para saturar el sistema oxidativo de ácidos grasos (Zammit, 1984). Por lo que respecta a la capacidad de oxidación de ácidos grasos podemos considerar cuatro procesos en la oxidación de un acil-CoA; el transporte al interior del orgánulo, la β-oxidación, la cetogénesis, y el ciclo de los ácidos tricarboxílicos. El transporte y la β-oxidación tienen lugar tanto en los peroxisomas como en las mitocondrias, mientras que la cetogénesis y el ciclo de los ácidos tricarboxílicos se llevan a cabo exclusivamente en el interior de las mitocondrias.

Transporte

Los ácidos grasos de cadena corta o media pueden penetrar libremente en la mitocondria independientemente de carnitina y ser posteriormente activados a ésteres de CoA en la matriz mitocondrial por la acción de acil-CoA sintetasas específicas de ácidos grasos de cadena corta y media donde, finalmente, sufrirán el proceso de β-oxidación (Bieber, 1988). Sin embargo, los acil-CoA de cadena larga son capaces de atravesar solo la membrana mitocondrial externa; para poder atravesar la membrana mitocondrial interna deben esterificarse con carnitina para formar un complejo acilcarnitina. Esta reacción de síntesis del complejo acilcarnitina la lleva a cabo una familia de enzimas denominadas en su conjunto carnitina aciltransferasas. El hígado de los mamíferos contiene tres actividades carnitina aciltransferasa atendiendo a la longitud de cadena del acil-CoA sustrato (Bieber, 1988): una actividad carnitina aciltransferasa específica de acil-CoA de cadena corta (actividad carnitina acetiltransferasa, CAT), una actividad carnitina aciltransferasa específica de acil-CoA de cadena intermedia (actividad carnitina octanoiltransferasa, COT) y una actividad carnitina aciltransferasa específica de acil-CoA de cadena larga (carnitina palmitolitransferasa, CPT). Estas tres actividades enzimáticas se encuentran localizadas en las mitocondrias y en los peroxisomas y en el retículo endoplásmico. Sin embargo, existe una cierta evidencia hoy en día de que las actividades COT y CPT son en realidad dos actividades de una misma enzima, que recibe en general el nombre de CPT y que se estudia en detalle en un próximo apartado.

β-oxidación

El conocimiento de la β-oxidación de ácidos grasos ha aumentado en gran medida durante los últimos años debido a la caracterización de distintas enzimas implicadas en esta ruta y a la descripción de una serie de defectos hereditarios asociados a ella (Schulz, 1991; Bennet, 1994; Middleton, 1994; Vockley, 1994; Eaton *et al.*, 1996). La ruta comprende dos etapas oxidativas (las reacciones catalizadas por la acil-CoA deshidrogenasa y la L-3-hidroxiacil-CoA deshidrogenasa), por lo que un posible factor regulador podría ser la relación entre las concentraciones intramitocondriales de $NADH/NAD^+$ (Grunnet y Kondrup, 1986; Latipää *et al.*, 1986; Eaton *et al.*, 1994). Aunque existen otra serie de efectores que pueden actuar a distintos niveles en la ruta β-oxidativa (Wang *et al.*, 1991; He *et al.*, 1992; Bennet, 1994; Eaton *et al.*, 1996), no existen evidencias directas de que las enzimas de la β-oxidación estén sometidas a regulación alostérica o a modificación covalente *in vivo*.

Cetogénesis

La formación de cuerpos cetónicos constituye el destino metabólico principal del acetil-CoA producido por la β-oxidación de ácidos grasos en el hígado. Así la contribución del ciclo de los ácidos tricarboxílicos a la utilización de este acetil-CoA es bastante reducida (menos del 10%) en situaciones en las cuales la disponibilidad de glucosa es la adecuada y prácticamente nula en estados catabólicos tales como el ayuno y la diabetes, en los que los ácidos grasos oxidados por el hígado se desvían prácticamente en su totalidad hacia la síntesis de cuerpos cetónicos (McGarry y Foster, 1980; Zammit, 1984; Sugden *et al.*, 1989). Algunos estudios han sugerido la hipótesis de la canalización de sustratos entre las enzimas de la ruta β-oxidativa y la cetogénica, así como entre la oxida-

ción de piruvato y el ciclo de los ácidos tricarboxílicos. Así, el acetil-CoA producido en la β-oxidación de ácidos grasos es desviado de manera preferencial hacia la ruta cetogénica, mientras que el formado por la piruvato deshidrogenasa es principalmente utilizado por el ciclo de los ácidos tricarboxílicos (Baranyai y Blum, 1989; Norsten y Cronholm, 1990; Des Rosiers *et al.*, 1991). Actualmente está bien establecido que la cetogénesis hepática está controlada tanto a corto como a largo plazo por el estado nutricional y hormonal del animal (McGarry y Foster, 1980; Zammit, 1994).

El primer paso de la formación de cuerpos cetónicos es la condensación de dos moléculas de acetil-CoA para generar acetoacetil-CoA, en reacción catalizada por la acetil-CoA acetiltransferasa. La formación de acetoacetil-CoA catalizada por la acetiltransferasa es inhibida por uno de sus productos, la CoA libre, que disminuye la afinidad de la enzima por su sustrato, el acetil-CoA (Zammit, 1994). Éste podría constituir un mecanismo para la regulación de la cetogénesis a través de cambios en la relación intramitocondrial de [acetil-CoA]/[CoA] (Wang *et al.*, 1991).

El acetoacetil-CoA es un importante metabolito implicado en el control del metabolismo mitocondrial de ácidos grasos en hígado. Se ha observado que el acetoacetil-CoA inhibe *in vitro* a la acil-CoA deshidrogenasa, la primera enzima implicada en la β-oxidación de ácidos grasos, y que ejerce inhibición por producto sobre la acetil-CoA acetiltransferasa y por sustrato sobre la 3-hidroxi-3-metilglutaril-CoA (HMG-CoA) sintasa (Lowe y Tubbs, 1985). La HMG-CoA sintasa mitocondrial cataliza la síntesis de HMG-CoA para la formación de cuerpos cetónicos y parece representar la principal etapa limitante del flujo en la cetogénesis a partir de acetil-CoA (Quant *et al.*, 1990; 1993). La actividad de la HMG-CoA sintasa mitocondrial está controlada por dos mecanismos que operan de manera coordinada: (i) modificaciones a corto plazo de las moléculas de enzima preexistentes a través de un proceso de modificación covalente (succinilación / desuccinilación) (Quant *et al.*, 1990; Zammit, 1994); (ii) cambios a largo plazo que afectan a los niveles de mRNA que codifica para la enzima y también a la cantidad de proteína inmunorreactiva (Casals *et al.*, 1992; Rodríguez *et al.*, 1994).

Ciclo de los ácidos tricarboxílicos

La oxidación completa de acetil-CoA a CO_2 tiene lugar en la matriz mitocondrial por las enzimas del ciclo de los ácidos tricarboxílicos. Como ya se ha mencionado en el apartado anterior, la contribución del ciclo de los ácidos tricarboxílicos a la utilización del acetil-CoA producido por la β-oxidación hepática de ácidos grasos es prácticamente nula comparada con la utilización de dicho acetil-CoA para la síntesis de cuerpos cetónicos. Los mecanismos de control del ciclo son muy variados e incluyen modulación alostérica, control por carga energética y potencial redox, regulación por iones Ca^{2+} y control por volumen mitocondrial. El lector interesado puede consultar una serie de revisiones publicadas en los últimos años (Halestrap, 1989; McCormack y Denton, 1990; McCormack *et al.*, 1990; Brown, 1992; Halestrap, 1994).

Oxidación de ácidos grasos en peroxisomas

Los peroxisomas poseen un equipamiento enzimático particular para β-oxidar ácidos grasos, ácidos dicarboxílicos, prostaglandinas, ácidos 5β-colestanóicos hidroxilados y diversos análogos de ácidos grasos (Osmundsen *et al.*, 1991; Osumi, 1993; Reddy y Mannaerts, 1994; Singh, 1997). Comparada con la β-oxidación mitocondrial, la β-oxidación en peroxisomas posee una especificidad de sustrato más amplia, siendo especialmente activa con ácidos grasos de cadena muy larga (Osmundsen *et al.*, 1991; Reddy y Mannaerts, 1994). De esta manera, los peroxisomas acortarían la cadena hidrocarbonada de los ácidos grasos que, debido a su longitud, difícilmente podrían ser oxidados en la mitocondria (Osmundsen *et al.*, 1991; Pourfarzam y Barlett, 1992). Los productos de la oxidación en los peroxisomas son transferidos al citoplasma y posteriormente a la matriz mitocondrial, donde continúan su metabolización (Bieber, 1988; Osmundsen *et al.*, 1991; Mannaerts y van Veldhoven, 1993). La contribución de la β-oxidación en peroxisomas a la oxidación total de ácidos grasos de cadena larga en hepatocitos depende tanto de la concentración como de la longitud de cadena de los ácidos grasos utilizados (Skorin *et al.*, 1992). En ratas alimentadas oscila en un rango desde el 20 al 35% si se utiliza palmitato u oleato como sustrato (Rongstad, 1991; Guzmán y Geelen, 1992; Skorin *et al.*, 1992). La β-oxidación en peroxisomas puede ser fácilmente inducida por la dieta, así como por la administración a largo plazo de una serie de agentes hipolipidémicos y xenobióticos (osmundsen *et al.*, 1991; Moody *et al.*, 1992; Reddy y Mannaerts, 1994).

CARNITINA PALMITOILTRANSFERASA-I

Como se ha mencionado anteriormente, los ácidos grasos de cadena larga no pueden atravesar la membrana mitocondrial interna. Para poder hacerlo, los acil-CoA procedentes de dichos ácidos grasos deben ser transformados en acilcarnitinas que, finalmente, son transportadas al interior de la mitocondria. Las CPT catalizan de forma reversi-

ble la síntesis de complejos acilcarnitina a partir de una molécula de acil-CoA de longitud de cadena intermedia o larga y una molécula de carnitina. En las mitocondrias existen dos actividades CPT, que suelen denominarse CPT-I y CPT-II, con distinta localización en la barrera mitocondrial (Murthy y Pande, 1987). Así, hoy en día se admite el siguiente modelo de traslocación de ácidos grasos de cadena larga al interior de la mitocondria (McGarry y Brown, 1997): la acil-CoA sintetasa de la cara citoplásmica de la membrana mitocondrial externa activaría un ácido graso de cadena larga a su respectivo éster de CoA. En estrecho contacto con la acil-CoA sintetasa estaría la CPT-I que mediaría la síntesis de acilcarnitina a partir del acil-CoA y la carnitina. El complejo acilcarnitina difundiría a través del espacio intermembrana hasta llegar a la cara externa de la membrana mitocondrial interna, o alcanzaría esta a través de los puntos de contacto entre las dos membranas mitocondriales (Kerner y Bieber, 1990) y allí la acción sucesiva de la translocasa de carnitina:acilcarnitina y de la CPT-II permitiría la entrada del resto de acil-CoA en la matriz mitocondrial (Fig. 4) A nivel fisiológico, la CPT de mayor importancia reguladora es la CPT-I (McGarry y Brown, 1997). La CPT-I es la enzima que se considera clave en el proceso de oxidación de ácidos grasos de cadena larga tanto en hígado (Zammit, 1986) como en los tejidos extrahepáticos (Saggerson, 1986), puesto que cataliza la principal etapa reguladora del flujo a través de dicha ruta (Drynan *et al.*, 1996; Spurway *et al.*, 1997) A continuación abordaremos el estudio de las propiedades estructurales y reguladoras que presentan las CPT, haciendo especial incapié en la CPT-I.

Estructura e Isoformas

Durante bastante tiempo el estudio de las propiedades cinéticas y reguladoras de la CPT-I tuvo que enfrentarse a tres grandes dificultades: (i) La caracterización molecular de la enzima, principalmente debido a la creencia errónea (pero clásicamente aceptada) de que la enzima se encontraba localizada en la membrana mitocondrial interna (Murthy y Pande, 1987). (ii) La presencia de peroxisomas en las preparaciones convencionales de mitocondrias y la consiguiente contaminación cruzada con la CPT de peroxisomas, fácilmente

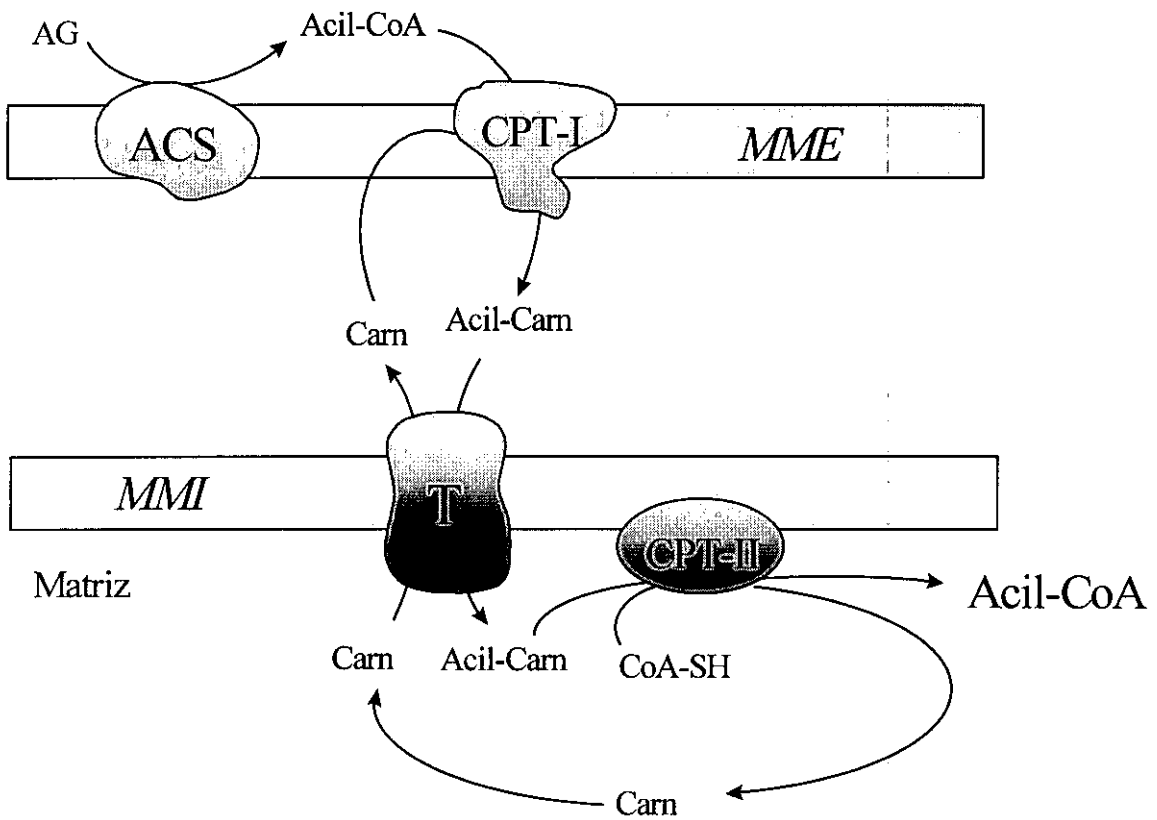

Figura 4. Traslocación de ácidos grasos a la matriz mitocondrial. Abreviaturas: ACS, acil-CoA sintetasa;; AG, ácido graso; Carn, carnitina; MME, membrana mitocondrial externa; MMI, membrana mitocondrial interna; T, translocasa de carnitina:acilcarnitina

solubilizable (Healy *et al.*, 1988; Ramsay, 1988). (iii) La solubilización de la proteína a partir de las membranas mitocondriales con la consiguiente pérdida de de actividad enzimática y/o de sensibilidad a la inhibición por malonil-CoA (Murthy y Pande, 1987; Woeltje *et al.*, 1987; McGarry *et al.*, 1991). Así, al comienzo de la presente década había una serie de cuestiones planteadas y no resueltas en torno a la CPT-I tales como: (i) Si la CPT-I y la CPT-II eran la misma o diferentes proteínas. (ii) Si existían diferentes isoformas de ambas enzimas en los distintos tejidos. (iii) Cómo se producía la interacción de los inhibidores reversibles (como el malonil-CoA) o irreversibles (como el tetradecilglicidil-CoA o el etomoxir -CoA) con la CPT-I. Clásicamente ha habido dos escuelas que proponían respuestas diferentes a estas cuestiones. Una de ellas sostenía que la CPT-I y la CPT-II eran la misma proteína (al menos en un tejido dado), pero que la primera tenía asociada una subunidad reguladoracon la que interaccionarían los diferentes inhibidores que, de esta manera, suprimirían la actividad del componente catalítico (Zammit *et al.*, 1989; Kerner y Bieber, 1990; Ghanimineja y Saggerson, 1990ab; Woldegiorgis *et al.*, 1992). Una visión alternativa proponía que la CPT-I y la CPT-II eran proteínas diferentes y que la primera poseería, dentro de la misma cadena polipeptídica, tanto el centro activo como el sitio regulador de la enzima (Woeltje *et al.*, 1987; Declercq *et al.*, 1987; Woeltje *et al.*, 1990ab; Murthy y Pande, 1990; McGarry *et al*, 1991). Finalmente la aplicación de las técnicas de la biología molecular ha permitido clarificar este escenario mediante la determinación de las estructuras primarias de ambas enzimas.

Estructura primaria de la CPT-II

Primeramente se caracterizó la estructura primaria de la CPT-II. Gracias a que esta proteína puede ser solubilizada en forma activa mediante el empleo de detergentes fue posible purificar una actividad CPT insensible a malonil-CoA (Woeltje *et al.*, 1990a) y finalmente llevar a cabo la clonación del cDNA de la CPT-II de hígado de rata (Woeltje *et al.*, 1990b) y humano (Finocchiaro *et al.*, 1991) que, en ambos casos, permitió predecir un tamaño de 658 aminoácidos y un peso molecular de 71 KDa para la CPT-II. Diferentes estudios mostraron que el mRNA de la CPT-II era idéntico en tamaño (Woeltje *et al.*, 1990a; McGarry *et al.*, 1991) e inmunológicamente indistinguible (Kolodziej *et al.*, 1992) en toda una serie de tejidos, indicando que en todos ellos se expresaba la misma proteína.

El gen de la CPT-II (el único de los genes que codifican para las CPTs que ha sido analizado en profundidad hasta el momento) (Verderio *et al.*, 1995), está compuesto de 5 exones, uno de los cuales es excepcionalmente largo. En estudios realizados con los genes humano y murino de la CPT-II (Gelb, 1993; Verderio *et al.*, 1995) se han localizado potenciales elementos reguladores en la región promotora que podrían incluir: un sitio Sp-1, una secuencia consenso para la unión de factores de respuesta a insulina y, finalmente, una secuencia que podría ser un sitio de unión de receptores de hormonas esteroideas/tiroideas.

Estructura primaria de la CPT-I

Hígado

La caracterización de la CPT-I fue bastante más difícil debido a dos propiedades de la enzima que ya habían sido previamente observadas: su estrecha asociación a la membrana mitocondrial externa y la pérdida de actividad catalítica que experimenta cuando es separada de su entorno nativo (McGarry *et al.*, 1989; Zammit *et al.*, 1989). La estrategia que finalmente se empleó fue la de tratar mitocondrias de hígado de rata con [^{3}H]-etomoxir-CoA (que como ya se había señalado anteriormente es un inhibidor irreversible y específico de la CPT-I) para marcar covalentemente dicha enzima. Posteriormente se trataron las membranas mitocondriales con una mezcla de proteasas. Así, se pudo obtener u fragmento de CPT-I todavía marcado y que pudo ser purificado a partir de geles de electroforesis en cantidades suficientes como para llevar a cabo su secuenciación (Esser *et al.*, 1993a). Con esta información, se secuenciaron oligonucleótidos con los que se realizó la búsqueda del gen de la CPT-I en una genoteca de cDNA de hígado de rata. Finalmente, el clon aislado permitió predecir una proteína de 773 aminoácidos (88 KDa) que, una vez expresada en células COS, produjo una inducción de entre 10 y 20 veces de una actividad CPT inhibible por malonil-CoA y sensible al tratamiento con detergentes (Esser *et al.*, 1993b). La expresión de esta proteína en levaduras (que carecen de actividad CPT endógena) confirmó plenamente los resultados anteriores (Brown *et al.*, 1994; De Vries *et al.*, 1997). Estos experimentos establecieron claramente que: (i) la CPT-I está constituida por un único polipéptido en el que residen tanto el dominio catalítico como el inhibidor y, (ii) que la CPT-I y la CPT-II son proteínas diferentes. A diferencia de lo que ocurre con la CPT-II, que presenta una secuencia señal en el amino terminal para poder ser transportada al interior de la mitocondria, la CPT-I al igual que otras proteínas de la membrana mitocondrial externa, carece de esa secuencia (Brown *et al.*, 1994).

A partir del cDNA de la CPT-I de hígado de rata fue posible aislar su equivalente en una genoteca de cDNA de hígado humano (Britton *et al.*, 1995). Al igual que ocurría en el caso de la CPT-II

Característica	L-CPT I	M-CPT I	CPT II
Peso Molecular	≈ 88 KDa	≈ 88 KDa	≈ 70 KDa
IC_{50} para el Malonil-CoA	≈ 2.5 μM	0.03 μM	-
K_m para la Carnitina	≈ 30 μM	≈ 500 μM	≈ 120 μM
Cromosoma humano	11q13	22q13.3	1p32
Expresión en tejidos			
Hígado	++++	-	+
Músculo esquelético	(+)	++++	+
Corazón	+	+++	+
Riñón	++++	(+)	+
Pulmón	++++	(+)	+
Bazo	++++	-	+
Intestino	++++	-	+
Páncreas	++++	-	+
Adiposo (pardo)	(+)	++++	+
Adiposo (blanco)	+	+++	+
Ovario	++++	(+)	+
Testículos	(+)	++++	+
Fibroblastos humanos	++++	-	+
Deficiencia humana descrita	Sí	No	Sí

Tabla 1. Características de las CPT mitocondriales. Los niveles relativos de expresión en cada órgano están basados en análisis por *northern blot* (y en algunos casos por marcaje con [H^3] etomoxir-CoA). (+) Solo trazas en comparación con la isoforma alternativa. (-) Indetectable. Los datos de expresión en tejidos se refieren a rata excepto en el caso de los fibroblastos. La K_m para la carnitina de la CPT-II fue determinada tras solubilización de las mitocondrias en octilglucósido y por tanto no debe ser comparada directamente con los valores de CPT-I.

tanto la secuencia obtenida a partir del cDNA de CPT-I humana como la estructura primaria (773 aminoácidos) presentaron una gran homología (82 y 88% de identidad, respectivamente) con respecto a la enzima de rata.

Músculo esquelético

Que la CPT-I de hígado (L) y músculo (M) son proteínas diferentes se sospechaba desde hacía ya tiempo ya que presentaban una sensibilidad a malonil-CoA muy diferente (IC_{50}≈ 2.7 μM y 0.03 μM en hígado y músculo esquelético, respectivamente) y valores muy diferentes de K_m para la carnitina (30 μM y 500 μM, respectivamente) (McGarry *et al*, 1983). Y además, tras su marcaje con inhibidores específicos de la CPT-I ambas proteínas migraban con pesos moleculares aparentes distintos (88 KDa (L) y 82 KDa (M) (Woeltje *et al.*, 1990a; Weis *et al.*, 1994a). La clonación del gen de CPT-I de músculo esquelético (Yamazaki *et al.*, 1995; Esser *et al.*, 1996) confirmó que dicho tejido presenta, en efecto, una isoforma diferente de CPT-I y cuyo peso molecular era sorprendentemente de 88 KDa. El hecho de que la isoforma hepática y muscular tengan movilidades electrofo réticas tan diferentes, a pesar de poseer un peso molecular casi idéntico, se ha sugerido que se debe, más a la estructura primaria de la M-CPT I que a posibles modificaciones postraduccionales de la misma (McGarry y Brown, 1997). Ambas

isoformas presentan una amplia distribución en diferentes tejidos (McGarry y Brown, 1997) (Tabla 1). La isoforma L-CPT I es la principal (o única) en hígado, riñón, pulmón, bazo, intestino, ovario y pancreas (tanto en los islotes como en los acinos), mientras que la forma M-CPT I predomina en músculo esquelético y testículos. Especialmente interesante es el caso del corazón, donde la isoforma de músculo se expresa mayoritariamente pero donde también hay una presencia significativa de la isoforma hepática (Weis *et al.*, 1994) que podría dar cuenta de las propiedades cinéticas intermedias que presentan las preparaciones de

corazón (en términos de IC_{50} para el malonil-CoA y K_m para la carnitina) con respecto a las observadas en hígado y en músculo esquelético (McGarry *et al.*, 1983).

Correlación estructura función

Aunque todavía no se ha determinado la estructura de ninguna de las CPT de forma inequívoca, sí existen datos aportados por estudios de expresión en levaduras, por comparación de secuencias o por utilización de anticuerpos específicos frente a distintos dominios de estas proteínas que permiten conocer algunos datos acerca de la topología de estas enzimas.

Topología en la membrana

La CPT-II se encuentra débilmente asociada a la cara interna de la membrana mitocondrial interna y puede ser solubilizada fácilmente en su forma activa (Woeltje *et al.*, 1987). En concordancia con esta observación, la CPT-II no presenta ninguna secuencia de aminoácidos indicativa de la presencia de fragmentos transmembranares (Woeltje *et al.*, 1990b).

La forma de asociación a la membrana externa mitocondrial de la CPT-I parece considerablemente diferente. El hecho de que la enzima no pueda solubilizarse en su forma activa mediante la utilización de detergentes suaves ya es indicativo de una fuerte asociación a la membrana. La secuencia deducida tanto para la isoforma L como para la M de la CPT-I de rata contiene dos dominios hidrofóbicos entre los aminoácidos 53 y 75 (H1) y 103 y 122 (H2). Aunque se ha cuestionado que H1 pudiera ser un fragmento transmembranar debido a la presencia de dos prolinas en las posiciones 56 y 59 de la secuencia de aminoácidos de la proteína (aminoácidos poco habituales en este tipo de dominios), diferentes datos parecen indicar que realmente adopta esta estructura. La presencia de 5 aminoácidos con carga positiva altamente conservados en el extremo amino terminal de la secuencia H1, junto a la conservación de distribuciones de carga de un aminoácido positivo y uno negativo en el extremo carboxilo terminal de dicho dominio, son características de proteínas de membrana que presentan el amino terminal hacia la cara citoplásmica (Kolodziej y Zammit, 1993). Así, se ha propuesto que la CPT-I tendría dos fragmentos transmembranares (H1 y H2) de tal forma que tanto el extremo amino como el carboxilo terminal se situarían hacia la cara citoplásmica de la membrana mitocondrial externa y que el corto bucle de 27 aminoácidos que une H1 y H2, estaría situado hacia el espacio intermembrana (Fraser *et al.*, 1997) (Fig 5). De acuerdo con esta hipótesis, una serie de estudios realizados con malonil-CoA y octanoil-CoA asociados a agarosa (para imposibilitar que pudiesen atravesar la membrana mitocon-

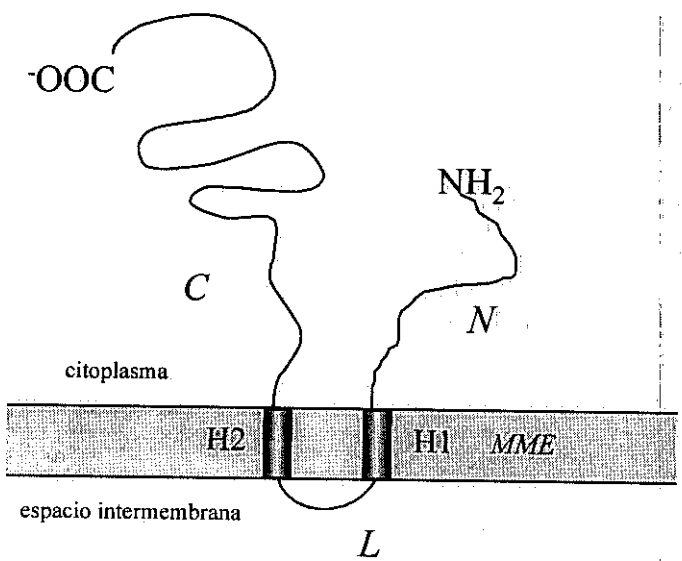

Figura 5 Topología de la CPT-I en la memebrana mitocondrial externa. En la figura se exponen las principales características de la topología de la CPT-I: (i) La proteína tiene dos fragmentos transmembranares (H1 y H2); tanto el extremo N como C terminal de la proteína están expuestos en la cara citosólica de la membrana mitocondrial externa. Las letras *C*, *N* y*L*, indican la localización de los fragmentos frente a los cuales se han desarrollado anticuerpos específicos.

drial externa) mostraba que ambos compuestos podían seguir actuando como inhibidor y sustrato de la CPT-I respectivamente, indicando que la localización de los dominios regulador y catalítico de la CPT-I es citosólica (Fraser, *et al.*, 1996).

Sitios de unión del sustrato y del malonilCoA en la CPT-I

Si bien inicialmente se consideró que la inhibición de la actividad CPT-I que produce el malonil-CoA podría ser competitiva, diferentes análisis mostraron que, en gran parte, esa inhibición es alostérica (Cook *et al.*, 1994). El tratamiento de mitocondrias de hígado de rata con diferentes proteasas produce una pérdida de sensibilidad a malonil-CoA de la CPT-I sin que la actividad catalítica se vea afectada (Frase *et al.*, 1996; Kashfi y Cook, 1992), indicando que existen dos dominios diferentes, uno catalítico y otro alostérico, en la enzima. Además, la presencia en el medio de malonil-CoA durante el tratamiento con proteasas previene tanto la desensibilización como la inactivación de la enzima (Kashfi y Cook, 1991). Se ha propuesto (ver apartado siguiente) que la presencia de malonil-CoA en el medio puede inducir un cambio de conformación en la CPT-I que favorezca la unión posterior del propio malonil-CoA y que quizá haga a la enzima menos suceptible a la acción de las proteasas (Zammit, 1996).

La diferencia de tamaño existente entre la CPT-I y la CPT-II se debe fundamentalmente a la presencia de un extremo amino terminal mucho más largo en la primera. Así, las dos proteínas presentan una alta homología de secuencia excepto en los primeros 170 aminoácidos de la CPT-I, en los que se localizarían los dos fragmentos transmembranares que la CPT-II no posee. Puesto que la diferencia más destacable entre ambas proteínas estriba en la capacidad de ser inhibida por malonil-CoA de la CPT-I pero no de la CPT-II, es posible especular que el dominio de unión para malonil-CoA pudiera residir en ese extremo amino terminal de la CPT-I, mientras que el centro activo se encontraría en el extremo carboxilo terminal común con la CPT-II. Diferentes datos apoyan esta hipótesis. Así, la expresión en levaduras de una L-CPT I truncada a la que faltaban los primeros 82 aminoácidos y que presenta una notable disminución en la sensibilidad a malonil-CoA ($IC_{50}\approx80$ μM para la proteína truncada y 5 μM para la nativa) (Brown *et al.*, 1994) indica que el extremo amino terminal podría estar implicado de alguna manera en la formación del dominio de unión a malonil-CoA. De acuerdo con esto, el tratamiento con proteinasa K de mitocondrias de hígado escinde el extremo amino terminal de la CPT-I produciendo una pérdida de sensibilidad a malonil-CoA. Sin embargo, cuando la proteinasa K tiene acceso también a la cara interna de la membrana mitocondrial externa, la pérdida de sensibilidad a malonil-CoA de la CPT-I es bastante más rápida, indicando que otras interacciones entre los dominios amino terminal y carboxilo terminal, entre el dominio H1 y la membrana, y/o entre los dominios H1 y H2 podrían estar implicadas en determinar la sensibilidad de la CPT-I a malonil-CoA (Fraser *et al.*, 1997). El hecho de que ambos dominios sean importantes a la hora de determinar la función de la enzima se apoya también en la observación de que, aunque el dominio catalítico de la CPT-I parece residir en el gran extremo carboxilo terminal de la enzima, la rotura del extremo amino terminal por proteinasa K también induce una fuerte disminución de la actividad catalítica (Fraser *et al.*, 1997)

Regulación a corto plazo

Regulación por malonil-CoA

Hígado

El descubrimiento de la inhibición de la CPT-I por malonil-CoA (McGarry *et al.*, 1977; McGarry *et al.*, 1978; McGarry y Foster, 1980) atrajo una enorme atención hacia el estudio de la regulación de esta enzima. Como se menciona en el apartado de síntesis *de novo* de ácidos grasos, el malonil-CoA es precisamente el producto de la reacción catalizada por la ACC, que constituye la etapa limitante de la síntesis de ácidos grasos (Geelen *et al.*, 1980; Wakil *et al.*, 1983). De esta manera, se consigue un control coordinado de la síntesis y la oxidación de ácidos grasos en el hígado (McGarry y Foster, 1980; Zammit, 1984). Puesto que los niveles de malonil-CoA en la célula son dependientes de la actividad ACC, se ven afectados notablemente por los cambios a corto plazo que diferentes mecanismos producen sobre dicha actividad enzimática (Zammit, 1994). Así, los niveles de malonil-CoA hepáticos se ven afectados por alteraciones en el estado hormonal y nutricional del animal (McGarry y Foster, 1980; Zammit, 1984). Especialmente determinante en la actividad ACC y en los niveles de malonil-CoA, y por tanto en la actividad CPT-I, es el cociente insulina/glucagón. Cuando este cociente es alto, los niveles de malonil-CoA también los son y la actividad CPT-I está inhibida, y viceversa (Zammit, 1994).

Músculo esquelético y corazón

El descubrimiento de que diferentes tejidos no lipogénicos, como el corazón y el músculo esquelético, también contienen malonil-CoA, y que sus niveles oscilan en función del estado nutricional del organismo indicaban que este metabolito también podría estar desempeñando un importante papel regulador en otros tejidos (Saggerson, 1986). De hecho, la isoforma de CPT-I mayoritariamente

presente en músculo cardiaco y esquelético (M-CPT I), es mucho más sensible a la presencia de malonil-CoA que la isoforma hepática (Saggerson, 1986). La idea de que el malonil-CoA y la CPT-I constituyen un importante punto regulador del metabolismo energético en músculo esquelético y en corazón fue confirmado por el descubrimiento de que ambos tejidos contienen ACC. La isoforma de músculo esquelético y corazón es la de 280 KDa (ó ACC2) distinta de la de 265 (ó ACC1) mayoritaria en hígado (Thampy, 1989; Bianchi *et al.*, 1990). Diversos experimentos han llevado a la conclusión de que los niveles de malonil-CoA en corazón dependen principalmente de la disponibilidad de acetil-CoA citosólico, de forma que el papel del malonil-CoA sería el de regulador de la CPT-I más que el de precursor para la síntesis de ácidos grasos (Awan y Saggerson, 1993; Saddik *et al.*, 1993; Lopaschuk *et al.*, 1994; Kudo *et al.*, 1995; Ha *et al.*, 1996; Stanley *et al.*, 1996). La situación en músculo esquelético es menos clara y aún existen datos contradictorios con respecto al posible papel que el malonil-CoA pudiera estar desempeñando en este tejido (McGarry y Brown, 1997).

Célula β

Un tercer tipo celular extrahepático en el que la regulación de la CPT-I mediada por malonil-CoA parece tener un papel fisiológico importante es la célula β del pancreas. Se ha propuesto que tras un incremento de la concentración de glucosa en sangre, este azúcar es metabolizado por la célula β a través de la ruta glicolítica, de tal forma que parte del piruvato sería desviado hacia la síntesis de malonil-CoA. Este malonil-CoA inhibiría a la CPT-I, lo que provocaría una acumulación de acil-CoAs de cadena larga en el citoplasma de la célula β. Se ha propuesto que estos acil-CoA actúan estimulando la exocitosis de los gránulos de insulina acumulados en estas células (Newgard y McGarry, 1995; McGarry y Brown, 1997). De esta forma, el malonil-CoA y la CPT-I podrían también desempeñar una función esencial en el mecanismo implicado en la secreción de insulina.

Sensibilidad a malonil-CoA

El malonil-CoA, por si mismo, no solo inhibe la actividad CPT-I, sino que también podría determinar la sensibilidad de la CPT-I a la inhibición por malonil-CoA. De esta manera, tanto la preincubación de hepatocitos intactos con agentes que incrementan los niveles intracelulares de malonil-CoA (Guzmán y Castro, 1989b) como la exposición de mitocondrias aisladas de hepatocitos a concentraciones fisiológicas de malonil-CoA (Zammit, 1983) hacen que la CPT-I sea más sensible al malonil-CoA en el posterior ensayo. Es más, se ha observado una fuerte correlación entre la sensibilidad de la CPT-I hepática a la inhibición por malonil-CoA y la concentración de malonil-CoA a la que la enzima está expuesta *in vivo* antes del aislamiento de las mitocondrias para el ensayo enzimático (Robinson y Zammit, 1982). Estas observaciones sugieren que este fenómeno podría también constituir la base de un importante mecanismo regulador a través del cual la respuesta de la CPT-I a pequeños cambios en la concentración de malonil-CoA hepático pudiera verse amplificada (Zammit, 1994).

En cualquier caso, la importancia fisiológica de este mecanismo está ampliamente admitida, ya que en diferentes situaciones fisiopatológicas la sensibilidad de la CPT-I a malonil-CoA se ve modificada. Así, los cambios inducidos por la dieta y el estado hormonal en el flujo a través de la ruta oxidativa de los ácidos grasos están normalmente acompañados por variaciones paralelas en la actividad específica y la sensibilidad a malonil-CoA de la CPT-I de hígado de rata. Por ejemplo en estados cetóticos tales como el ayuno (Bremer, 1981), diabetes (Cook y Gamble, 1987; Grantham y Zammit, 1988) e hipertiroidismo (Stakkestad y Bremer, 1983), la actividad específica de la CPT-I aumenta, mientras que la sensibilidad de la enzima a inhibición por malonil-CoA disminuye. Por el contrario, en estados lipogénicos tales como hipotiroidismo (Saggerson y Carpenter, 1986), realimentación tras el ayuno (Grantham y Zammit, 1986) o alimentación crónica con etanol (Guzmán *et al.*, 1987) ocurre lo contrario. La importancia de la sensibilidad a malonil-CoA de la CPT-I en el control de la oxidación de ácidos grasos de cadena larga ha sido inequivocamente demostrada en hepatocitos aislados (Prip-Buus *et al.*, 1990).

Cambios conformacionales y malonil-CoA

La posibilidad de que puedan existir diferentes conformaciones en la CPT-I relacionadas con la unión de malonil-CoA ha venido sugerida por una serie de observaciones, por ejemplo, la sensibilización a malonil-CoA de la CPT-I inducida por la preincubación en presencia de concentraciones fisiológicas de malonil-CoA se mantiene cuando las mitocondrias se incuban a 4°C (aún en ausencia de malonil-CoA) mientras que la incubación de la enzima durante unos minutos a 37°C revierte completamente esta sensibilización a malonil-CoA (Zammit, 1983; Zammit *et al.*, 1984). Además, la unión de malonil-CoA a la enzima parece inducir un fuerte cambio conformacional puesto que su presencia protege a la enzima de la acción de proteasas (Kashfi y Cook, 1992).

Se ha propuesto que la CPT-I podría adoptar diferentes conformaciones en un comportamiento que ha sido sugerido para otras enzimas monoméricas (Ricard *et al.*, 1974). De esta forma la unión

del malonil-CoA produciría un cambio conformacional que además facilitaría la unión posterior de otra molécula de malonil-CoA (Fig. 6). El pH (Fafournoux *et al.*, 1987) o la composición lipídica de la membrana (Zammit, 1994) podrían contribuir asimismo a determinar la conformación de la enzima en la membrana mitocondrial externa y por tanto, su sensibilidad a malonil-CoA.

Regulación independiente de malonil-CoA

Durante bastante tiempo se consideró que el único mecanismo de regulación a corto plazo de la actividad CPT-I sería aquel dependiente de los agonistas. Por ejemplo, la insulina y el éster de forbol 4β-forbol 12β-miristato 13α-acetato (PMA) inhiben la actividad CPT-I, mientras que el vanadato, el ácido okadaico y los agentes que incrementan los niveles de cAMP tienen el efecto opuesto (Guzmán y Geelen, 1988; Guzmán y Castro, 1990; Guzmán y Geelen, 1992). En todos estos estudios se observó una relación cualitativa entre los cambios en la actividad CPT-I y los cambios en la velocidad de oxidación mitocondrial de palmitato, confirmando que la CPT-I es una enzima clave en el control de la oxidación de ácidos grasos de cadena larga por mitocondrias hepáticas (Tabla 2). Es importante resaltar que el ensayo de la acti-

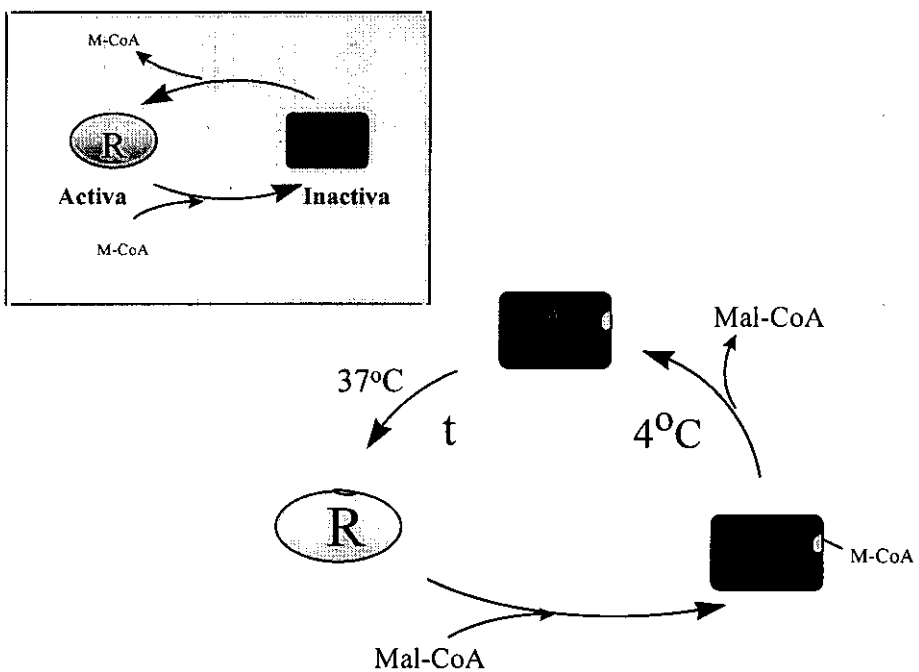

Figura 6. La CPT-I podría sufrir cambios conformacionales en función de la existencia o no de malonil-CoA en el medio. La CPT-I podría adoptar al menos dos conformaciones diferentes en función de la presencia de malonil-CoA en el medio. Abreviaturas: M-CoA, malonil-CoA; R, conformación relajada (más activa); T, conformaci\n tensa (menos activa).

niveles de malonil-CoA que como hemos visto anteriormente determinan tanto la actividad catalítica de la enzima como su sensibilidad al propio malonil-CoA. Ésto podría ser debido al hecho de que la modulación a corto plazo de la CPT-I se perdiera durante el proceso de rotura celular y posterior aislamiento de las mitocondrias para llevar a cabo el correspondiente ensayo enzimático. Sin embargo, la puesta a punto de métodos de determinación de la actividad CPT *in situ* mediante la utilización de células permeabilizadas (Stephens y Harris, 1987; Guzmán y Geelen, 1988) (Fig. 7) permitió demostrar que la CPT-I hepática se halla controlada a corto plazo por diferentes tipos de

vidad CPT-I en células permeabilizadas implica una notoria dilución del contenido del citoplasma celular en el medio de ensayo y con ello del malonil-CoA presente en el mismo, por lo que los cambios observados en la actividad CPT-I no se deberían a modificaciones en los niveles de malonil-CoA, sino a otro(s) posible(s) mecanismo(s) implicado(s) en la regulación a corto plazo de la CPT-I. Dado que diversos agonistas capaces de modificar a corto plazo la actividad CPT-I, como por ejemplo el glucagón (que incrementa los niveles intracelulares de cAMP, induciendo así la activación de la PKA) median su acción a través de la activación de proteínas quinasas, se especuló con la posibili-

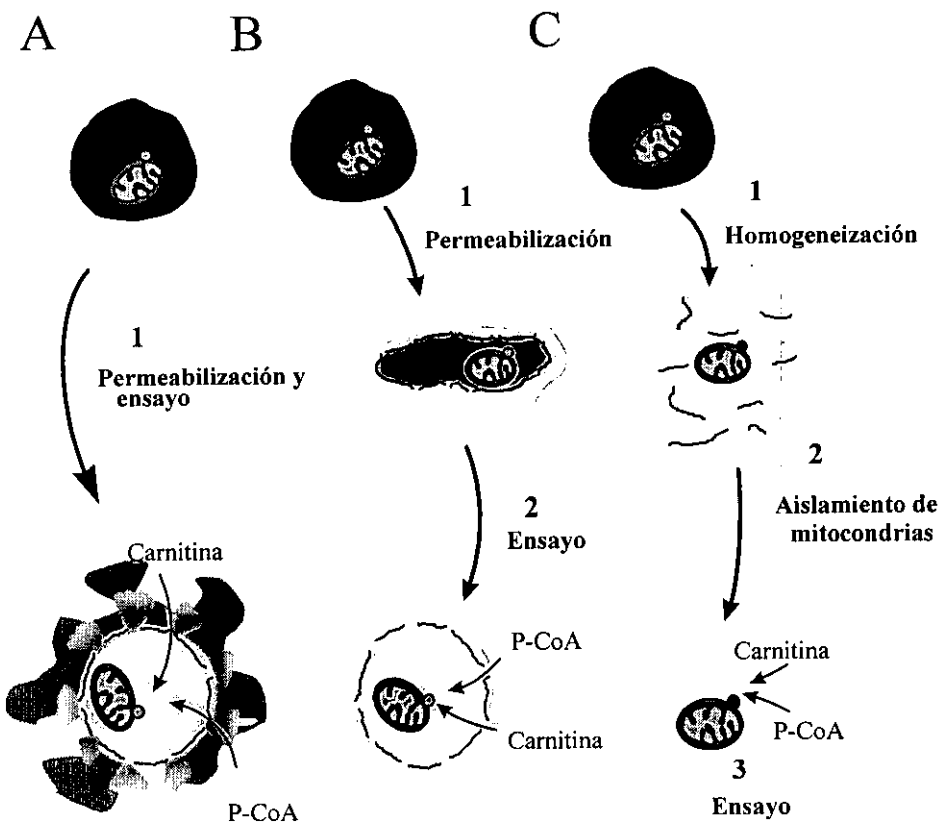

Figura 7. Esquema de los diferentes tipos de ensayo de actividad CPT-I. Cuando la actividad CPT-I es determinada tras el aislamiento de mitocondrias, (método C), las modificaciones que diferentes agonistas pueden inducir en dicha actividad enzimática se pierden. Por el contrario, la utilización de los métodos de permeabilización con digitonina, directo (A) o indirecto (B), permiten establecer los cambios que estos compuestos inducen a corto plazo en la actividad CPT-I.

dad de que la CPT-I pudiera ser directamente fosforilada por acción de algunas de estas quinasas (Harano *et al.*, 1985). Además, el ácido okadaico, un compuesto que inhibe las proteínas fosfatasas 1 y 2A, produciendo así un fuerte aumento en el grado de fosforilación de numerosas proteínas celulares, induce asimismo una notable estimulación de la actividad CPT-I (Guzmán y Castro, 1991; Guzmán y Geelen, 1992). Sin embargo, diversos experimentos demostraron que la CPT-I no es directamente fosforilada ni por acción de la PKA ni de la AMPK (Guzmán *et al.*, 1994), indicando que las modificaciones a corto plazo de la actividad CPT-I deben estar mediadas a través de otro(s) mecanismo(s). La clarificación de esos posibles mecanismos es el principal objetivo de la presente memoria.

Regulación a largo plazo

El estudio de la regulación de la expresión de los genes de las CPT aún no se ha llevado a cabo en profundidad y hasta el momento se ha restringido al estudio de cómo diversos factores nutricionales y hormonales pueden afectar a la expresión de las CPT de hígado de rata. Se sabe que diferentes situaciones que estimulan la cetogénesis en hígado, tales como el ayuno, la diabetes, el hipertiroidismo o el tratamiento con agentes proliferadores de peroxisomas, inducen tanto un aumento en los niveles de proteína inmunorreactiva como de mRNA de CPT-I y CPT-II (Kolodziej *et al.*, 1992; Park *et al.*, 1995; McGarry y Brown, 1997). Otra situación fisiológica que afecta de manera selectiva a la expresión de los genes de CPT-I y CPT-II se produce durante el periodo perinatal en la rata. En el hígado fetal, tanto los niveles de mRNA como la actividad CPT-I son muy bajos en el periodo anterior al nacimiento. Sin embargo, ambos aumentan bruscamente durante el primer día de vida extrauterina y permanecen elevados durante todo el periodo de lactancia (Saggerson y Carpenter, 1982; Thumelin *et al.*, 1994; Asins *et al.*, 1995). Tras el destete, la transcripción del gen de la CPT-I se ve fuertemente atenuado si se suministra a la cría una dieta rica en hidratos de carbono, mientras que si la dieta es rica en grasas los niveles de mRNA de CPT-I se man-

Preincubación	Actividad CPT-I (%)	Oxidación de palmitato (%)
Sin adiciones	100	100
Glucagón 10 nM	127±7[a]	136±4[b]
Dibutiril cAMP 50 μM	131±3[b]	137±3[b]
Forskolina 50 μM	138±5[b]	140±5[b]
Insulina 85 nM	84±8[a]	85±1[a]
EGF 100 nM	86±4[a]	86±3[a]
Vasopresina 100 nM	82±7[a]	88±3[a]
PMA 1μM	76±7[b]	68±9[b]
Vanadato 2 mM	145±16[b]	142±11[b]
Etanol 20 mM	73±11[b]	66±8[b]
Acetaldehido	81±6[a]	71±11[b]
Acido okadaico 0.5 μM	154±15[b]	149±12[b]

Tabla 2. Efecto de diferentes agonistas sobre la actividad CPT-I la oxidación de palmitato. Después de un periodo de incubación de 10-30 min. en ausencia o presencia de las adiciones indicadas, se tomaron alícuotas de las suspensiones de hepatocitos para determinar la actividad CPT-I en hepatocitos permeabilizados con digitonina y la velocidad de oxidación de palmitato en hepatocitos intactos. Significativamente diferente de las incubaciones sin adiciones: [a]$P<0.05$; [b]$P<0.01$.

tienen elevados (Thumelin *et al.*, 1994). Por el contrario, durante todo el periodo prenatal-lactancia-destete los niveles de mRNA y la actividad CPT-II permanecen elevados independientemente del contenido de la dieta suministrada (Thumelin *et al.*, 1994; Asins *et al.*, 1995). Así, durante el periodo de transición fetal-neonatal, tanto la actividad CPT-I como los niveles de mRNA se encuentran aumentados. Además, la sensibilidad a malonil-CoA de la enzima también se encuentra disminuida (Decaux *et al.*, 1988). Todo ello favorece que la oxidación de ácidos grasos y la cetogénesis se produzcan de manera más efectiva, lo que se corresponde con el mayor porcentaje en grasas de la alimentación durante el periodo de lactancia.

En cuanto a los posibles mecanismos que podrían estar mediando este aumento de la expresión de la CPT-I, recientemente se ha determinado en hepatocitos fetales en cultivo que tanto el dibutiril-cAMP como los ácidos grasos de cadena larga inducen un aumento en los niveles de mRNA de CPT-I mientras que no modifican los de la CPT-II (Chatelain *et al.*, 1996). Se ha sugerido que la disminución en los niveles de insulina en sangre durante el periodo de lactancia, lo que se relaciona con un aumento de la concentración de cAMP y ácidos grasos de cadena larga en el hígado podría mediar el incremento en los niveles de mRNA de CPT-I (Chatelain *et al.*, 1996; McGarry y Brown, 1997). Sin embargo, los agentes proliferadores de peroxisomas estimulan la transcripción tanto del gen de la CPT-I como del de la CPT-II, indicando que los ácidos grasos de cadena larga y dichos compuestos podrían actuar a través de diferentes receptores activados por ácidos grasos (Chatelain *et al.*, 1996; McGarry y Brown, 1997).

CPTs extramitocondriales

Se sabe desde hace tiempo que no solo las mitocondrias, sino también otros orgánulos subcelulares como el retículo endoplásmico y los peroxisomas, contienen enzimas con actividad CPT. En los microsomas de hígado de rata se han localizado dos proteínas distintas con actividad CPT. Una de ellas se halla asociada a membrana y otra es soluble y está localizada en el lumen del retículo endoplásmico. La primera presenta propiedades reguladoras semejantes a las de la CPT-I, puesto que se inhibe reversiblemente por malonil-CoA e irrever-

siblemente por etomoxir-CoA. La forma soluble no presenta este tipo de regulación, a semejanza de lo que ocurre con la CPT-II (Lilly *et al.*, 1990; Murthy y Pande, 1994a; Broadway Saggerson, 1995). A pesar de su aparente similitud funcional con la CPT-I (88 KDa), la enzima unida a membrana es una proteína diferente (47 KDa), al igual que ocurre con la forma soluble (54 KDa), que difiere de la CPT-II (71 KDa). Además, la forma asociada a membrana parece ser idéntica a una proteína de estrés (GRP58) cuyo cDNA había sido previamente clonado (Murthy y Pande, 1994b).

En cuanto al posible papel que las CPT microsomales pudieran desempeñar se ha propuesto que pudieran intervenir en la acilación de proteínas destinadas a la secreción y/o al ensamblaje delas VLDL (Broadway y Saggerson, 1995). Puesto que este proceso podría ocurrir, al menos en parte, en el interior del retículo endoplásmico, el aporte de acil-CoA al lumen de dicho orgánulo podría requerir de una CPT implicada en el proceso de transporte de los acil-CoA a través de la membrana del retículo endoplásmico.

En los peroxisomas también parecen existir dos actividades CPT. Al igual que ocurre en el caso de los microsomas, existe una actividad CPT insensible a malonil-CoA (Derrick y Ramsay, 1989) que, dada su preferencia por acil-CoA de cadena intermedia como sustratos ha sido denominada carnitina octanoiltransferasa (COT). Esta proteína, puede ser solubilizada en forma activa con cierta facilidad, pero difiere en su peso molecular (≈63 KDa) estructura primaria y reconocimiento por anticuerpos de las otras dos CPT insensibles a malonil-CoA (la CPT-II y la CPT soluble de microsomas) (Ramsay, 1988;Pande *et al.*, 1992). También se ha determinado la existencia de una CPT sensible a malonil-CoA que estaría fuertemente unida a la membrana del peroxisoma (Singh *et al.*, 1996). Actualmente se acepta que los peroxisomas no requieren de carnitina para llevar a cabo la oxidación de acil-CoAs, por lo que se ha propuesto que la CPT de estos orgánulos podría estar implicada más bien en la exportación de acil-CoAs de cadena intermedia, que son los principales productos de la oxidación de ácidos grasos en peroxisomas (Kunau *et al.*, 1995). Es en cualquier caso curioso que las mitocondrias, el retículo endoplásmico y los peroxisomas compartan un sistema similar de transporte de ácidos grasos a pesar de que las proteínas que constituyen dichos sistemas sean tan diferentes.

OBJETIVOS Y CONTENIDO

El hígado desempeña una función fundamental en la distribución de sustratos a los distintos tejidos del organismo (Capítulo 1). En lo que al metabolismo de ácidos grasos se refiere, el hígado puede llevar a cabo la síntesis y oxidación de estas moléculas, así como su exportación a otros tejidos del organismo. De esta manera, la coordinación de todos esos procesos depende de la acción de una serie de efectores celulares, que son liberados en respuesta a diferentes situaciones fisiopatológicas.

La principal etapa reguladora del flujo a través de la ruta de oxidación de ácidos grasos es la catalizada por la carnitina palmitoltransferasa I. Desde que en 1977 se descubriera la inhibición mediada por malonil-CoA de la CPT-I este mecanismo que permite explicar la regulación coordinada de la síntesis y oxidación de ácidos grasos, ha sido considerado como el principal proceso implicado en la regulación a corto plazo de la CPT-I. Sin embargo, estudios llevados a cabo en nuestro laboratorio a mediados de la década de los 80 con hepatocitos permeabilizados en los que es posible determinar la actividad CPT-I *in situ* sin necesidad de recurrir al aislamiento de mitocondrias, pusieron de manifiesto que diversos factores pueden modificar la actividad de la CPT-I de manera estable e independiente de malonil-CoA. Puesto que dichos experimentos no permitieron dilucidar el mecanismo implicado en la regulación independiente de malonil-CoA de la CPT-I, en la presente Tesis Doctoral se ha llevado a cabo un estudio detallado sobre las posibles bases bioquímicas de la regulación a corto plazo e independiente de malonil-CoA de la CPT-I.

En el primer bloque de resultados de este trabajo (Capítulo 2.1), se describen los efectos del ATP extracelular y los cambios en el volumen celular sobre la actividad CPT-I y la oxidación de ácidos grasos que, en ambos casos parecen utilizar esta vía de regulación "independiente de malonil-CoA". Una vez que la existencia de esta regulación de la CPT-I pareció confirmarse, en un segundo bloque de resultados (Capítulo 2.2), estudiamos qué mecanismos intracelulares podrían determinar estas modificaciones de la actividad de la enzima. Para ello utilizamos el ácido okadaico como herramienta que permitiera establecer si en dicha regulación de la CPT-I estaban interviniendo procesos de fosforilación desfosforilación. Por último en un tercer bloque (Capítulo 2.3) se apuntan las posibles implicaciones fisiológicas de este mecanismo, tanto a nivel de su potencial implicación en procesos de malignización celular como de su coordinación con el mecanismo dependiente de malonil-CoA ya previamente establecido. Finalmente, la presente memoria incluye una discusión general de los resultados obtenidos, en la que se exponen las conclusiones finales del trabajo realizado (Capítulo 3).

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2. Resultados y Discusión

2.1. Efectos del ATP extracelular y de los cambios en el volumen celular sobre la actividad CPT-I

INTRODUCCION

Como ya se ha señalado anteriormente en el apartado de Introducción General, las diferentes situaciones fisiopatológicas en las que se puede encontrar el organismo, conducen a la liberación de una gran variedad de mediadores extracelulares que ejercen su efecto sobre el metabolismo hepático. En lo que a la regulación de la oxidación de ácidos grasos se refiere, la insulina y los factores de crecimiento inhiben mientras que el glucagón activa a la CPT-I, la enzima limitante del flujo a través de esta ruta (Guzmán y Geelen, 1993). En el presente capítulo, se estudian los efectos que otros mediadores (tales como los agentes que movilizan Ca^{2+}), o situaciones fisiológicas (cambios en el volumen celular), pudieran ejercen sobre la actividad CPT-I.

El ATP extracelular, que puede ser liberado en respuesta a diferentes estímulos por diversos tejidos (El-Moatassim *et al.*, 1992), puede ejercer sus efectos a través de toda una serie de receptores (Windscheif, 1996). A nivel hepático, el ATP extracelular actúa sobre el receptor P_{2y}, estimulando la hidrólisis de fosfoinosítidos (Windscheif, 1996) y mediando así un incremento en la concentración citosólica de Ca^{2+} y de diacilglicerol. Por ello, se utilizó este compuesto como modelo para estudiar los efectos que este tipo de agentes pudieran ejercer sobre la actividad CPT-I.

Los cambios en el volumen celular, también constituyen un importante mecanismo regulador del metabolismo hepático (Häussinger, 1996), de tal manera que el hinchamiento de los hepatocitos se encuentra relacionado con la activación de rutas anabólicas, mientras que su deshinchamiento lo está con la de rutas catabólicas (Häussinger, 1996). Los posibles efectos de los cambios en el volumen celular sobre la actividad CPT-I no son aún conocidos. Los resultados que se exponen a continuación confirman que la actividad CPT-I también puede verse controlada a corto plazo por agonistas que movilizan Ca^{2+} y por cambios en el volumen celular.

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Effects of extracellular ATP on hepatic fatty acid metabolism

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Guzmán, Manuel, Guillermo Velasco, and José Castro. Effects of extracellular ATP on hepatic fatty acid metabolism. *Am. J. Physiol.* 270 (*Gastrointest. Liver Physiol.* 33): G701–G707, 1996.—Incubation of rat hepatocytes with extracellular ATP inhibited acetyl-CoA carboxylase (ACC) activity and fatty acid synthesis de novo, with a concomitant decrease of intracellular malonyl-CoA concentration. However, both carnitine *O*-palmitoyltransferase I (CPT-I) activity and ketogenesis from palmitate were inhibited in parallel by extracellular ATP. The inhibitory effect of extracellular ATP on ACC and CPT-I activities was not evident in Ca^{2+}-depleted hepatocytes. Incubation of hepatocytes with thapsigargin, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (BHQ), or A-23187, compounds that increase cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), depressed ACC activity, whereas CPT-I activity was unaffected. The phorbol ester 4β-phorbol 12β-myristate 13α-acetate (PMA) increased ACC activity, whereas it decreased CPT-I activity in a nonadditive manner with respect to extracellular ATP. The inhibitory effect of extracellular ATP on ACC activity was also evident in the presence of bisindolylmaleimide, a specific inhibitor of protein kinase C (PKC), whereas this compound abolished the extracellular ATP-mediated inhibition of CPT-I. In addition, the PMA-induced inhibition of CPT-I was not potentiated by thapsigargin, BHQ, or A-23187. Results thus show *1*) that the intracellular concentration of malonyl-CoA is not the factor responsible for the inhibition of hepatic long-chain fatty acid oxidation by extracellular ATP, and *2*) that the inhibition of ACC by extracellular ATP may be mediated by an elevation of $[Ca^{2+}]_i$, whereas CPT-I may be inhibited by extracellular ATP through a PKC-dependent mechanism.

adenosine 5′-triphosphate; fatty acid metabolism; protein kinase C; hepatocyte

THE BINDING OF MANY hormones and growth factors to their plasma membrane receptors is coupled through phospholipase C activation to the formation of two second messengers, diacylglycerol, which activates protein kinase C (PKC) (1, 21), and inositol 1,4,5-trisphosphate, which releases Ca^{2+} from intracellular stores, thereby transiently raising cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) (1). In hepatocytes, receptor-mediated phosphoinositide breakdown occurs in response to a number of Ca^{2+}-mobilizing effectors such as vasopressin, angiotensin, α_1-adrenergic agents, and extracellular ATP (6). ATP is released to the extracellular medium from several cell types, including neurons, platelets, and chromaffin cells of the adrenal medulla (8, 9); it interacts with specific receptors (P_2-purinergic receptors) on the surface of many different cells, in which it regulates many physiological processes (8, 9). In isolated hepatocytes or perfused liver, similar to other Ca^{2+}-mobilizing effectors, extracellular ATP notably stimulates glycogenolysis, gluconeogenesis, tricarboxylic acid cycle activity, and ureagenesis (8, 9).

In contrast to this knowledge on the effects of extracellular ATP on hepatic carbohydrate and nitrogen metabolism, as far as we know the effects of extracellular ATP on hepatic fatty acid metabolism have not yet been studied. Thus in the present study we examine in detail the effects of extracellular ATP on the different fatty acid-metabolizing pathways in rat hepatocytes. In addition, it has been suggested that the regulation of some hepatic enzymes by Ca^{2+}-mobilizing agents may be solely mediated by the diacylglycerol limb (27) or the inositol 1,4,5-trisphosphate limb (8, 18, 23, 24) of membrane phosphoinositide hydrolysis. Thus we also studied the contribution of these two components of phospholipid breakdown to the effects of extracellular ATP on hepatic fatty acid metabolism.

MATERIALS AND METHODS

Isolation and incubation of hepatocytes. Male Wistar rats (250–300 g) with free access to food and water were used throughout this study. All the animal protocols described below followed the guidelines of the Spanish Ministry of Health. Hepatocytes were isolated by the collagenase perfusion method described by Beynen et al. (2). To minimize glycogenolysis, glucose (20 mM) was added to all the buffers employed in the isolation procedure (5). Because lipogenesis is markedly depressed just after hepatocyte isolation, cells were incubated for 15 min at 37°C in a gyratory metabolic shaker and subsequently filtered through nylon mesh before use (5). Cell viability, as determined by trypan blue exclusion, always exceeded 90% in the final hepatocyte suspension.

Except for $[Ca^{2+}]_i$ determinations (see below), hepatocytes were incubated in Krebs-Henseleit bicarbonate buffer (Ca^{2+} concn 2.5 mM) supplemented with 10 mM glucose and 1% (wt/vol) defatted and dialyzed bovine serum albumin. Incubations (4–6 mg of cellular protein/ml) were performed in a total volume of 2 ml at 37°C, with constant shaking (85 oscillations/min) and under an atmosphere of O_2-CO_2 of 19:1.

Ca^{2+}-depleted hepatocytes were used in some experiments (see Table 2). For this purpose, after isolation, hepatocytes were washed twice in Ca^{2+}-free Krebs-Henseleit bicarbonate buffer supplemented with 0.5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid and further incubated in this medium for 20 min under the aforementioned conditions before use (22).

Rates of fatty acid synthesis, esterification, and oxidation. For the determination of rates of fatty acid synthesis de novo, reactions were started by the addition to cell incubations of either [1-^{14}C]acetate (0.1 Ci/mol, 3 mM final concn) or ^3H_2O (0.4 Ci/l in the final incubation). Total fatty acids were extracted according to the method of Tijburg et al. (25).

For the determination of rates of fatty acid esterification into triacylglycerols and phospholipids, reactions were started by the addition to cell incubations of either [U-^{14}C]palmitate (0.05 Ci/mol, 0.4 mM final concn) bound to albumin or

[U-^{14}C]glycerol (0.2 Ci/mol, 0.5 mM final concn). Cellular triacylglycerols and phospholipids were isolated by thin-layer chromatography and quantified as described by Tijburg et al. (25).

For the determination of rates of fatty acid oxidation, reactions were started by the addition to cell incubations of [1-^{14}C]fatty acid (either palmitate or octanoate, 0.05 Ci/mol, 0.4 mM final concn) bound to albumin. Oxidation products (both acid-soluble products and CO_2) were extracted and quantified exactly as described before (10). Ketone bodies routinely represent 80–90% of total acid-soluble products (10).

Enzymatic assays. Acetyl-CoA carboxylase (ACC) activity was determined in digitonin-permeabilized hepatocytes as the incorporation of radiolabeled acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase (FAS) reaction. This method avoids the number of interferences inherent to the classic bicarbonate-fixation assay of ACC activity (5). To measure enzyme activity, 100 µl of hepatocyte suspension was added to 100 µl of prewarmed digitonin-containing assay medium exactly as described by Bijleveld and Geelen (5).

FAS activity was monitored in digitonin-permeabilized hepatocytes as described previously (5).

Carnitine *O*-palmitoyltransferase I (CPT-I) activity was determined in digitonin-permeabilized hepatocytes as the tetradecylglycidate (TDGA)-sensitive incorporation of radiolabeled L-carnitine into palmitoylcarnitine. In brief, hepatocytes were preincubated for 20 min in the absence or presence of 5 µM TDGA, a potent and specific inhibitor of CPT-I (7, 12). Aliquots were removed from both sets of incubations to monitor CPT-1 activity. For that purpose, 100 µl of hepatocyte suspension was added to 100 µl of prewarmed digitonin-containing assay medium exactly as described by Guzmán and Geelen (12). Peroxisomal CPT activity was determined in the same incubations as the TDGA-insensitive, malonyl-CoA-sensitive hepatocellular CPT activity (12).

Lactate dehydrogenase activity was determined by a standard spectrophotometric method (29).

Determination of $[Ca^{2+}]_i$. Hepatocytes (2–3 mg of cellular protein/ml) were incubated with 6 µM indo 1-acetoxymethyl ester (indo 1-AM) in bicarbonate-free Krebs-Henseleit medium supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-NaOH (pH 7.4), 10 mM glucose, and 1% (wt/vol) defatted and dialyzed bovine serum albumin. The incubation was performed at 37°C for 45 min with constant shaking (85 oscillations/min).

The fluorescence of indo 1-loaded cells was excited by a 5-W laser tuned to 345–365 nm, and the emitted fluorescence was simultaneously measured at 395 ± 12.5 nm (for Ca^{2+}-bound indo 1) and 488 ± 5 nm (for Ca^{2+}-free indo 1) in a FACStar Plus (Becton Dickinson) flow cytometer.

Other analytical procedures. Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid cell extracts by a radioenzymatic method as described by Beynen et al. (2). Protein was determined by the method of Lowry et al. (19), with bovine serum albumin as standard.

Materials. [1-^{14}C]acetyl-CoA (54 Ci/mol), [1-^{14}C]acetate (56 Ci/mol), ^3H_2O (5 Ci/mol), [1-^{14}C]palmitic acid (58 Ci/mol), [1-^{14}C]octanoic acid (32 Ci/mol), [U-^{14}C]glycerol (171 Ci/mol), and L-[Me-^{14}C]carnitine (54 Ci/mol) were supplied by Amersham International (Amersham, Bucks, UK). Digitonin, collagenase (type I), 4β-phorbol 12β-myristate 13α-acetate (PMA), and ATP were purchased from Sigma Chemical (St. Louis, MO). TDGA was a gift from Dr. M. J. H. Geelen, Utrecht University, The Netherlands. Bisindolylmaleimide, thapsigargin, A-23187, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (BHQ), and indo 1-AM were from Calbiochem (San Diego, CA).

Statistical analysis. Unless otherwise indicated, results are means ± SD of the number of animals indicated in every case. Cell incubations and/or enzyme assays were always carried out in triplicate. Statistical analysis was performed by Student's *t*-test.

RESULTS

The effects of extracellular ATP on fatty acid synthesis and oxidation were studied in isolated rat hepatocytes. In addition, digitonin-permeabilized hepatocytes were used for the study of the effects exerted by extracellular ATP on the activity of ACC (a key regulatory enzyme of fatty acid synthesis de novo in the liver) and CPT-I (an important regulatory site of hepatic fatty acid oxidation). Exposure of hepatocytes to the concentration of digitonin used in our system (~40 µg/mg of cellular protein) liberates more than 95% of total lactate dehydrogenase (a cytosolic marker enzyme) within 15 s (11, 12). The integrity of the mitochondrial outer and inner membranes is proved by the fact that less than 1% of total monoamine oxidase (a mitochondrial outer membrane marker enzyme) and of total glutamate dehydrogenase (a mitochondrial matrix marker enzyme) is released from the permeabilized cells after 1 min of exposure to digitonin (11, 12).

Because extracellular ATP acts on hepatic metabolism in a very rapid fashion (8, 9), and because it is actively hydrolyzed by plasma membrane ecto-adenosinetriphosphatases (8, 9, 18), the incubation times used in the present study were very short. These incubation times were observed to allow maximal effects on the parameters experimentally determined (results not shown). In the case of measurements of rates of fatty acid synthesis, esterification, and oxidation, incubations were carried out for up to 5 min to achieve adequate incorporation of the radiolabeled substrates into products.

Prolonged exposure of rat hepatocytes to high doses of ATP has been shown to produce cytotoxic effects (31). However, we did not observe any cytotoxic effect of this compound in our short-term cell incubations. Thus challenge of hepatocytes to 200 µM ATP for 5 min did not induce any significant variation of cell viability, as determined by both trypan blue exclusion and lactate dehydrogenase release.

Effects of extracellular ATP on lipogenesis. Incubation of hepatocytes with ATP markedly decreased the rate of fatty acid synthesis de novo when [^{14}C]acetate was used as a substrate (Table 1). Similarly, extracellular ATP depressed fatty acid synthesis de novo by 39.3 ± 6.4% ($n = 3$, $P < 0.01$) when ^3H_2O was used as a substrate. This decrease correlated well with the extracellular ATP-mediated inhibition of ACC activity (Table 1). Likewise, the intracellular concentration of malonyl-CoA, the product of the carboxylase-catalyzed reaction and a physiological inhibitor of CPT-I (20), was decreased in parallel by extracellular ATP (Table 1). FAS activity was not affected by extracellular ATP (Table 1).

Because ATP is a substrate for ACC and it is not removed from the medium before assays are conducted,

Table 1. *Effect of extracellular ATP on hepatic fatty acid synthesis*

Parameter	n	Additions to the Incubations: None	Additions to the Incubations: 100 μM ATP
Rate of fatty acid synthesis	6	25.3 ± 0.6	16.8 ± 2.1*
ACC activity	9	0.74 ± 0.08	0.45 ± 0.16*
FAS activity	9	2.2 ± 0.3	2.3 ± 0.4
Malonyl-CoA concn	4	0.089 ± 0.011	0.055 ± 0.006*

Hepatocytes were incubated in absence or in presence of 100 μM ATP for 1 min. Then, cells were used for measurement of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) activities, as well as malonyl-CoA concentration. The rate of fatty acid synthesis de novo was monitored in parallel cell incubations. In this case, [^{14}C]acetate (3 mM final concn) and ATP (100 μM final concn) were simultaneously added to the incubations, which were allowed to run for 5 min. Enzyme activities are expressed as nanomoles of product per minute per milligram cellular protein. Malonyl-CoA levels are expressed as nanomoles per milligram cellular protein. Rates of fatty acid synthesis are expressed as nanomoles acetyl units per hour per milligram cellular protein. Significantly different from incubations with no additions: *$P < 0.01$.

it might be argued that the ATP added to cell incubations might have a direct effect on ACC activity. However, hepatocytes are incubated with or without 100 μM ATP, and to determine ACC activity 100 μl of hepatocyte suspension was added to 100 μl of assay mixture containing 4.0 mM ATP substrate (see MATERIALS AND METHODS and Ref. 5). Hence, ACC activity is assumed to be unchanged when determined with either 2.0 or 2.05 mM ATP as substrate.

With regard to fatty acid esterification, no significant effect of extracellular ATP was detected on the incorporation of exogenous [^{14}C]palmitate into cellular triacylglycerols and phospholipids. Thus the rate of palmitate esterification (in nmol of [^{14}C]palmitate into product·h^{-1}·mg cellular protein^{-1}; n = 3) was 11.5 ± 1.3 (incubations without ATP) or 10.7 ± 1.4 nmol·h^{-1}·mg protein^{-1} (incubations with 100 μM ATP) in the case of triacylglycerols, and 29.3 ± 2.6 (incubations without ATP) or 28.6 ± 4.1 nmol·h^{-1}·mg protein^{-1} (incubations with 100 μM ATP) in the case of phospholipids. Similarly, extracellular ATP had no effect on the incorporation of [^{14}C]glycerol into cellular lipids. Thus the rate of glycerol incorporation into lipids (in nmol of [^{14}C]glycerol into product·h^{-1}·mg cellular protein^{-1}, n = 3) was 7.4 ± 1.1 (incubations without ATP) or 7.0 ± 0.9 nmol·h^{-1}·mg protein^{-1} (incubations with 100 μM ATP) in the case of triacylglycerols, and 17.8 ± 3.5 (incubations without ATP) or 17.2 ± 1.6 nmol·h^{-1}·mg protein^{-1} (incubations with 100 μM ATP) in the case of phospholipids.

Extracellular ATP might have an effect on fatty acid release from cellular lipids. To test that possibility, hepatocyte lipids were prelabeled by incubating the cells for 5 min in the presence of 0.4 mM [^{14}C]palmitate. Hepatocytes were subsequently washed and further incubated for 5 min in a radioisotope-free medium in the absence or presence of 100 μM ATP. However, under these conditions fatty acid release from hepatocellular lipids was negligible in both control and ATP-containing incubations.

Effects of extracellular ATP on fatty acid oxidation. The rate of ketogenesis from palmitate was depressed by the addition of ATP to the hepatocyte incubation medium (Fig. 1). However, extracellular ATP had no significant effect on the rate of ketogenesis from octanoate (Fig. 1). It is well established that palmitate is transported into mitochondria by a carnitine-dependent process, whereas octanoate may enter mitochondria independently of carnitine (4, 20, 30). Therefore, this result suggests that the target for extracellular ATP action might be CPT-I; other components of the fatty acid-translocation system, namely long-chain acyl-CoA synthetase, carnitine:acylcarnitine translocase, and CPT-II, are generally not considered to play a significant regulatory role in the transport of long-chain fatty acids into the mitochondrial matrix (4, 13, 20, 30). As can be seen in Fig. 1, hepatic CPT-I activity was decreased by extracellular ATP. However, extracellular ATP did not exert any effect on peroxisomal CPT activity (0.53 ± 0.09 and 0.52 ± 0.06 nmol product·min^{-1}·mg cellular protein^{-1} in incubations without additions or with 100 μM ATP, respectively).

To test whether ATP might have a direct effect on CPT-I, enzyme activity was determined in isolated liver mitochondria incubated in the presence of up to 0.5 mM ATP. CPT-I activity was also determined in the permeabilized cell assay after hepatocyte preincubation with no additions except 100 μM ATP added to the digitonin-containing assay mix. However, we could not detect any effect of ATP on CPT-I activity in either of the two assays.

In our isolated hepatocyte system, acid-soluble products routinely represent 85–90% of total oxidation

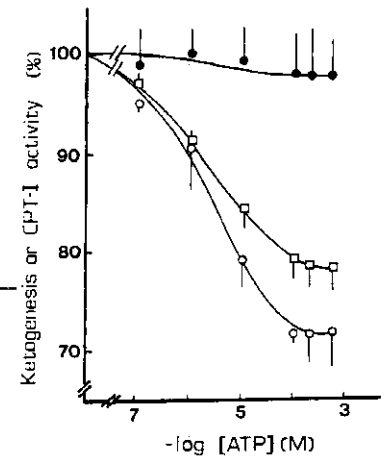

Fig. 1. Effect of extracellular ATP on ketogenesis from palmitate and octanoate, and on the activity of carnitine *O*-palmitoyltransferase I (CPT-I). For determination of the rate of ketogenesis, hepatocytes were incubated for 5 min with either 0.4 mM [^{14}C]palmitate (○) or 0.4 mM [^{14}C]octanoate (●) in presence of increasing concentrations of ATP. Maximal (100%) values of hepatic ketogenesis for [^{14}C]palmitate and [^{14}C]octanoate were 67.4 ± 3.8 and 141.7 ± 16.1 nmol fatty acid·h^{-1}·mg cellular protein^{-1} into acid-soluble products, respectively. For determination of CPT-I activity (□), hepatocytes were incubated for 1 min in presence of increasing concentrations of ATP. 100% Value of CPT-I activity was 1.86 ± 0.24 nmol product·min^{-1}·mg cellular protein^{-1}. Results correspond to 4 different animals. Note scale on y-axis.

Table 2. *Lack of effect of extracellular ATP on fatty acid synthesis and oxidation in Ca^{2+}-depleted hepatocytes*

Parameter	Additions to the Incubations	
	None	100 μM ATP
ACC activity	0.44 ± 0.08	0.48 ± 0.06
Fatty acid synthesis	17.2 ± 4.6	16.8 ± 5.8
CPT-I activity	1.39 ± 0.24	1.38 ± 0.04
Ketogenesis from palmitate	51.2 ± 1.1	50.2 ± 6.4

Ca^{2+}-depleted hepatocytes were incubated in absence or in presence of 100 μM ATP for 1 min. Then, cells were used for measurement of ACC and carnitine *O*-palmitoyltransferase (CPT-I) activities. Rates of fatty acid synthesis de novo and ketogenesis from palmitate were monitored in parallel cell incubations. In this case, either [^{14}C]acetate (3 mM final concn) or [^{14}C]palmitate (0.4 mM final concn), together with ATP (100 μM final concn), were simultaneously added to incubations, which were allowed to run for 5 min. Enzyme activities are expressed as nanomoles of product per minute per milligram cellular protein. Rates of fatty acid synthesis are expressed as nanomoles acetyl units per hour per milligram cellular protein. Rates of ketogenesis are expressed as nanomoles [^{14}C]palmitate into acid-soluble products per hour per milligram cellular protein. Results correspond to 4 different animals.

products when [^{14}C]palmitate is used as a substrate. Nevertheless, we also determined the effect of extracellular ATP on fatty acid oxidation to CO_2 by hepatocytes. Extracellular ATP had a dual effect on palmitate oxidation, since ketone body formation was reduced (see above), whereas CO_2 production was notably enhanced. Thus CO_2 production was 10.2 ± 2.9 (incubations without ATP) or 16.2 ± 4.0 nmol·h^{-1}·mg cellular protein^{-1} (incubations with 100 μM ATP) ($n = 4$, $P < 0.01$). In any case, palmitate oxidation to total products was significantly inhibited by extracellular ATP. Values obtained in this case were 82.1 ± 9.2 nmol·h^{-1}·mg cellular protein^{-1} for incubations without ATP and 67.5 ± 2.5 nmol·h^{-1}·mg cellular protein^{-1} for incubations with 100 μM ATP ($n = 4$, $P < 0.01$).

Modulation of the effects of extracellular ATP by compounds that change $[Ca^{2+}]_i$ and PKC activity. It has been suggested that the regulation of some hepatic enzymes by Ca^{2+}-mobilizing hormones may be solely mediated by the diacylglycerol limb (27) or the inositol 1,4,5-trisphosphate limb (8, 18, 23, 24) of membrane phosphoinositide hydrolysis. Hence, we attempted to determine the contribution of these two components to the aforementioned effects of extracellular ATP on ACC and CPT-I. For this purpose we used Ca^{2+}-depleted hepatocytes; depletion of intracellular Ca^{2+} stores under these conditions was assessed by the lack of effect of thapsigargin and BHQ on ACC activity (see below). In addition, we used a number of compounds that specifically *1*) increase $[Ca^{2+}]_i$ (the intracellular Ca^{2+} releasers thapsigargin and BHQ, and the Ca^{2+} ionophore A-23187) (9) or *2*) modulate PKC activity (a PKC stimulator such as the phorbol ester PMA and a specific enzyme inhibitor such as bisindolylmaleimide) (1, 21, 26).

The inhibition induced by extracellular ATP on ACC activity and fatty acid synthesis de novo was not evident in Ca^{2+}-depleted hepatocytes (Table 2), indicating that Ca^{2+} should be involved in these effects. Incubation of hepatocytes with thapsigargin, BHQ, or A-23187 decreased ACC activity both alone and in combination with extracellular ATP (Fig. 2). PMA increased ACC activity, whereas bisindolylmaleimide did not significantly affect enzyme activity (Fig. 2). Furthermore, the effect of extracellular ATP on ACC activity was also evident in the presence of the PKC inhibitor (Fig. 2).

The inhibition of ACC by agents that increase $[Ca^{2+}]_i$ (Fig. 2) showed a good relation with the magnitude of the elevation of $[Ca^{2+}]_i$ (Table 3). Thus extracellular ATP, thapsigargin, and BHQ, compounds that trigger the release of Ca^{2+} from intracellular stores as well as the capacitative influx of extracellular Ca^{2+} (1, 6, 9), induced a more remarkable elevation of $[Ca^{2+}]_i$ than the ionophore A-23187 (Table 3). When thapsigargin or BHQ was added to the incubation medium together with extracellular ATP, the magnitude of the elevation of $[Ca^{2+}]_i$ (Table 3) and the inhibition of ACC (Fig. 2) were slightly enhanced compared with the action of the three compounds separately. The effect of A-23187 on

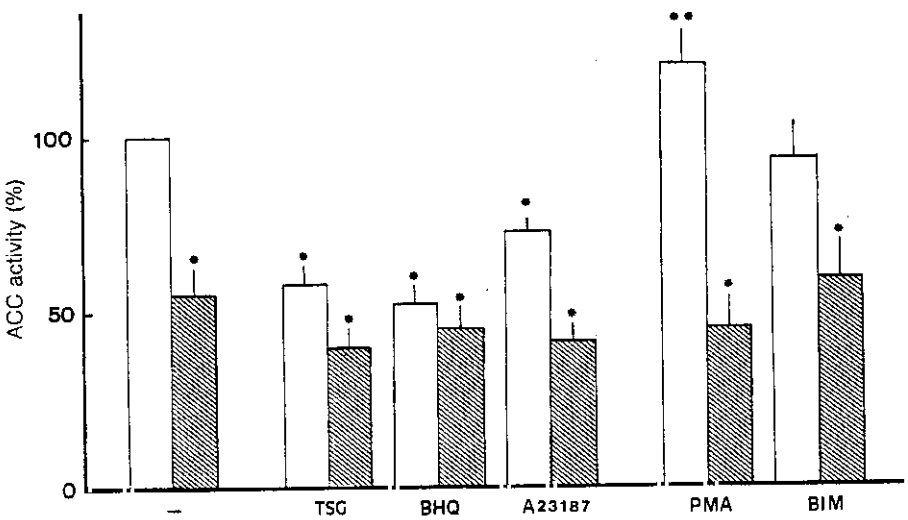

Fig. 2. Modulation of the effects of extracellular ATP on acetyl-CoA carboxylase (ACC) activity by compounds that change cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and protein kinase C (PKC) activity. Hepatocytes were preincubated for 5 min with no additions (−) or with (in μM) 0.1 thapsigargin (TSG), 10 2,5-di-(*t*-butyl)-1,4-benzohydroquinone (BHQ), 2 A-23187, 1 4β-phorbol 12β-myristate 13α-acetate (PMA), or 2 bisindolylmaleimide (BIM). Hepatocytes were further incubated for 1 min in absence (open bars) or presence (hatched bars) of 100 μM ATP. Then, ACC activity was determined. Results are % of activity relative to incubations with no additions and correspond to 3–4 different animals. Maximal (100%) value of ACC activity was 0.80 ± 0.11 nmol product·min^{-1}·mg cellular protein^{-1}. Significantly different from incubations with no additions: $*P < 0.01$; $**P < 0.05$.

Table 3. *Effect of extracellular ATP, thapsigargin, BHQ, and A-23187 on $[Ca^{2+}]_i$ in isolated hepatocytes*

Additions to the Incubations	Fluorescence Intensity, %
None	100
100 μM ATP	296
0.1 μM thapsigargin	304
0.1 μM thapsigargin + 100 μM ATP	371
10 μM BHQ	297
10 μM BHQ + 100 μM ATP	355
2 μM A-23187	230
2 μM A-23187 + 100 μM ATP	383

After indo 1 loading, hepatocytes were incubated with the additions indicated at 37°C, and the intracellular indo 1-emitted fluorescence light was determined by flow cytometry. Data are % of incubations with no additions (% of control) and represent maximum value of fluorescence intensity at 395 ± 12.5 nm relative to that at 488 ± 5 nm. These peak values were routinely obtained 45–75 s after addition of the different Ca^{2+}-mobilizing agents. Data correspond to a representative experiment that was repeated 3 times with similar results. BHQ, 2,5-di-(*t*-butyl)-1,4-benzohydroquinone.

both ACC activity (Fig. 2) and $[Ca^{2+}]_i$ (Table 3) was markedly potentiated by extracellular ATP.

Extracellular ATP was unable to depress CPT-I activity and ketogenesis from palmitate in Ca^{2+}-depleted hepatocytes (Table 2). Although this may indicate that Ca^{2+} should be involved in these effects, hepatocyte incubation with thapsigargin, BHQ, or A-23187 had no effect on CPT-I activity (Fig. 3). On the other hand, PMA decreased CPT-I activity in a nonadditive and quantitatively similar manner with respect to extracellular ATP (Fig. 3). Moreover, bisindolylmaleimide abolished the extracellular ATP-mediated inhibition of CPT-I activity (Fig. 3). Bisindolylmaleimide also antagonized the extracellular ATP-dependent inhibition of CPT-I when thapsigargin, BHQ, or A-23187 was present in the incubation medium (results not shown). The dependency of the antiketogenic effect of extracellular ATP on Ca^{2+} (Table 2) may thus reside in a step prior to PKC activation. Interestingly, the PMA-induced inhibition of CPT-I activity was not evident in Ca^{2+}-depleted hepatocytes (results not shown). Moreover, the PMA-induced inhibition of CPT-I activity observed in Ca^{2+}-containing hepatocytes (Fig. 3) was not potentiated by BHQ, thapsigargin, or A-23187. Values of CPT-I activity (as a percentage of incubations with no additions, $n = 3$) were 73 ± 4% (incubations with 1 μM PMA), 73 ± 3% (incubations with 1 μM PMA plus 10 μM BHQ), 72 ± 5% (incubations with 1 μM PMA plus 0.1 μM thapsigargin), and 75 ± 5% (incubations with 1 μM PMA plus 2 μM A-23187).

DISCUSSION

In the present study we show that extracellular ATP markedly affects hepatic fatty acid metabolism. The parallel inhibition of ACC and fatty acid synthesis de novo by extracellular ATP supports the general view that ACC is a key regulatory point of the fatty acid-synthesizing process (13). Malonyl-CoA, the product of the reaction catalyzed by ACC, is a physiological inhibitor of CPT-I and plays an essential role in the coordinate control of fatty acid synthesis and oxidation in the liver (4, 13, 20, 30). The extracellular ATP-induced depression of hepatic ACC activity led to a parallel decrease in malonyl-CoA content. Because CPT-I activity and palmitate oxidation were inhibited by extracellular ATP, this would indicate that the intracellular concentration of malonyl-CoA is not the factor responsible for the regulation of hepatic long-chain fatty acid oxidation under these conditions. It should also be pointed out that to measure CPT-I activity we have permeabilized the plasma membrane, and this has caused the cytosol to leak out of the cell, leading to a large dilution of cytosolic components, including malonyl-CoA (11, 12). In this respect it is noteworthy that the short-term modulation of CPT-I by extracellular ATP (results not shown), hepatocyte swelling (15), or the phosphatase inhibitor okadaic acid (14) is very stable, since they survive hepatocyte permeabilization, extensive washing of the permeabilized cells, and subsequent incubation of the permeabilized cells at 37°C for at least 10 min. Thus, although inhibition of CPT-I by malonyl-CoA is a well-described property of the enzyme (4, 13, 20, 30), other types of regulatory mechanisms may be involved in the short-term control of hepatic CPT-I by cellular effectors (cf. Ref. 14).

The malonyl-CoA-independent inhibition of hepatic CPT-I activity by extracellular ATP is accompanied by a dual effect on mitochondrial fatty acid oxidation, since

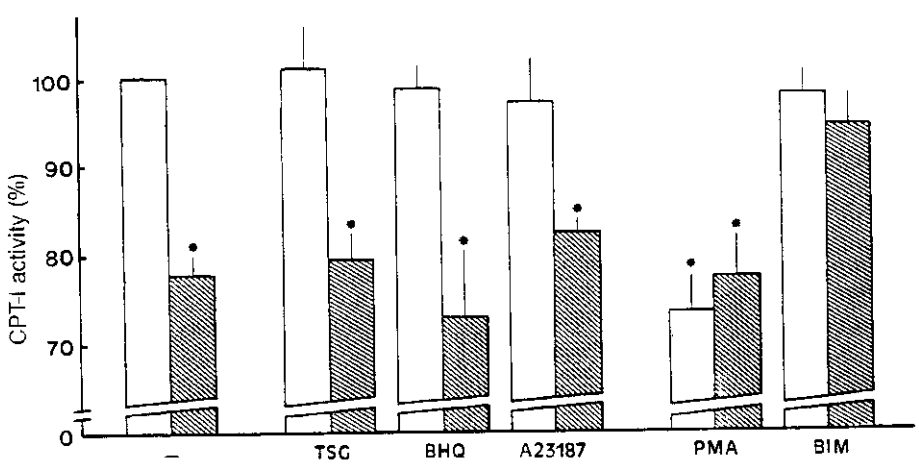

Fig. 3. Modulation of effects of extracellular ATP on CPT-I activity by compounds that change $[Ca^{2+}]_i$ and PKC activity. Hepatocytes were preincubated for 5 min with no additions (–) or with (in μM) 0.1 TSG, 10 BHQ, 2 A-23187, 1 PMA, or 2 BIM. Hepatocytes were further incubated for 1 min in absence (open bars) or presence (hatched bars) of 100 μM ATP, and then CPT-I activity was determined. Results are % of activity relative to incubations with no additions and correspond to 3–4 different animals. 100% Value of CPT-I activity was 1.77 ± 0.35 nmol product·min^{-1}·mg cellular $protein^{-1}$. Note scale on *y*-axis. Significantly different from incubations with no additions: $^*P < 0.01$.

ketone body formation is depressed while CO_2 production is enhanced. Thus extracellular ATP may reduce the entry of fatty acyl-CoA into hepatic mitochondria and divert mitochondrial acetyl-CoA into the tricarboxylic acid cycle at the expense of the ketogenic pathway. The stimulation of CO_2 formation has also been observed in the case of other Ca^{2+}-mobilizing agents such as vasopressin and α_1-adrenergic agents (e.g., see Refs. 6, 11, 22), whereas the inhibition of hepatic CPT-I activity and ketogenesis by vasopressin (11, 22) and α_1-adrenergic agonists (results not shown) has been described as well. However, others have reported that α_1-adrenergic agents stimulate or have no effect on hepatic ketogenesis (cf. Ref. 13). In any case, our results are in agreement with the notion that apart from CPT-I other factors may exert control over the fatty acid-oxidative process in the liver at the intramitochondrial level (see Refs. 13 and 30 for review).

The effects of extracellular ATP and other Ca^{2+}-mobilizing effectors on liver metabolism are believed to be dependent on receptor-mediated breakdown of membrane phosphatidylinositol 4,5-bisphosphate to produce diacylglycerol and inositol 1,4,5-trisphosphate (1, 6). Extracellular ATP is also able to trigger the receptor-mediated hydrolysis of phosphatidylcholine and phosphatidylethanolamine by membrane phospholipases (8, 9). In some cell types, such as platelets and various secretory cells, the diacylglycerol and the inositol 1,4,5-trisphosphate limbs of phospholipid hydrolysis act synergically (1). However, this is not the case with hepatocytes. Thus it has been shown that the stimulation of glycogen breakdown, gluconeogenesis, and the tricarboxylic acid cycle induced by Ca^{2+}-mobilizing agents in rat liver solely depends on the inositol 1,4,5-trisphosphate component of membrane phosphoinositide hydrolysis, i.e., on the elevation of $[Ca^{2+}]_i$ (6, 8, 9). However, the effects of extracellular ATP on hepatic fatty acid metabolism, as shown in the present paper, seem to be more complex (Fig. 4). Thus the inhibition of ACC (and hence of fatty acid synthesis de novo) by extracellular ATP may be mediated by the elevation of $[Ca^{2+}]_i$. This is in line with the observations that the serine residues phosphorylated on ACC upon hepatocyte treatment with phorbol esters do not correspond with sites phosphorylated on the purified enzyme by PKC, and that PKC-induced phosphorylation of purified ACC does not correlate with changes in enzyme activity (16). In addition, and similar to extracellular ATP, α_1-adrenergic agents inhibit fatty acid synthesis as well as acetyl-CoA carboxylase activity in hepatocytes, and this effect may be mediated by Ca^{2+}/calmodulin-dependent protein kinase II (28). In contrast, the inhibition of CPT-I (and hence of ketogenesis from long-chain fatty acids) by extracellular ATP may be solely dependent on the diacylglycerol limb of the bifurcated mechanism. The latter effect would in turn require a certain level of Ca^{2+} in the cytosol (as shown by the lack of PMA effect in Ca^{2+}-depleted hepatocytes), but it would not be synergic to elevations in $[Ca^{2+}]_i$ (as shown by the lack of potentiation of the PMA effect by BHQ, thapsigargin, and A-23187).

Because PMA inhibits CPT-I activity, it might be argued that inhibition of PKC by bisindolylmaleimide should cause an activation of CPT-I. However, it should be pointed out that the basal activity of hepatocellular PKC is very low because most of the enzyme is present in the soluble fraction (e.g., see Ref. 28). Therefore, inhibition of this marginal activity of PKC should not have any important effect on CPT-I activity. In contrast, bisindolylmaleimide should be expected to abolish PKC-mediated events when PKC is fully activated by hepatocyte incubation with phorbol esters. Thus we have observed that bisindolylmaleimide is able to block the PMA-induced inhibition of CPT-I activity (results not shown), indicating that the inhibitory effect of PMA on CPT-I is mediated by activation of PKC.

Extracellular ATP has been shown to trigger in vitro a number of biological responses in different cell lines of

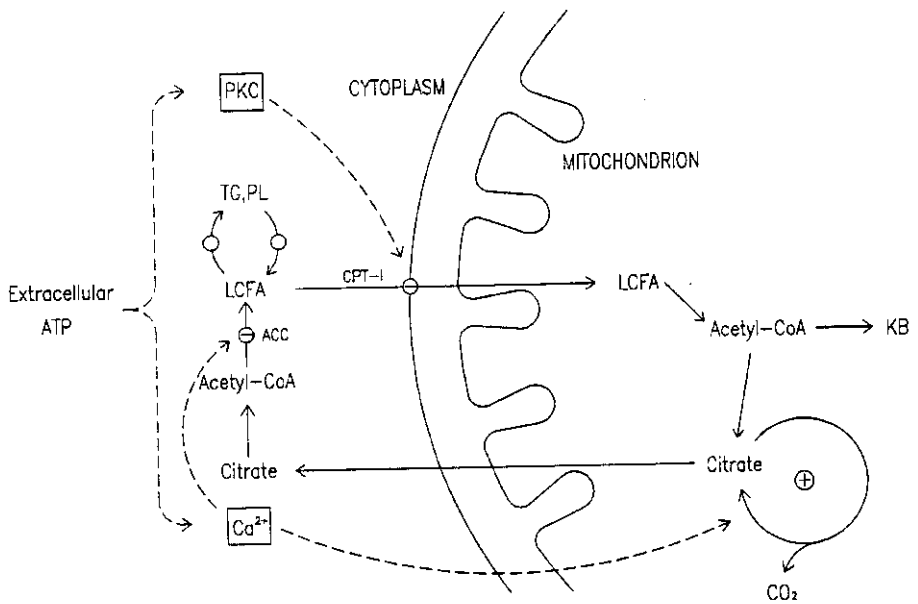

Fig. 4. Diagram showing effects of extracellular ATP on hepatic fatty acid metabolism. Inositol 1,4,5-trisphosphate limb of phosphoinositide hydrolysis (i.e., the Ca^{2+} connection) may be responsible for inhibition of ACC (and hence of fatty acid synthesis de novo) and stimulation of tricarboxylic acid cycle. The diacylglycerol component of phospholipid breakdown may modulate the inhibition of CPT-I (and hence of ketogenesis from long-chain fatty acids). Effect of extracellular ATP is shown as +, activation; −, inhibition; or ○, no effect. KB, ketone bodies; LCFA, long-chain fatty acids; PL, phospholipids; TG, triacylglycerols.

the cardiovascular tract and the nervous system that have been extrapolated to the possible situation in vivo (6, 9). In the case of liver tissue, however, the studies reported to date have been restricted to the use of isolated hepatocytes or perfused liver, so the possible role of ATP in vivo remains to be fully elucidated (6, 9). The present results suggest a potential physiological role for extracellular ATP in the modulation of hepatic metabolism. Thus, in line with our observations, sympathetic activation of the liver, which may involve both catecholamines and extracellular ATP (8, 9), has been shown to depress ketogenesis (3). In addition, stress mediators such as vasopressin, catecholamines, and (potentially) extracellular ATP exert similar effects on hepatic carbohydrate (8, 9) and lipid metabolism (the present paper) by increasing glucose ouput from the liver to the bloodstream and by inhibiting hepatic fatty acid utilization, thus increasing the supply of substrates for extrahepatic tissues in stress situations.

In conclusion, our results show that extracellular ATP exerts striking effects on hepatic fatty acid metabolism by simultaneously inhibiting fatty acid synthesis (and ACC activity) and ketogenesis from long-chain fatty acids (and CPT-I activity). Results also indicate that the inhibition of ACC by extracellular ATP is mediated by an elevation of cytosolic free Ca^{2+} concentration, whereas CPT-I may be inhibited by extracellular ATP through a PKC-dependent mechanism. This scenario may be even more complicated, since extracellular ATP has been observed to decrease the volume of liver cells, and this may cause profound alterations in hepatic metabolism (17).

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Inhibition of carnitine palmitoyltransferase I by hepatocyte swelling

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Abstract

Incubation of hepatocytes under conditions known to increase their volume, i.e. with amino acids (glutamine, proline) or in hypo-osmotic medium, decreased carnitine palmitoyl-transferase I (CPT-I) activity. This effect of hepatocyte swelling was antagonized by okadaic acid and dibutyryl-cAMP. Physiological concentrations of glutamate inhibited CPT-I activity in digitonin-permeabilized hepatocytes but not in isolated mitochondria. Results suggest that the amino acid-induced inhibition of CPT-I shares a common mechanism with the amino acid-induced stimulation of acetyl-CoA carboxylase and glycogen synthase [(1993) Eur. J. Biochem. 217, 1083–1089].

Key words: Carnitine palmitoyltransferase I; Hepatocyte swelling; Glutamine; Ketogenesis

1. Introduction

Evidence is rapidly accumulating that changes in hepatocyte volume play an important role in the control of hepatocellular metabolic function [1,2]. Hepatocyte swelling induced by several amino acids, notably glutamine and proline, or by hypotonicity have a number of anabolic and anti-catabolic effects, such as stimulation of glycogen [3,4], lipid [4] and protein synthesis [5], or inhibition of glycogenolysis [6] and proteolysis [7]. Ketogenesis is also inhibited by hepatocyte swelling [8]. Although the mechanism responsible for this effect has not been described, amino acid-induced inhibition of hepatic ketogenesis was observed to be independent of changes in the concentration of malonyl-CoA [8], a physiological inhibitor of the key regulatory enzyme in the transport of long-chain fatty acids into the mitochondrial matrix, viz. carnitine palmitoyltransferase I (CPT-I) [9–12]. Changes in the kinetic characteristics of CPT-I that occur in parallel with, or independently of, intracellular malonyl-CoA concentration have been shown to be involved in a number of short- and long-term alterations of hepatic ketogenesis [9–12]. Therefore, the present work was undertaken to test whether changes in the intrinsic properties of CPT-I are involved in the amino acid-induced inhibition of hepatic ketogenesis.

2. Experimental

2.1. Hepatocyte isolation and incubation

Male Wistar rats (220–250 g) were used throughout this study. Hepatocytes were isolated as described in [13] and incubated in Krebs–Henseleit bicarbonate buffer supplemented with 10 mM glucose and 1% (w/v) defatted and dialysed bovine serum albumin. Incubations (4–6 mg of cellular protein/ml) were carried out in a total volume of 2 ml at 37°C under an atmosphere of O_2/CO_2 (19:1). The osmolarity of the medium (305 mOsm under iso-osmotic conditions) was varied to 225 mOsm (hypo-osmotic medium) or to 385 mOsm (hyper-osmotic medium) by changing NaCl concentration.

2.2. Rate of ketogenesis

The rate of ketogenesis was monitored in incubations containing 0.4 mM [1-^{14}C]palmitate (0.1 Ci/mol) bound to albumin. Reactions were stopped with 0.5 ml of 2 M perchloric acid and ketone bodies were extracted and quantified as described before [14,15]. Ketone bodies routinely accounted for 85–90% of total oxidation products.

2.3. CPT-I assay

CPT-I activity was determined as the tetradecylglycydate (TDGA)-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine by three different methods (A, B and C). TDGA is a potent, specific and irreversible inhibitor of CPT-I [14,16]. In brief, hepatocytes were incubated in the absence or in the presence of 5 μM TDGA. Aliquots were removed from both sets of incubations in order to monitor CPT activity. In methods A and B, CPT activity was measured in digitonin-permeabilized hepatocytes. Both methods were performed using the same detergent/cell protein ratio (ca. 40 μg digitonin/mg cell protein). In method A ('one-step assay'), 100 μl of hepatocyte suspension was added to 100 μl of prewarmed digitonin-containing assay medium exactly as described in [15], and so the cell permeabilization and enzyme assay were performed at the same time. In method B ('two-step assay'), hepatocytes were permeabilized and thoroughly washed prior to determination of enzyme activity. Thus, 1.0 ml of hepatocyte suspension was added to 1.0 ml of prewarmed medium containing 0.20 mg digitonin, 5 mM Tris-HCl (pH 7.4), 150 mM KCl, 5 mM EDTA and 5 mM EGTA (Cl^- medium). The resulting mix was gently shaken for 5 s and rapidly diluted by transfer to tubes containing 40 ml of ice-cold Cl^- medium. Permeabilized cells were sedimented by centrifugation at 350 × *g* for 15 s, and pellets were resuspended in 1.0 ml of prewarmed digitonin-free Cl^- medium. The permeabilized-cell

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suspensions were incubated at 37°C for 5–15 min and then CPT activity was monitored. Finally, the third method (method C) measures enzyme activity in mitochondria isolated from hepatocyte suspensions as described in [17]. When the direct effect of glutamate on CPT-I activity was studied, iso-osmotic controls were always run in parallel by replacing potassium glutamate for identical concentrations of KCl.

3. Results and discussion

Incubation of isolated hepatocytes in conditions known to increase their volume, i.e. with amino acids (10 mM glutamine or 10 mM proline) or in hypo-osmotic (225 mOsm) medium, decreased CPT-I activity, as measured in digitonin-permeabilized cells by method A (Table 1). This was accompanied by an inhibition of hepatic ketogenesis from palmitate in parallel cell incubations (Table 1). In contrast, a slight increase in CPT-I activity and in ketogenesis from palmitate was observed in hepatocytes incubated in hyper-osmotic (385 mOsm) medium (Table 1).

The inhibition of CPT-I and ketogenesis by hepatocyte swelling could result from an increase in the intracellular levels of malonyl-CoA, a physiological inhibitor of CPT-I [9–12]. However, Baquet et al. have shown that the amino acid-induced inhibition of hepatic ketogenesis can occur independently of increases in malonyl-CoA concentration [8]. In the present study, the simultaneous permeabilization of the plasma membrane and assay of CPT-I activity (method A) is assumed to have diluted intracellular malonyl-CoA [14,15]. Consequently, it is possible that the observed changes in CPT-I activity are due to stable post-translational modification of CPT-I, either directly [14,15,18] or indirectly [19]. Therefore, we tested whether factors that increase cell protein phosphorylation (e.g. dibutyryl-cAMP, okadaic acid) could prevent the observed effects of swelling. Table 1 shows that both agents were able to antagonize the effects of swelling on CPT-I activity.

In another set of experiments, hepatocytes were incubated with glutamine and/or okadaic acid, and CPT-I activity was determined in digitonin-permeabilized hepatocytes by method B (Fig. 1). The changes induced by these cellular effectors on CPT-I activity survived permeabilization of hepatocytes, extensive washing of the permeabilized cells, and subsequent incubation of the per-

Table 1
Inhibition of CPT-I activity and ketogenesis by hepatocyte swelling and its reversal by okadaic acid and dibutyryl-cAMP

Cell incubation	Percentage of incubations with no additions	
	CPT-I activity	Rate of ketogenesis
Hypo-osmotic medium (n = 6)	78.5 ± 5.4[a]	81.9 ± 7.0[a]
Hyper-osmotic medium (n = 4)	118.1 ± 3.7[a]	116.0 ± 4.5[a]
10 mM glutamine (n = 6)	74.7 ± 6.0[a]	78.4 ± 5.3[a]
10 mM proline (n = 4)	68.9 ± 8.3[a]	74.7 ± 8.1[a]
0.5 μM okadaic acid (n = 6)	154.6 ± 14.7[a]	148.6 ± 12.1[a]
50 μM dibutyryl-cAMP (n = 6)	137.2 ± 5.1[a]	132.4 ± 5.7[a]
Hypo-osmotic medium + 0.5 μM okadaic acid (n = 6)	149.6 ± 10.2[a]	140.7 ± 8.4[a]
Hypo-osmotic medium + 50 μM dibutyryl-cAMP (n = 4)	136.4 ± 5.7[a]	133.9 ± 3.2[a]
10 mM glutamine + 0.5 μM okadaic acid (n = 6)	142.0 ± 8.5[a]	143.4 ± 9.3[a]
10 mM glutamine + 50 μM dibutyryl-cAMP (n =4)	133.2 ± 5.0[a]	128.1 ± 4.4[a]
10 mM proline + 0.5 μM okadaic acid (n = 4)	145.9 ± 6.9[a]	139.5 ± 10.4[a]
10 mM proline + 50 μM dibutyryl-cAMP n = 4)	126.8 ± 4.0[a]	126.1 ± 3.7[a]

Hepatocytes were incubated for 30 min in the presence of the additions indicated. Then, part of the cells were used for measurement of CPT-I activity in digitonin-permeabilized hepatocytes by method A. The rest of the cells were used for determination of the rate of ketogenesis from [1-^{14}C]palmitate. Results represent the means ± S.D. of the number of cell preparations indicated in every case. 100% values of CPT-I activity and ketogenesis were 2.16 ± 0.43 nmol product/min × mg cell protein and 78.5 ± 8.0 nmol palmitate into product/h × mg cell protein, respectively. As determined by the Student's t-test. [a]Significantly different ($P < 0.01$) from incubations with no additions.

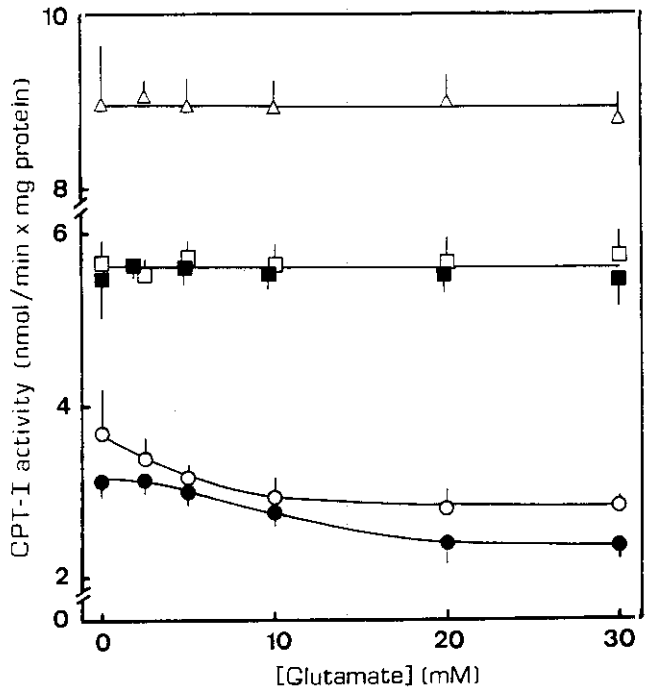

Fig. 1. Inhibition of CPT-I activity by glutamate in digitonin-permeabilized hepatocytes and its reversal by hepatocyte pretreatment with okadaic acid. Hepatocytes were incubated for 30 min with no additions (○) or in the presence of either 10 mM glutamine (●), 0.5 μM okadaic acid (□), or 10 mM glutamine plus 0.5 μM okadaic acid (■). Then, cells were permeabilized, washed in an excess of Cl^- medium, and incubated at 37°C for 5–15 min with increasing concentrations of glutamate. CPT-I activity was determined in those permeabilized cells by method B. Alternatively, CPT-I activity was determined by method C in mitochondria isolated from hepatocytes incubated with no additions (△). Results represent the means ± S.D. of 4 cell preparations.

meabilized cells at 37°C for 5–15 min, again indicating that the modulation of enzyme activity is stable. Therefore, although the inhibition of CPT-I by malonyl-CoA is a well-described property of the enzyme [9–12], other types of regulatory mechanisms could be involved in the control of hepatic CPT-I by hepatocyte swelling. Since the changes observed in CPT-I activity are stable in spite of the absence of fluoride in the medium, they are unlikely to be due to changes in the phosphorylation state of CPT-I.

A mechanism linking amino acid-induced hepatocyte swelling to stimulation of glycogen synthesis and lipogenesis has been recently suggested. Thus, the increase in the intracellular concentration of glutamate and (to a lesser extent) aspartate that is observed in swollen hepatocytes after incubation with glutamine or proline seem to be responsible, at least in part, for the stimulation of protein phosphatase(s) involved in the activation of glycogen synthase (and hence of glycogen synthesis) [20] and acetyl-CoA carboxylase (and hence of lipogenesis) [21]. Therefore, we wondered whether a similar mechanism could be responsible for the inhibition of CPT-I (and hence of ketogenesis independently of malonyl-CoA concentration) by hepatocyte swelling. From data presented in Fig. 1 it may be inferred that (i) concentrations of glutamate found in hepatocytes incubated with glutamine or proline [20] inhibited CPT-I when enzyme activity was assayed by method B in digitonin-permeabilized hepatocytes; (ii) preincubation of hepatocytes with glutamine induced a certain desensitization of the CPT-I enzyme toward glutamate; (iii) preincubation of hepatocytes with okadaic acid rendered CPT-I insensitive to the inhibitory effect of glutamate. Therefore, results suggest that the amino acid-induced inhibition of hepatic CPT-I may result from a glutamate-dependent mechanism related to that involved in the activation of glycogen synthase [20] and acetyl-CoA carboxylase [21].

It has recently been shown that the okadaic acid-induced stimulation of CPT-I is retained when mitochondria are still associated with other cellular fractions, e.g. in permeabilized cell ghosts and in crude cellular homogenates, but not when mitochondria are isolated for determination of enzyme activity [19]. Likewise, the inhibition of CPT-I by glutamate observed in permeabilized hepatocytes (Table 1 and Fig. 1) was not evident when enzyme activity was assayed by method C in mitochondria isolated from hepatocyte suspensions (Fig. 1). Okadaic acid is known to disrupt the cytoskeleton [22], and some effects of hepatocyte swelling are abolished by hepatocyte preincubation with colchicine, i.e. they seem to depend on the integrity of the cytoskeleton [23]. However, when we preincubated hepatocytes with colchicine or cytochalasin B, the effects of glutamine and okadaic acid on CPT-I activity were still evident (results not shown), suggesting that the integrity of the cytoskeleton is not necessary for the modulation of CPT-I activity by these cellular effectors. Our current research focuses on the characterization of the extra-mitochondrial cell components, presumably non-diffusible and possibly membranous, which seem to be required for the effects of glutamine or okadaic acid on CPT-I activity to be observed.

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DISCUSIÓN.

En el presente capítulo, se describen los efectos del ATP extracelular y de los cambios en el volumen celular sobre la actividad CPT-I. En ambos casos, y al igual que ocurría en el caso de la insulina, el glucagón o los factores de crecimiento, dichos cambios en la actividad CPT-I no parecen ser debidos únicamente a cambios en la concentración intracelular de malonil-CoA. En el caso del ATP extracelular, éste inhibe simultaneamente tanto la síntesis como la oxidación de ácidos grasos, de tal manera que a pesar de que los niveles de malonil-CoA se encuentran disminuidos, la actividad CPT-I se encuentra inhibida. Las modificaciones que los cambios en el volumen celular ejercen sobre la actividad CPT-I tampoco parecen deberse a cambios en los niveles de malonil-CoA, puesto que el metodo utilizado para determinar la actividad CPT-I (hepatocitos permeabilizados con digitonina) implica la dilución extrema del contenido del citoplasma en el medio extracelular. De esta manera, diferentes tipos de agonistas que ejercen sus efectos a través de muy diferentes vías de transducción de señales, son capaces de modificar la actividad CPT-I mediante un mecanismo independiente de los niveles de malonil-CoA.

El efecto inhibitorio del ATP extracelular sobre la actividad CPT-I podría estar mediado por la activación de la PKC. Por otra parte, la inhibición de la CPT-I mediada por hinchamiento celular, parece verse mimetizada por ácido glutámico, sugiriendo que, tal y como se ha propuesto para la glucógeno sintasa y la acetil-CoA-carboxilasa (Baquet *et al.*, 1993), este aminoácido podría activar una proteína fosfatasa. La activación de esta proteína fosfatasa estaría implicada en la estimulación de ambas enzimas (Baquet *et al.*, 1993) y quizá también en la inhibición de la CPT-I. Todas estas observaciones apuntan a que la modulación de la actividad de proteínas quinasas y fosfatasas podría desempeñar una función importante en la regulación de la actividad CPT-I. De hecho, la incubación de los hepatocitos con ácido okadaico un inhibidor de las proteína fosfatasas de tipo 1 y 2A induce un fuerte incremento en dicha actividad enzimática (Guzmán y Geelen, 1992), aunque este aumento no parece deberse a una fosforilación directa de la enzima (Guzmán *et al.*, 1994). Así, consideramos la posibilidad de que las diferentes vías de transducción de la señal pudiesen afectar a la actividad CPT-I mediante cambios en el grado de fosforilación de potenciales proteínas reguladoras. En la búsqueda de esas posibles dianas, algunas observaciones pueden resultar interesantes:

i) Tanto los cambios en el volumen celular, en los que el propio ATP extracelular podría estar implicado, ya que se ha propuesto que podría actuar como una señal paracrina secretada por la célula en respuesta al hinchamiento celular (Wang *et al.*, 1996), como el tratamiento de los hepatocitos con ácido okadaico, inducen cambios importantes en la morfologíay organización intracelular de la célula.

ii) Los cambios en la actividad CPT-I que inducen todos los efectores celulares estudiados se preservan en células permeabilizadas, cuya organización tridimensional no se ve básicamente alterada (Fiskum *et al.*, 1980), pero se pierden tras el aislamiento de mitocondrias, indicando que puede ser necesaria la presencia de elementos extramitocondriales para que este tipo de regulación de la CPT-I se verifique.

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2.2. Mecanismo de regulación a corto plazo (independiente de malonil-CoA) de la CPT-I

INTRODUCCIÓN

Una vez puesto de manifiesto que muy diferentes agonistas son capaces de modificar la actividad CPT-I mediante un mecanismo independiente de malonil-CoA (Capítulo 2.1), intentamos determinar cuáles podrían ser las bases bioquímicas de dicho mecanismo. Para ello, utilizamos el ácido okadaico como una herramienta que nos permitiera profundizar en el estudio de potenciales dianas cuya fosforilación fuese inducida por este compuesto y que a su vez pudiesen regular a la CPT-I. Dado que, tanto el ácido okadaico como el db-cAMP o el vanadato (compuestos todos ellos que inducen un incremento en la actividad de la CPT-I) afectan a la morfologíaa celular y a la organización del citoesqueleto, primeramente decidimos estudiar el posible papel del mismo como factor extramitocondrial implicado en la regulación de la CPT-I.

El citoesqueleto está constituido por tres tipos de elementos, los microtúbulos, los microfilamentos y los filamentos intermedios (Tabla 1) que se encuentran estrechamente relacionados entre sí. Aunque hasta hace tiempo se consideraba que el citoesqueleto únicamente poseía una función estructural, cada vez son más los datos que indican que también desempeña un importante papel regulador (De Loof *et al.*, 1996). Las proteínas de los diferentes elementos del citoesqueleto pueden verse fosforiladas por la acción de diferentes proteína quinasas, entre las que la Ca^{2+}/CMPKII es quizá la más importante (Toivola *et al.*,

Tipo	*Composición*	*Drogas (desorganizadoras)*
Microtúbulos	α,β-tubulina MAP's	Colchicina Nocodazol
Microfilamentos	Actina	Citocalasina B
IDPN	Citoqueratinas desmina, vimentina proteínas fibrilares	IDPN

Tabla 1. Componentes del citoesqueleto. Abreviaturas: MAP's, proteínas asociadas a microtúbulos; IDPN, 3,3'-iminodipropionitrilo; IF, filamentos intermedios

1997). Esos procesos de fosforilación/desforilación afectan notablemente al grado de polimerización/organización del citoesqueleto, de tal manera que las interacciones en las que éste pudiera estar implicado también se verían alteradas. Por ello también procedimos a estudiar si el tratamiento con ácido okadaico podría inducir la activación de alguna proteína quinasa y, dadas sus características, la Ca^{2+}/CMPKII es quizá el candidato más probable, tanto por su capacidad de fosforilar a diferentes proteínas del citoesqueleto como por sus propiedades reguladoras: En determinadas condiciones de activación, la Ca^{2+}/CMPKII se autofosforila, alcanzando así un estado autónomo (activado) que es independiente de la presencia de Ca^{2+} y de calmodulina (Braun y Schulman, 1995) (Fig. 1).

Los diferentes estudios realizados y que se exponen a continuación, nos permiten proponer un modelo de regulación a corto plazo (independiente de malonil-CoA) de la CPT-I.

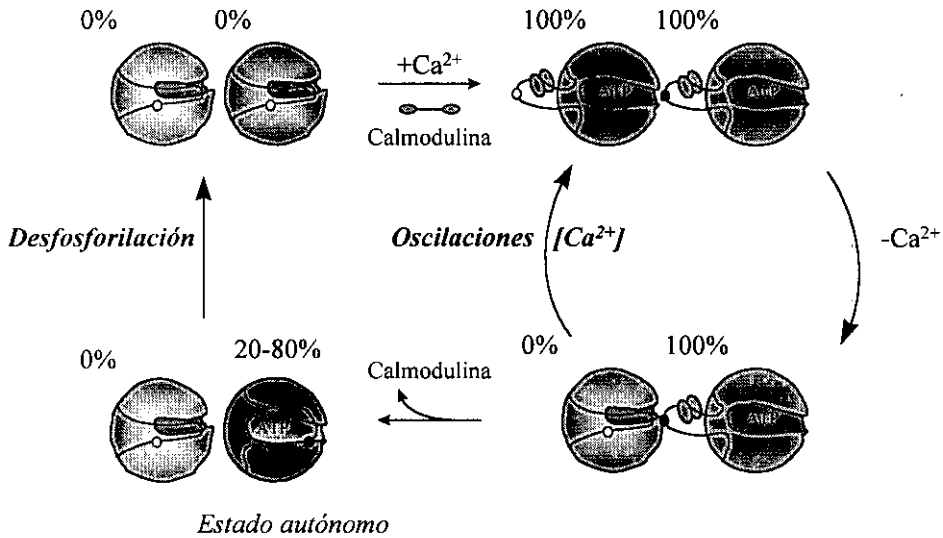

Figura 1. Estados de activación de la Ca^{2+}/CMPKII. La autofosforilación de la Ca^{2+}/CMPKII conduce a un estado autónomo que conserva parte de la actividad de la quinasa, siendo esta activación independiente de la presencia de Ca^{2+} y calmodulina en el medio.

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Are Cytoskeletal Components Involved in the Control of Hepatic Carnitine Palmitoyltransferase I Activity?

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The present work was undertaken to test whether cytoskeletal components are involved in the control of rat-liver carnitine palmitoyltransferase I (CPT-I) activity by cellular effectors. The microtubule stabilizer taxol abolished the changes in CPT-I activity induced by the effectors tested. Taxol also prevented OA-induced shrinkage of hepatocytes as well as the enhanced release of lactate dehydrogenase from digitonin-permeabilized hepatocytes. On the basis of its relative sensitivity to tautomycin and OA, the modulation of CPT-I activity seemed to involve mostly protein phosphatase 1. These data suggest that the short-term control of hepatic CPT-I by cellular effectors may involve modulation of interactions between CPT-I and cytoskeletal components.

Carnitine palmitoyltransferase I (CPT-I), the mitochondrial outer membrane carnitine palmitoyltransferase, catalyzes the pace-setting step of long-chain fatty acid translocation into the mitochondrial matrix (1-3). The phosphatase inhibitor okadaic acid (OA) is able to stimulate by up to 50% hepatic CPT-I activity as well as palmitate oxidation (4,5). This observation led to the suggestion that, apart from modulation of rat-liver CPT-I activity by malonyl-CoA, a phosphorylation-dephosphorylation mechanism might be involved in the short-term control of this enzyme (3). However, further research showed that the increase of CPT-I activity observed in OA-treated hepatocytes was not due to direct phosphorylation of the CPT-I enzyme, but may involve interactions between the mitochondrial outer membrane and extra-mitochondrial cell components (6).

A number of reports have recently described the existence of specific interactions between the mitochondrial outer membrane and cytoskeletal elements (7-9). In the context of CPT-I regulation, OA and vanadate, which activate hepatic CPT-I (5,10), have been shown to disrupt the cytoskeleton of hepatocytes (11-13). Furthermore, CPT-I activity is affected by changes in hepatocyte volume (14), and several responses of hepatocytes to changes in cell volume are dependent on microtubule dynamics (15,16). Therefore, the present work was undertaken to test whether cytoskeletal components may be involved in the control of hepatic CPT-I activity by OA and other short-term effectors of cellular metabolism.

MATERIALS AND METHODS

Male Wistar rats (250-300 g) which had free access to food and water were used throughout in this study. Hepatocytes were isolated and incubated as described in (17). In some experiments, the osmolarity of the medium (305 mOsm in the normal, iso-osmotic Krebs-Henseleit bicarbonate buffer) was increased to 385 mOsm (hyper-osmotic medium) by changing the NaCl concentration. This was achieved by adding 10 μl of 4.0 M NaCl per ml of cell incubation.

After incubation of the hepatocytes with the additions indicated in each case, CPT-I activity was routinely determined in digitonin-permeabilized hepatocytes exactly as described before (5).

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TABLE 1
Taxol Prevents Short-Term Modulation of CPT-I Activity

Taxol	Other additions	CPT-I activity (%)
–	None (6)	100
+	None (6)	98 ± 5
–	0.5 μM OA (6)	151 ± 15*
+	0.5 μM OA (6)	97 ± 8
–	50 μM dibutyryl-cAMP (4)	136 ± 13*
+	50 μM dibutyryl-cAMP (4)	104 ± 2
–	1.0 mM vanadate (4)	143 ± 16*
+	1.0 mM vanadate (4)	105 ± 7
–	10 mM glutamine (6)	83 ± 10**
+	10 mM glutamine (6)	101 ± 6
–	385 mOsm medium (6)	116 ± 7**
+	385 mOsm medium (6)	99 ± 6

Hepatocytes were preincubated for 30 min in the absence or in the presence of 10 μM taxol, followed by 15 (OA, dibutyryl-cAMP, vanadate) or 30 (glutamine, hyper-osmotic medium) additional min with the additions indicated. Subsequently, CPT-I activity was determined in digitonin-permeabilized hepatocytes. Values correspond to the number of hepatocyte preparations indicated in parentheses. 100% value of CPT-I activity was 1.56 ± 0.18 nmol/min per mg of cellular protein. Versus incubations with no additions: *$P < 0.01$; **$P < 0.05$.

Hepatocyte volume was estimated from the wet to dry cell weight ratio as described in (18). Lactate dehydrogenase activity was determined in permeabilized cells as described before (6).

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

Sources of chemicals as in (5) and (6).

RESULTS

The possibility that cytoskeletal components may be involved in the short-term modulation of CPT-I activity was tested by the use of taxol. This complex diterpenoid binds to tubulin and stabilizes microtubules, preventing the disassembly of microtubules in a very efficient fashion (*cf.* ref. 19). Interestingly, stimulation of hepatic CPT-I activity induced by OA, dibutyryl-cAMP or vanadate were completely abolished by pretreatment of hepatocytes with taxol (Table 1). Likewise, changes in CPT-I activity produced by hepatocyte swelling or shrinkage were also prevented by taxol (Table 1). Hence, blockade of microtubule dynamics prevents short-term modulation of CPT-I activity by a number of cellular effectors. Since OA produced the most pronounced change of CPT-I activity, a dose-response of the combined effects of taxol and OA on CPT-I activity was determined (Fig. 1).

The possible relationship between hepatocyte volume, microtubule stability and CPT-I activity was further studied. OA decreased hepatocyte volume by 7% (Table 2), a magnitude similar to that observed after hepatocyte treatment with glucagon or dibutyryl-cAMP (20). More important, taxol prevented the OA-induced shrinkage of hepatocytes (Table 2). When hepatocytes were incubated in a hyper-osmotic (385 mOsm) medium, a 12% decrease in cell volume was observed. This shrinkage was also prevented by taxol (Table 2).

One of the alterations observed after OA-induced disruption of the cytoskeleton is enhanced cell fragility (6,11,21). In an attempt to quantify hepatocyte fragility, we determined the percentage of lactate dehydrogenase retained in the permeabilized cells after permeabilization of the plasma membrane with digitonin (*cf.* ref. 6). As shown in Table 2, treatment of hepato-

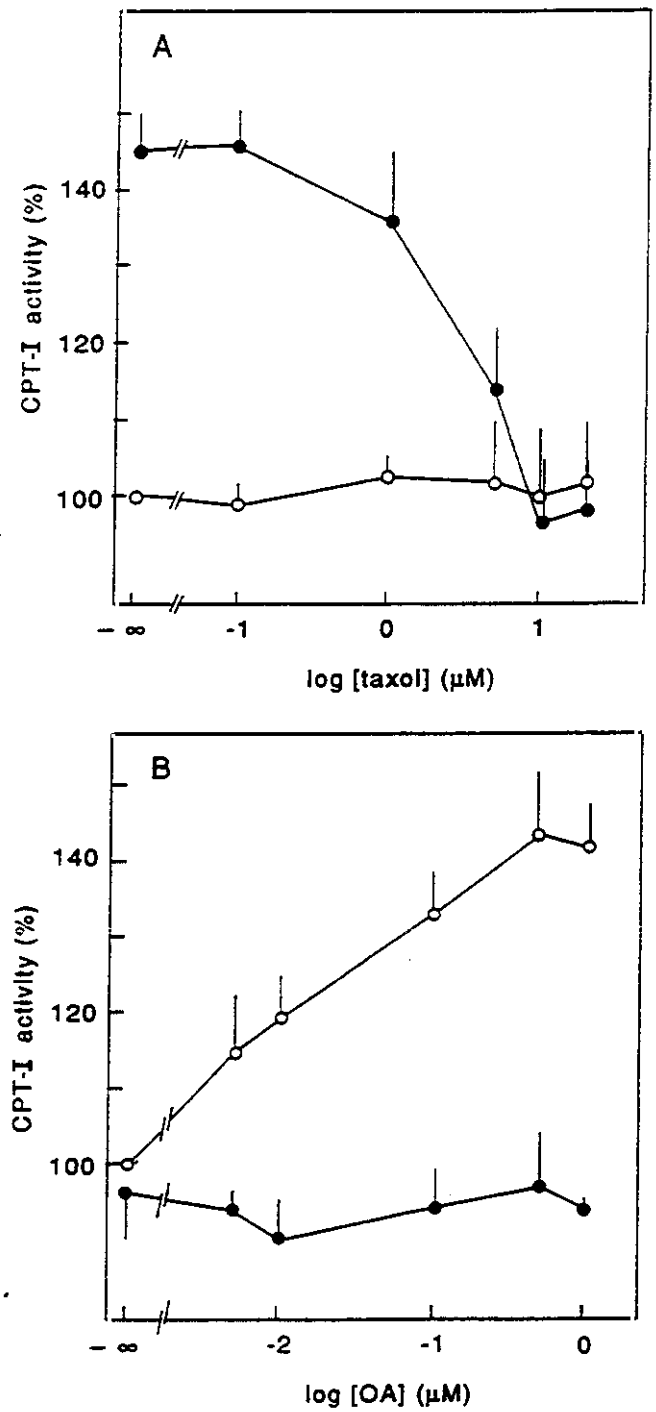

FIG. 1. Taxol antagonizes the OA-induced stimulation of CPT-I. *Panel A:* Hepatocytes were preincubated for 30 min with varying concentrations of taxol, followed by 15 additional min in the absence (○) or in the presence of 0.5 μM OA (●). *Panel B:* Hepatocytes were preincubated for 30 min in the absence (○) or in the presence of 10 μM taxol (●), followed by 15 additional min with varying concentrations of OA. In both panels, CPT-I activity was determined in digitonin-permeabilized hepatocytes following the incubations of the cells in the presence or absence of the indicated agonists. In both cases values correspond to 3 separate hepatocyte preparations.

cytes with OA resulted in a decreased retention of lactate dehydrogenase in the permeabilized cells. Once again, taxol prevented this effect of OA (Table 2).

Although OA is a more potent inhibitor of protein phosphatase 2A than of protein phosphatase 1, at the doses employed in the present study (0.5 μM) OA is supposed to completely inhibit both protein phosphatases (22,23). To study whether the stimulatory

TABLE 2
Taxol Prevents Hepatocyte Shrinkage as Well as Release of Lactate Dehydrogenase from Permeabilized Hepatocytes

Taxol	OA	385 mOsm medium	Wet weight: Dry weight	Lactate dehydrogenase retained in cell ghosts
–	–	–	3.93 ± 0.07 (100)	6.5 ± 1.1
–	+	–	3.67 ± 0.03* (93.4)	2.0 ± 0.3*
+	–	–	3.94 ± 0.02 (100.3)	6.7 ± 1.4
+	+	–	3.92 ± 0.04 (99.7)	6.6 ± 0.9
–	–	+	3.47 ± 0.07* (88.3)	n.d.
+	–	+	3.86 ± 0.08 (98.2)	n.d.

Hepatocytes were preincubated for 30 min in the absence or in the presence of 10 μM taxol, followed by 15 additional min with or without 0.5 μM OA or 30 additional min in hyperosmotic medium. Then, part of the hepatocytes was used to determine the wet to dry cell weight ratio. The percentage as compared to incubations with no additions is shown in parentheses. The rest of the cells was permeabilized with ca. 40 μg digitonin/mg protein and the percentage of lactate dehydrogenase retained by the permeabilized cells was determined. Values correspond to 4 separate hepatocyte preparations. *$P < 0.01$ versus incubations with no additions. n.d.: not determined.

effect of OA on CPT-I activity was mostly due to inhibiton of phosphatase 1 or 2A, we compared the effect of OA with that of tautomycin, which inhibits phosphatase 1 more efficiently than phosphatase 2A (23). As shown in Fig. 2, tautomycin was quite more potent in stimulating hepatic CPT-I activity (50% of activation at 4 nM) than OA (50% activation at 18 nM), indicating that protein phosphatase 1 is more important than protein

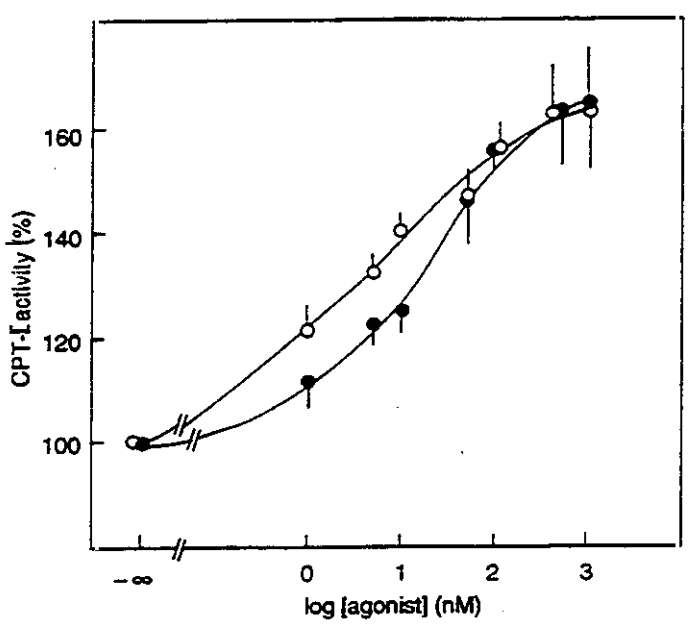

FIG. 2. Tautomycin is more potent than OA in stimulating CPT-I. Hepatocytes were incubated for 15 min in the presence of varying concentrations of tautomycin (○) or OA (●). Subsequently, CPT-I activity was determined in a cell-permeabilized system. Values correspond to 4 separate hepatocyte incubations.

phosphatase 2A in the control of CPT-I activity. Interestingly, taxol also prevented the tautomycin-induced activation of CPT-I.

DISCUSSION

Several studies performed by our group using digitonin-permeabilized hepatocytes led to the suggestion that a mechanism of phosphorylation-dephosphorylation might be involved in the short-term control of rat liver CPT-I activity (reviewed in ref. 3). However, further research showed that the increase in CPT-I activity observed in OA-treated hepatocytes was not due to direct phosphorylation of the CPT-I enzyme, but may involve interactions between the mitochondrial outer membrane and extra-mitochondrial, non-diffusible cell components (6). Data on the effects of taxol presented in the present report indicate that the extra-mitochondrial cell components potentially involved in the short-term control of CPT-I activity might reside in the cytoskeleton. In addition, the data on the stimulation of CPT-I by OA and tautomycin suggest that protein phosphatase 1 is more important than protein phosphatase 2A in the short-term control of CPT-I. This is in agreement with the observation that phosphatase 1 seems to be the main protein phosphatase involved in the regulation of the phosphorylation state of the cytoskeleton, and in turn in the control of cytoskeletal integrity (21,24).

The nature of the putative cytoskeletal component(s) that might be involved in controlling CPT-I activity is still unknown. A first possibility could be that the control of CPT-I activity by those potential interactions between mitochondria and the cytoskeleton merely reflected a physical phenomenon, *i.e.* CPT-I activity might be dependent on mitochondrial shape, stretching or contraction of the mitochondrial outer membrane, etc. (*cf.* ref. 7). A second possibility could be that modulation of CPT-I activity involved the specific interaction between CPT-I and regulatory cytoskeletal protein(s). This notion is supported by the observation that the mere disruption of microtubules by 2-methoxy 5-(2,3,4-trimethoxyphenyl) 2,4,6-cycloheptatrien-1-one or colchicine or the mere disruption of actin microfilaments by cytochalasin B does not affect CPT-I activity (unpublished work). OA and other phosphatase inhibitors produce hyperphosphorylation and consequently disruption of microtubules, actin microfilaments and intermediate filaments in several cell lines, including hepatocytes (21,25,26). Whether these cytoskeletal changes are related to the effects of OA on hepatic CPT-I activity is as yet an open question.

ACKNOWLEDGMENTS

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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, Costorphine 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 deinhibition 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 osmolarity 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-CH_2Cl (15 μg/ml), Tos-Lys-CH_2Cl (18 μg/ml), tosyl-L-arginine methyl ester (18 μg/ml), pepstatin (5 μg/ml), trypsin inhibitor (50 μg/ml), leupeptin (5 μg/ml), benzamidine (0.8 mg/ml), aprotinin (0.4 kIU/ml) and 2-mercaptoethanol (5 μl/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 ± 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 μM 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 μM OA	CPT-I (nmol/min per mg of protein) One-step assay	Two-step assay
None	−	1.47 ± 0.21 ($n = 11$) (100)	2.27 ± 0.25 ($n = 4$) (100)
	+	2.15 ± 0.34* ($n = 11$) (146)	3.54 ± 0.43* ($n = 4$) (156)
10 μM K-252a	−	1.54 ± 0.15 ($n = 6$) (105)	2.21 ± 0.35 ($n = 3$) (97)
	+	1.50 ± 0.13 ($n = 6$) (102)	2.34 ± 0.17 ($n = 3$) (103)
30 μM KN-62	−	1.53 ± 0.10 ($n = 11$) (104)	2.28 ± 0.37 ($n = 4$) (100)
	+	1.50 ± 0.08 ($n = 11$) (102)	2.41 ± 0.26 ($n = 4$) (106)
2 μM bisindolylmaleimide	−	1.44 ± 0.16 ($n = 4$) (98)	n.d.
	+	2.16 ± 0.18* ($n = 4$) (147)	n.d.
100 μM H-7	−	1.51 ± 0.13 ($n = 5$) (103)	n.d.
	+	2.18 ± 0.33* ($n = 5$) (148)	n.d.

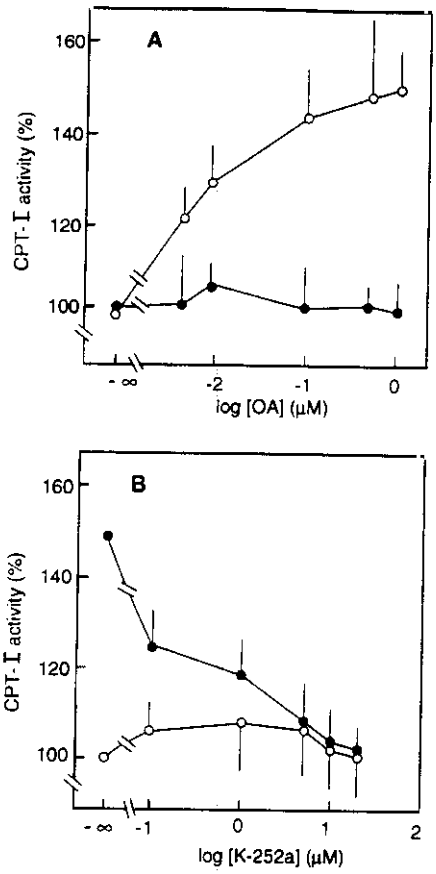

Figure 1 Effect of K-252a on OA-induced stimulation of CPT-I

(**A**) Hepatocytes were preincubated for 15 min in the absence (○) or presence (●) of 10 μM 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 μM OA. CPT-I activity was determined by the one-step assay. In both cases values correspond to three separate hepatocyte preparations.

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 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

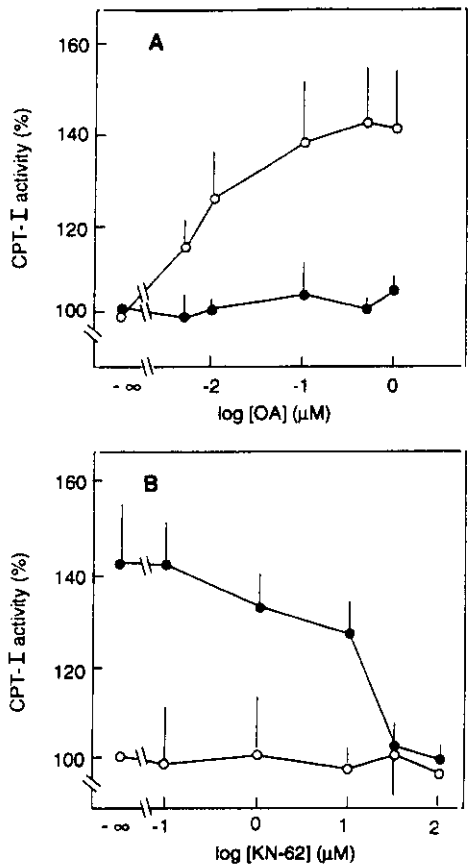

Figure 2 Effect of KN-62 on OA-induced stimulation of CPT-I

(**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

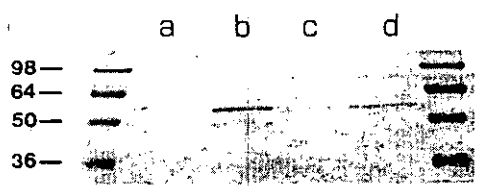

Figure 3 Immunodetection of hepatocyte Ca^{2+}/CM-PKII

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.

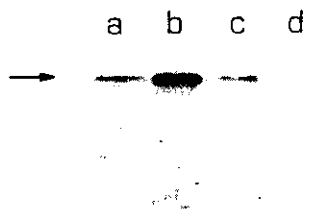

Figure 4 Effect of KN-62 on OA-induced phosphorylation of Ca^{2+}/CM-PKII

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 ± 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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Malonyl-CoA-independent Acute Control of Hepatic Carnitine Palmitoyltransferase I Activity

Role of Ca^{2+}/calmodulin-dependent Protein Kinase II and Cytoskeletal Components*

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The mechanism of malonyl-CoA-independent acute control of hepatic carnitine palmitoyltransferase I (CPT-I) activity was investigated. In a first series of experiments, the possible involvement of the cytoskeleton in the control of CPT-I activity was studied. (i) Treatment of permeabilized hepatocytes with trypsin in very mild conditions produced a ca. 50% stimulation of CPT-I. This effect was not observed in cells that had been pretreated with okadaic acid (OA) and seemed to be due to the action of trypsin on cell component(s) distinct from CPT-I. (ii) Incubation of intact hepatocytes with 3,3'-iminodipropionitrile (IDPN), a disruptor of intermediate filaments, increased CPT-I activity in a non-additive manner with respect to OA. Taxol, a stabilizer of the cytoskeleton, prevented the OA- and IDPN-induced stimulation of CPT-I. (iii) CPT-I activity in isolated mitochondria was depressed in a dose-dependent fashion by the addition of a total-cytoskeleton fraction and a cytokeratin-enriched cytoskeletal fraction, the latter being 3 times more potent than the former. In a second series of experiments, the possible link between Ca^{2+}/calmodulin-dependent protein kinase II (Ca^{2+}/CM-PKII) and the cytoskeleton was studied in the context of CPT-I regulation. (i) Purified Ca^{2+}/CM-PKII activated CPT-I in permeabilized hepatocytes but not in isolated mitochondria. (ii) Purified Ca^{2+}/CM-PKII abrogated the inhibition of CPT-I induced by a cytokeratin-enriched fraction. (iii) The Ca^{2+}/CM-PKII inhibitor KN-62 prevented the OA-induced phosphorylation of cytokeratins in intact hepatocytes. Results thus support a novel mechanism of short-term control of hepatic CPT-I activity which may rely on the cascade Ca^{2+}/CM-PKII activation -> Cytokeratin phosphorylation -> CPT-I de-inhibition.

Mitochondrial fatty acid oxidation in liver provides a major source of energy to this organ and supplies extrahepatic tissues with ketone bodies as a glucose-replacing fuel (1,2). Carnitine palmitoyltransferase I (CPT-I), the outer-mitochondrial-membrane carnitine palmitoyltransferase, catalyzes the pace-setting step of long-chain fatty acid translocation into the mitochondrial matrix (1-5). Moreover, recent determination of flux control coefficients of the enzymes involved in hepatic long-chain fatty acid oxidation shows that CPT-I plays a pivotal role in controlling the flux through this pathway under different substrate concentrations and patho-physiological states (6,7). CPT-I is subjected to long-term regulation in response to alterations in the nutritional and hormonal status of the animal (1,2,5). Short-term control of CPT-I activity involves inhibition by malonyl-CoA, the product of the reaction catalyzed by acetyl-CoA carboxylase (8). Since the latter enzyme is a key regulatory site of fatty acid synthesis *de novo* (cf. 1-5), malonyl-CoA inhibition of CPT-I allows an elegant explanation for the coordinate control of the partition of hepatic fatty acids between esterification and oxidation. As a matter of fact, evidence has accumulated during the last two decades highlighting the physiological importance of malonyl-CoA inhibition of CPT-I not only in liver but also in extra-hepatic tissues (1,5).

During the last years, however, a novel mechanism of control of hepatic CPT-I activity has been put forward. Studies using permeabilized hepatocytes have shown that various agents exert short-term changes in CPT-I activity in parallel with changes in the rate of long-chain fatty acid oxidation (3,9). These short-term changes in hepatic CPT-I activity are assumed to be mediated by a malonyl-CoA-independent mechanism, since they survive cell permabilization, extensive washing of the permeabilized cells (to allow complete removal of malonyl-CoA) and subsequent preincubation of the cell ghosts at 37°C before determination of CPT-I activity (to allow equalization of the conformational state of CPT-I) (10). Evidence has also been presented showing that the stimulation of hepatic CPT-I by the phosphatase inhibitor okadaic acid (OA), used as a model compound to study the short-term regulation of CPT-I, does not involve the direct phosphorylation of CPT-I (10). It has been recently shown that the OA-induced stimulation of CPT-I is prevented by KN-62, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (Ca^{2+}/CM-PKII) (11), and by taxol, a stabilizer of the cytoskeleton (12). These observations suggest that both activation of Ca^{2+}/CM-PKII and disruption of the cytoskeleton may be necessary for the OA-induced stimulation of CPT-I to be demonstrated. It is conceivable

that these two processes may be related, since Ca^{2+}/CM-PKII is one of the protein kinases more actively involved in the control of the integrity of the cytoskeleton by phosphorylating cytoskeletal proteins (13). However, the events underlying this novel mechanism of control of CPT-I activity are as yet unknown. The present work was thus undertaken to study in detail the molecular basis of the malonyl-CoA-independent short-term control of hepatic CPT-I activity.

EXPERIMENTAL PROCEDURES

Materials - L-[*methyl*-^{3}H]carnitine, carrier-free [^{32}P]P_i, [1-^{14}C]acetyl-CoA, the ECL detection kit and the protein kinase C assay kit were from Amersham International (Amersham, Bucks, UK). Tetradecylglycidate was kindly donated by Dr. J.M. Lowenstein (Brandeis University, Waltham, MA). The anti-CPT-I antibody (raised against peptide residues 428-441 of rat liver CPT-I) was kindly given by Dr. Victor A. Zammit (Hannah Research Institute, Ayr, United Kingdom). Colchicine and cytochalasin B were kindly gifted by Dr. J.M. Andreu (CIB, Madrid, Spain). 3,3'-iminodipropionitrile (IDPN) was from Acros Chimica (Geel, Belgium). OA, KN-62, A23187 and classical protein kinase C were from Calbiochem (San Diego, CA). Ca^{2+}/CM-PKII was from Biomol (Plymouth Meeting, PA). cAMP-dependent protein kinase, trypsin and the monoclonal anti-cytokeratin antibody (clone K8.13) were from Sigma (St. Louis, MO).

Isolation and incubation of hepatocytes - Male Wistar rats (200-250 g) which had free access to food and water were used in all experiments. Hepatocytes were isolated by the collagenase perfusion method as described before (9). Hepatocytes were routinely incubated in Krebs-Henseleit bicarbonate buffer (pH 7.4) supplemented with 10 mM glucose and 1% (w/v) defatted and dialyzed bovine serum albumin. Incubations (4-6 mg of cellular protein/ml) were carried out at 37°C in a metabolic gyratory shaker (85 oscillations per min), under an atmosphere of O_2/CO_2 (19:1).

Assay of CPT-I activity in isolated mitochondria - Mitochondria were isolated either from hepatocytes and CPT-I activity was measured as the malonyl-CoA-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine exactly as described before (10). When CPT-I activity was determined in suspensions of mitochondria containing cytoskeletal fractions (Figs. 2 and 3), CPT activity was also determined in all the cytoskeletal fractions employed and then substracted from the CPT-I activity experimentally determined. Anyway, CPT activity determined in those cytoskeletal fractions was always marginal and on the basis of protein content never accounted for more than 5% of the CPT-I activity measured in mitochondrial suspensions. Preparations of mitochondria were practically devoid of peroxisomes, as judged from the low recovery of catalase activity (< 5%) in those preparations.

Assay of CPT-I activity in permeabilized hepatocytes - After incubation of the hepatocytes with the additions indicated in each case, CPT-I activity was determined in digitonin-permeabilized hepatocytes as the tetradecylglycidate-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine (9,10). In the "one-step assay" (Table II), cell permeabilization and assay of enzyme activity are simultaneously performed (9). In other experiments, however, CPT-I activity was determined by a more complex procedure (Fig. 1). In this "two-step assay", cells are permeabilized with digitonin, and then extensively washed with 40 volumes of a medium containing 10 mM Tris-HCl, pH 7.4, 150 mM KCl, 5 mM EDTA and 5 mM EGTA. The permeabilized-cell pellet was resuspended in that medium with the additions indicated and CPT-I activity was subsequently determined after preincubation at 37°C for 5 min. This was achieved exactly as described previously (10).

Since permeabilized hepatocytes also express CPT activity from peroxisomes and microsomes, the contribution of CPT-I to total hepatocellular tetradecylglycidate-sensitive CPT activity has been quantified. Thus, hepatocytes were incubated with 10 μM tetradecylglycidate for 30 min; purified mitochondria, peroxisomes and microsomes were isolated (9), and CPT activity was measured in these fractions. It turned out that at least 85% of total tetradecylglycidate-sensitive CPT activity experimentally determined corresponds to CPT-I, whereas microsomal CPT and peroxisomal CPT together make a minor contribution (< 15%) to the tetradecylglycidate-sensitive CPT pool under these conditions (ref. 9 and results not shown). Therefore, we believe that determination of CPT-I activity by this procedure is not prone to substantial error.

Western blot analysis of CPT-I - Mitochondrial fractions were subjected to SDS-PAGE using 1.0-mm thick 10% polyacrylamide gels. Stacking and resolving buffer pH values were adjusted to 6.8 and 8.8, respectively. Proteins were transferred from SDS gels onto nitrocellulose membranes. The blots were then blocked with 5% fat-free dried milk in PBS supplemented with 0.1% Tween 20. They were subsequently incubated with the anti-CPT-I antibody (1:5,000) in PBS/Tween 20 for 2 h at 4°C, and washed thorougly. The blots were then incubated with anti-sheep peroxidase-conjugated secondary antibody (1:10,000) for 1 h at room temperature, and finally subjected to luminography with an ECL detection kit.

Isolation of cytoskeletal fractions - Two cytoskeletal fractions were prepared according to van Bergen en Henegouwen et al. (14). Briefly, isolated hepatocytes were sedimented (2 min at 100 x g) and resuspended in a cytoskeleton stabilizing buffer (CSK buffer) consisting of 10 mM Pipes, pH 6.8, 0.25 M sucrose, 3 mM $MgCl_2$, 150 mM KCl and 1 mM EGTA. This buffer was supplemented with the following proteinase inhibitors: phenylmethanesulphonyl fluoride (75 μM), tosyl-L-phenylalanine chloromethyl ketone (2.5 μg/ml), tosyl-L-lysine chloromethyl ketone (2.5 μg/ml), tosyl-L-arginine methyl ester (2.5 μg/ml), pepstatin (0.8 μg/ml), trypsin inhibitor (7.5 μg/ml), leupeptin (0.8 μg/ml), benzamidine (115 μg/ml) and aprotinin (0.06 kallicrein inhibitory units/ml), together with 10 mM 2-mercaptoethanol. The fraction corresponding to total cytoskeleton (Fraction I) was obtained by incubation of hepatocytes in CSK buffer with 0.5% Triton X-100 at room temperature for 10 min and subsequent centrifugation at 20,000 x g for 1 min (14). The fraction enriched in intermediate filaments (Fraction II) was isolated by incubation of hepatocytes in CSK buffer with 0.5% Triton X-100 for 10 min at 4°C (which allows tubulin depolymerization) and in the presence of 0.6 M KI (which allows actin depolymerization). Collection of the intermediate filament-enriched fraction was performed as in (14). The two cytoskeletal fractions were finally resuspended in CSK buffer supplemented with the afore-mentioned proteinase inhibitors and characterized for their content in tubulin and cytokeratins exactly as in (14).

Immunoprecipitation of ^{32}P-labelled cytokeratins - After isolation, hepatocytes were washed twice in phosphate-free DMEM medium supplemented with 1% (w/v) defatted and dialyzed bovine serum albumin. Hepatocytes (6-8 mg of cellular protein in 1.5 ml of the aforementioned medium) were subsequently incubated in that medium at 37°C in a metabolic gyratory shaker (85 oscillations per min), under an atmosphere of O_2/CO_2 (19:1). Hepatocytes were labelled with 0.2 mCi of [^{32}P]P_i for 1 h and subsequently exposed to the additions indicated. One ml of cells was rapidly sedimented (5 s at 12,000 x g) and resuspended in 0.5 ml of 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 1% (v/v) Igepal, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS, supplemented with the afore-mentioned proteinase inhibitors. Samples were centrifuged (1 min at 12,000 x g) and 500 µl of the supernatant were treated with 1% (v/v) Triton X-100 for 1 min. After another centrifugation step, 200 µl of the resulting supernatant were taken for immunoprecipitation by incubating with a monoclonal anti-cytokeratin antibody bound to protein A-Sepharose in TTBS buffer (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl and 0.05% Tween 20). The latter was also supplemented with proteinase inhibitors. Immunoprecipitation of cytokeratins was performed as described before for acetyl-CoA carboxylase (15) and Ca^{2+}/CM-PKII (11). Samples were subjected to SDS-PAGE as described above. After fixing and drying of the gels, ^{32}P-labelled bands were visualized by autoradiography.

Incubation with protein kinases - Permeabilized hepatocytes or isolated mitochondria (1.5-2.0 mg protein/ml), supplemented or not with cytoskeletal fractions (as indicated in every case), were incubated at 30°C for 10 min in either of the following phosphorylation media, and aliquots of the incubations were subsequently taken to determine CPT-I activity as described above. (i) Ca^{2+}/CM-PKII phosphorylation medium contained 50 mM Hepes/KOH, pH 7.4, 100 mM KCl, 1 mM $CaCl_2$, 10 mM $MgCl_2$, 0.1 mM ATP, 30 ng/µl calmodulin, 50 nM OA and 0.6 ng/µl purified Ca^{2+}/CM-PKII, essentially as recomended by the supplier. (ii) cAMP-dependent protein kinase phosphorylation medium was exactly as described before (10). (iii) Protein kinase C phosphorylation medium contained 3 mU/µl purified protein kinase C and the assay components according to the supplier.

Determination of malonyl-CoA concentration - Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid extracts by a radioenzymatic method (15).

Statistical analysis - Results shown represent the means±S.D. of the number of hepatocyte preparations indicated in each case. Incubations of hepatocytes or mitochondria as well as enzyme assays were always carried out in triplicate. Statistical analysis was performed by Student's *t* test.

RESULTS

Effect of mild trypsin digestion on CPT-I activity - In a first set of experiments aimed at determining whether cytoskeletal components may be involved in the control of CPT-I activity (12), permeabilized hepatocytes were treated with trypsin in very mild conditions (low doses, 4°C, 2 min) and CPT-I activity was subsequently determined. As shown in Fig. 1, when hepatocytes were incubated in the presence of no additions and further permeabilized with digitonin, trypsin was able to stimulate CPT-I by ca. 50% in these cell ghosts. Preincubation of hepatocytes with OA led to a similar activation of CPT-I in the permeabilized-cell system (Fig. 1). However, trypsin was unable to produce any further stimulation of CPT-I in ghosts prepared from OA-pretreated hepatocytes (Fig. 1). The cytoskeletal stabilizer taxol has been shown to prevent the changes in hepatic CPT-I activity induced by a number of cellular effectors including OA (12). Likewise, when hepatocytes were pretreated with OA in combination with taxol, the stimulatory effect of trypsin was evident (Table I).

TABLE I

CPT-I activity in permeabilized hepatocytes and isolated mitochondria after mild trypsin treatment

Hepatocytes were preincubated for 45 min in the absence or in the presence of 10 µM taxol. Incubations were continued for 15 additional min with or without 0.5 µM OA. Cells were permeabilized with digitonin, and ghosts (1.5-2.0 mg protein/ml) were subsequently treated with or without 12.5 µg trypsin/ml for 2 min at 4°C. After trypsin removal as in Fig. 1, CPT-I activity was determined in permeabilized cells or in mitochondria isolated from those permeabilized hepatocytes. Results correspond to 4 different experiments.

		CPT-I activity (nmol/min x mg protein)	
Hepatocyte preincubation	Trypsin	Permeabilized hepatocytes	Isolated mitochondria
No additions	No	2.04±0.21	7.38±0.93
	Yes	3.01±0.13*	7.81±0.67
Taxol	No	1.98±0.34	8.05±1.33
	Yes	2.87±0.16*	7.94±0.41
OA	No	3.00±0.47*	8.02±0.72
	Yes	3.12±0.58*	7.32±0.50
Taxol + OA	No	2.11±0.30	7.57±0.88
	Yes	2.98±0.49*	8.07±1.05

*Significantly different ($P<0.01$) from incubations with no additions.

To test whether trypsin may cleave CPT-I itself under these digestion conditions, mitochondria were isolated from control and trypsin-treated permeabilized hepatocytes and CPT-I was subsequently detected by Western blotting (Fig. 1). As expected (5), a unique band of Mr=88 KDa was detected in the blots. In addition, no differences were observed between the two preparations of mitochondria (Fig. 1). Furthermore, CPT-I activity was determined in mitochondria isolated from permeabilized hepatocytes that had been treated with or without trypsin. As shown in Table I, no differences in CPT-I activity were evident among the different conditions.

It is worth noting that treatment of permeabilized hepatocytes with trypsin in the conditions employed herein had no effect on the recovery of total permeabilized-cell or total mitochondrial protein. Thus, when permeabilized hepatocytes at 1.6±0.2 mg protein (n=4) were treated or not with 17.5 µg trypsin for 2 min at 4°C and ghosts were collected after stopping trypsin action as described in legend to Fig. 1, 1.5±0.1 and 1.5±0.2 mg ghost protein were respectively recovered. Likewise, when mitochondria were isolated from those ghosts, 0.18±0.04 and 0.19±0.06 mg protein were recovered in mitochondria prepared from trypsin-treated and trypsin-untreated ghosts, respectively.

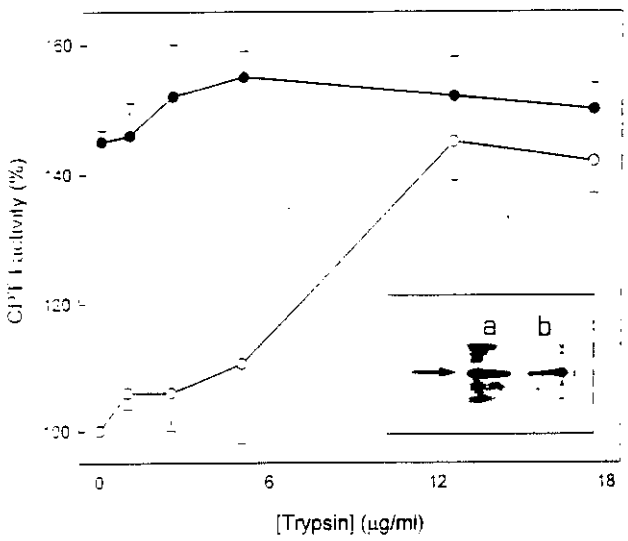


FIG. 1. **Effect of mild trypsin digestion on CPT-I activity in permeabilized hepatocytes.** Hepatocytes were preincubated for 15 min in the absence (o) or in the presence (●) of 0.5 µM OA. Cells were then permeabilized with digitonin and thorougly washed with 40 volumes of digitonin-free medium as described in Experimental Procedures. Permeabilized hepatocytes were subsequently resuspended at 1.5-2.0 mg protein/ml and treated with varying concentrations of trypsin at 4°C. Trypsin action was stopped after 2 min by addition of 10 mg/ml of bovine serum albumin and immediate washing with 40 volumes of trypsin-free medium. Then, CPT-I activity was determined in those permeabilized hepatocytes. One-hundred percent CPT-I activity was 1.87±0.20 nmol/min x mg protein. Results correspond to 4 different cell preparations. Inset: Mitochondria were isolated from permeabilized hepatocytes that had been treated without (lane a) or with (lane b) 17.5 µg/ml trypsin, and CPT-I was detected by Western blotting. The arrow points to the 88-KDa band.

Effect of disruptors of the cytoskeleton on CPT-I activity - OA and other phosphatase inhibitors produce hyperphosphorylation and consequently disruption of the cytoskeletal network in several cell types, including hepatocytes (e.g. 16,17). To test whether changes in the organization of the cytoskeleton may be related to parallel changes in CPT-I activity, hepatocytes were incubated with colchicine (a microtubule disruptor), cytochalasin B (a microfilament disruptor), and IDPN (an intermediate-filament disruptor) (18,19). As shown in Table II, neither colchicine nor cytochalasin B affected CPT-I activity. As a control to prove the biological activity of these two compounds on hepatocytes, cellular lipids were pre-labelled with [^{14}C]palmitate, and very-low-density-lipoprotein output into the medium was monitored (20). As previously described (20), disruption of microtubules with colchicine or disruption of microfilaments with cytochalasin B led to a strong inhibition (>90%) of the output of very-low-density-lipoprotein lipids into the medium and to a parallel accumulation of intracellular lipids.

TABLE II

Effect of disruptors of the cytoskeleton on CPT-I activity and malonyl-CoA levels

Hepatocytes were preincubated for 45 min in the absence or in the presence of the modulators of cytoskeletal integrity indicated. Incubations were continued for 15 additional min with or without 0.5 µM OA, and then aliquots were taken to determine CPT-I by the "one-step assay" as well as malonyl-CoA levels. One-hundred percent values of CPT-I activity and malonyl-CoA concentration were 1.29±0.22 nmol/min x mg protein and 73±12 pmol/mg protein, respectively. Results correspond to the number of experiments indicated in parentheses for CPT-I activity and to 3 different experiments for malonyl-CoA concentration.

Hepatocyte preincubation	OA	CPT-I activity (%)	[Malonyl-CoA] (%)
No additions	No	100±17	100±16
	Yes	154±15* (8)	7±5*
Colchicine (50 µM)	No	98±12 (4)	96±14
	Yes	155±11* (4)	5±6*
Cytochalasin B (10 µM)	No	96±11 (4)	104±21
	Yes	151±18* (4)	7±3*
IDPN (5 mM)	No	143±11* (6)	103±15
	Yes	166±15* (6)	6±6*
Taxol (10 µM)	No	102±9 (4)	101±12
	Yes	104±8 (4)	13±8*
Taxol (10 µM) + IDPN (5 mM)	No	96±6 (6)	101±9
	Yes	99±5 (6)	7±4*

*Significantly different (P<0.01) from incubations with no additions.

In contrast to colchicine and cytochalasin B, IDPN produced a significant increase in CPT-I activity (Table II). Interestingly, the effects of IDPN and OA were basically non-additive (Table II). Furthermore, stabilization of the cytoskeleton with taxol prevented the stimulation of CPT-I induced by IDPN and OA, either alone or in combination (Table II). It should be pointed out that neither taxol nor IDPN changed by themselves malonyl-CoA concentration in hepatocytes (Table II). In addition, neither of these two compunds affected the OA-induced decrease of intracellular malonyl-CoA levels (Table II).

Effect of cytoskeletal fractions on CPT-I activity - To further support the notion that cytoskeletal components may inhibit CPT-I, two cytoskeletal fractions were isolated (14) to study their possible inhibitory effect on CPT-I. Mitochondria were then incubated with the cytoskeletal fractions and CPT-I activity was determined. As shown in Fig. 2, the two cytoskeletal fractions produced a dose-dependent inhibition of CPT-I activity. In agreement with the effect of IDPN described above, the fraction that was more enriched in intermediate-filament components (Fraction II) produced a more potent inhibition of CPT-I (Fig. 2). Fifty percent inhibition of CPT-I by the two fractions occurred at total cytoskeletal protein:total mitochondrial protein ratios of 0.104±0.25 and 0.032±0.008 for Fraction I and Fraction II, respectively.

Effect of purified protein kinases on CPT-I activity - On the basis of the antagonistic effect exerted by KN-62, an inhibitor of Ca^{2+}/CM-PKII (21), the OA-dependent stimulation of CPT-I has been suggested to rely on the phosphorylation and subsequent activation of Ca^{2+}/CM-PKII (11). Hence, purified autophosphorylated Ca^{2+}/CM-PKII was directly added to isolated mitochondria or

permeabilized hepatocytes and CPT-I activity was determined. Ca^{2+}/CM-PKII was able to significantly ($P<0.01$) stimulate CPT-I in permeabilized cells (140±8% stimulation, n=4) but not in isolated mitochondria (6±7% stimulation, n=4). In contrast, addition of purified cAMP-dependent protein kinase or protein kinase C to permeabilized hepatocytes did not produce any change in CPT-I activity in either isolated mitochondria or permeabilized hepatocytes (data not shown).

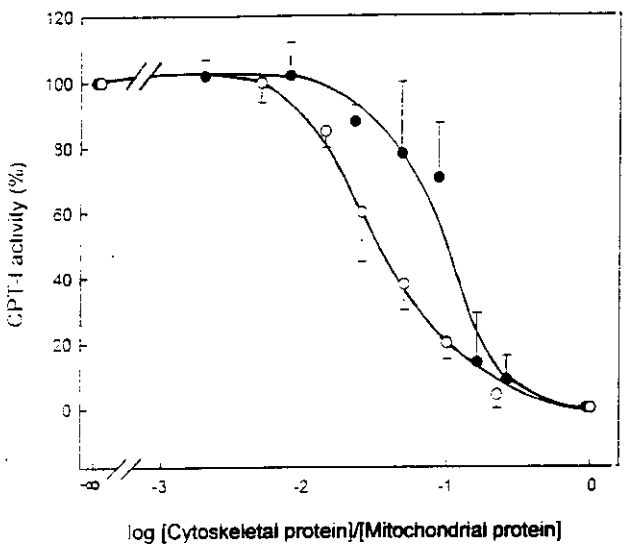


FIG. 2. **Effect of cytoskeletal fractions on CPT-I activity.** Hepatic mitochondria (1.5-2.0 mg protein/ml) were incubated for 30 min with varying amounts of cytoskeletal fractions (●, Fraction I; o, Fraction II) and CPT-I activity was subsequently determined as indicated in Experimental Procedures. One-hundred percent CPT-I activity was 8.65±1.11 nmol/min x mg protein. Results correspond to 4 different experiments.

In order to define the cell components that are sufficient for the malonyl-CoA-independent control of CPT-I to be demonstrated, we next attempted to reconstitute the whole-cell experimental system in a simple manner by incubating isolated mitochondria together with cytoskeletal Fraction II and purified Ca^{2+}/CM-PKII. As shown in Fig. 3, the inhibition of CPT-I produced by exposure of isolated mitochondria to cytoskeletal Fraction II was reverted by addition of exogenous Ca^{2+}/CM-PKII.

Phosphorylation of cytokeratins in intact hepatocytes - To obtain further evidence for the possible connection between Ca^{2+}/CM-PKII and intermediate filaments, experiments of hepatic cytokeratin phosphorylation were performed. The phosphorylation pattern of purified cytokeratins *in vitro* may not reflect their phosphorylation status in more physiological, intact-cell systems (22-24). Therefore, intact hepatocytes were labelled with $^{32}P_i$ and cytokeratins were immunoprecipitated. As shown in Fig. 4, two major cytokeratin bands were phosphorylated upon hepatocyte challenge to OA. These two bands were assigned to cyto-

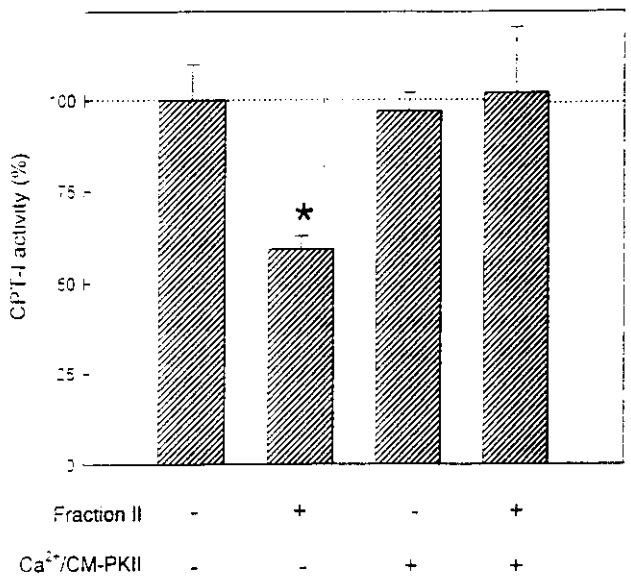


FIG. 3. **Effect of Ca^{2+}/CM-PKII on CPT-I activity.** Hepatic mitochondria (1.5-2.0 mg protein/ml) were preincubated for 30 min in the absence (-) or in the presence (+) of cytoskeletal Fraction II (0.05-0.06 mg protein/ml). Purified Ca^{2+}/CM-PKII was subsequently added (+) or not (-) to the incubations, which were run for 10 additional min. Aliquots were subsequently taken to determine CPT-I activity. Results correspond to 3 different experiments. *Significantly different ($P<0.01$) from incubations with no additions.

keratins 8 and 18 on the basis of their Mr (54 and 45 KDa, respectively) and high abundance in rat liver (e.g. 22-24). Moreover, the OA-induced phosphorylation of these two bands was prevented by KN-62, the Ca^{2+}/CM-PKII inhibitor that antagonizes the OA-induced stimulation of CPT-I (11). Nevertheless, the Ca^{2+} ionophore A23187 had no effect on cytokeratin phosphorylation in intact hepatocytes (Fig. 4, lanes e and f).

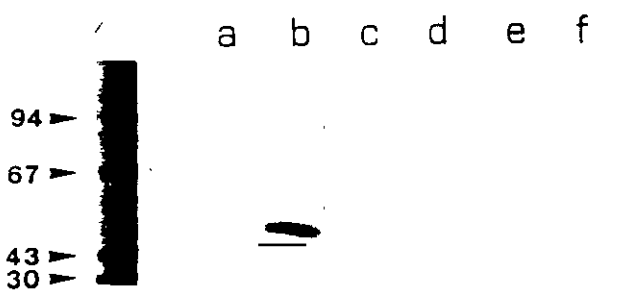


FIG. 4. **Phosphorylation of cytokeratins in intact hepatocytes.** Hepatocytes were loaded with $^{32}P_i$ as indicated in Experimental Procedures and further incubated for 15 min in the absence or in the presence of 30 μM KN-62. Incubations were continued for an additional 15-min period with or without 0.5 μM OA or 10 μM A23187. Cells were subsequently disrupted and cytokeratins were immunoprecipitated with a monoclonal anti-cytokeratin antibody. Immunoprecipitates were subjected to SDS-PAGE and autoradiography. Lane a: no additions; lane b: OA; lane c: KN-62; lane d: KN-62 plus OA; lane e: A23187; lane f: KN-62 plus A23187. Molecular-mass markers (in KDa) are shown on the left-hand side of the autoradiogram. The experiment was repeated three times and similar results were obtained.

DISCUSSION

The present work was undertaken to study the mechanism by which Ca^{2+}/CM-PKII (11) together with cytoskeletal structures (12) may be involved in the acute control of hepatic CPT-I, the enzyme that catalyzes the pace-setting step of long-chain fatty acid oxidation (1-5). Data presented in this report support the notion that hepatic CPT-I activity may be controlled in the short term not only by intracellular malonyl-CoA levels (5,8) but also by a malonyl-CoA-independent mechanism that may involve modulation of the interactions between CPT-I and cytoskeletal components. As will be discussed below, this novel mechanism of control of hepatic CPT-I activity may plausibly rely on the cascade Ca^{2+}/CM-PKII activation -> Cytokeratin phosphorylation -> CPT-I de-inhibition.

Involvement of cytoskeletal components in the control of CPT-I activity - A number of reports have recently described the existence of specific interactions between the mitochondrial outer membrane and cytoskeletal elements (25,26). In the context of CPT-I regulation, OA activates hepatic CPT-I (9) and disrupts the cytoskeleton of hepatocytes by causing the hyperphosphorylation of cytoskeletal proteins (16). Four observations in the present report provide additional evidence for the involvement of cytoskeletal components (most likely cytokeratin intermediate filaments) in the control of hepatic CPT-I activity. First, experiments of mild trypsin digestion suggest that CPT-I may become activated by cleavage of extra-mitochondrial cell component(s). In line with this observation, Fontaine et al. have recently reported that porin, the mitochondrial-outer-membrane pore-forming protein, also becomes activated in permeabilized hepatocytes upon mild trypsin digestion of extra-mitochondrial cell components (27). It is worth noting that the digestion conditions employed in the present paper were extremely milder than those previously used by Kashfi and Cook to study the effect of proteolysis on CPT-I (e.g. 28), and therefore the two types of experiments are not comparable. In line with our observations, Fraser et al. (29) did not observe any effect of trypsin on CPT-I under digestion conditions more or less comparable to ours. Interestingly, cell pretreatment with OA rendered CPT-I reluctant to activation by trypsin, suggesting that both OA and trypsin may share a common mechanism to relieve CPT-I from inhibition. Second, incubation of intact hepatocytes with IDPN increased CPT-I activity in a basically non-additive manner with respect to OA, suggesting a common mechanism of action. Third, CPT-I activity in isolated mitochondria was depressed in a dose-dependent fashion by the addition of a total-cytoskeleton fraction and a cytokeratin-enriched cytoskeletal fraction, the latter being 3 times more potent than the former. Fourth, taxol prevented the OA-induced desensitization of CPT-I to trypsin activation, as well as the OA- and IDPN-induced stimulation of CPT-I. In short, all these data suggest that disruption of interactions between CPT-I and cytoskeletal component(s) may relieve CPT-I from inhibition and therefore increase enzyme activity.

The possibility that CPT-I interacts with cytoskeletal components as put forward in this paper is in line with the current notion that the dynamics and intracellular distribution of mitochondria in living cells may result from specific interactions of mitochondria with components of the cytoskeleton (25,26). In the case of rat brain mitochondria, accruing evidence indicates that specific interactions occur between mitochondrial-outer-membrane proteins and cytoskeletal proteins, a well described example being the interaction between porin, microtubule-associated protein 2, and the neurofilamental proteins NF-H and NF-M (26,30). The existence of direct contact sites between intermediate filaments and the mitochondrial outer membrane has been reported not only in neurons, but also in smooth muscle myocytes (31) and adrenal cortex cells (32). As far as we know, although rat liver mitochondria have been shown to interact with microtubules (33), direct evidence for their interaction with intermediate filaments is still lacking.

It has been suggested that a function of the interactions between mitochondria and intermediate filaments may be to locate mitochondria in precise sites within the cell (25,26,34). This idea is based on experiments showing a parallel redistribution of mitochondria and intermediate filaments upon cell exposure to agents that disrupt intermediate filaments (34) or in certain stress situations (35). The mitochondrial alterations observed in desmin-null mice also support this hypothesis (36,37). Since the organization of intermediate filaments changes dramatically in a number of liver pathologies (38), the observations described in the present paper predict that CPT-I activity as affected by cytoskeletal components may change under patho-physiological situations in which the organization of the cytoskeleton is altered, e.g. in transformed cells. Thus, we have recently observed (authors' unpublished results) that CPT-I specific activity is similar in mitochondria isolated from hepatoma cells and normal hepatocytes, but just about half in permeabilized hepatocytes than in permeabilized hepatoma cells; in addition, CPT-I becomes reluctant to stimulation by OA in hepatoma cells. These observations support the notion that in hepatocytes OA releases CPT-I from certain constrictions imposed by extra-mitochondrial cell components that do not operate either in isolated mitochondria or in transformed liver cells.

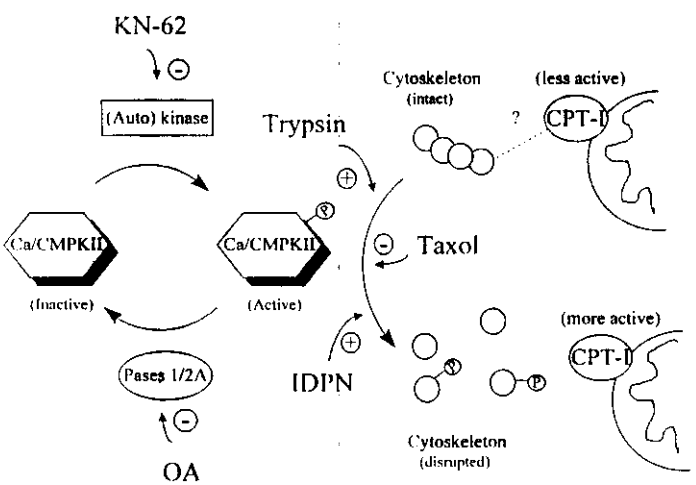
Involvement of Ca^{2+}/CM-PKII in the control of CPT-I activity - Previous experiments in our laboratories have shown that KN-62, an inhibitor of Ca^{2+}/CM-PKII (21), antagonizes the OA-induced stimulation of hepatic CPT-I activity (11). In contrast, neither H-7 -an inhibitor of cAMP-dependent protein kinase and protein kinase C- nor GF109203X -a protein kinase C inhibitor- were able to prevent the OA-induced stimulation of CPT-I (11). Likewise, inhibitors of the mitogen-activated protein kinase cascade such as wortmannin, apigenin and PD98059 are unable to prevent the OA-induced stimulation of CPT-I

(authors' unpublished results). Ca^{2+}/CM-PKII becomes constitutively activated when autophosphorylated in key serine or threonine residues on the autonomy site of the enzyme (13). Autophosphorylation is sufficient to disrupt the autoinhibitory domain of Ca^{2+}/CM-PKII, leading to a permanent de-inhibition of the kinase (13). It is thus conceivable that permanent activation of Ca^{2+}/CM-PKII ensues upon inhibition by OA of the phosphatases involved in the dephosphorylation (=deactivation) of Ca^{2+}/CM-PKII. Ca^{2+}/CM-PKII autophosphorylation has indeed been demonstrated in hepatocytes upon challenge to OA (13).

The present data point to a link between Ca^{2+}/CM-PKII and the cytoskeleton in the context of CPT-I regulation. This conclusion is based mostly on three observations. First, purified Ca^{2+}/CM-PKII was able to activate CPT-I in permeabilized cells but not in isolated mitochondria. This is in agreement with previous evidence against the involvement of direct phosphorylation in the OA-induced stimulation of CPT-I (10) and indicates that extra-mitochondrial cell components are required for the regulation of CPT-I activity by Ca^{2+}/CM-PKII. In this respect, it is worth noting that permeabilization of hepatocytes with digitonin seems to preserve quite well both the general morphology of the cell and the structure of the cytoskeleton (39), and therefore the potential interactions between the cytoskeleton and cell organelles may remain basically unaffected upon this type of manipulation. Second, when isolated mitochondria were incubated with a cytokeratin-enriched cytoskeletal fraction, purified Ca^{2+}/CM-PKII was able to abrogate the inhibition of CPT-I induced by that cytokeratin fraction. It is clear from these experiments that a simple reconstituted system composed by isolated mitochondria, a cytokeratin-enriched fraction and purified Ca^{2+}/CM-PKII may reflect the situation occurring in the intact hepatocyte, indicating that these three components are sufficient for the malonyl-CoA-independent acute control of CPT-I to be demonstrated *in vitro*. Third, the Ca^{2+}/CM-PKII inhibitor KN-62 prevented the OA-induced phosphorylation of cytokeratins in hepatocytes, pointing to a role of Ca^{2+}/CM-PKII on cytokeratin phosphorylation in intact hepatocytes (23,24). Phosphorylation of cytokeratin intermediate filaments by Ca^{2+}/CM-PKII in whole cells leads to the disruption of these structures (23,24), and therefore this observation fits with the OA- and IDPN-induced stimulation of CPT-I.

Additional evidence for the involvement of cytokeratins in the control of CPT-I activity is given by the lack of effect of A23187 on cytokeratin phosphorylation in hepatocytes. In this context, challenge of hepatocytes to OA leads to CPT-I activation and cytokeratin phosphorylation, whereas elevation of cytosolic free Ca^{2+} concentration by A23187 has no effect on either CPT-I activity (40) or cytokeratin phosphorylation (the present paper). The possibility that liver Ca^{2+}/CM-PKII may have a different pattern of activation by Ca^{2+}/calmodulin and by autophosphorylation than brain Ca^{2+}/CM-PKII (cf. 11) is as yet an open question.

It is worth noting that neither cAMP-protein protein kinase nor protein kinase C affected CPT-I activity in permeabilized hepatocytes in spite of their ability to phosphorylate cytokeratins *in vitro* (23,41,42). However, several lines of evidence indicate that neither of these two protein kinases play an important role in the direct control of intermediate filament integrity in intact hepatocytes. Thus, it has been shown that protein kinase C may be responsible for maintaining basal levels of phosphorylation on cytokeratins 8 and 18 without altering cytokeratin filament assembly (41). As a matter of fact, exposure of intact hepatocytes to phorbol esters induces cytokeratin phosphorylation but does not alter organization of intermediate filaments filament, which appear as fully assembled networks (42). In addition, phosphopeptide maps of hepatic cytokeratins phosphorylated *in vivo* and *in vitro* clearly indicate that cAMP-dependent protein kinase is not much involved in cytokeratin phosphorylation in intact cells (23). In contrast, and in line with data in the present paper, Ca^{2+}/CM-PKII has been shown to play a major role in the phosphorylation and functional integrity of hepatic cytokeratins *in vivo* (23). It has been also suggested that activation of Ca^{2+}/CM-PKII (and not of other protein kinases) is responsible for the OA-induced disruption of hepatocyte cytoskeleton (16). All this points to a functional involvement of this protein kinase in the control of the integrity of hepatic cytoskeleton.



SCHEME I. **Proposed model for the malonyl-CoA-independent acute control of hepatic CPT-I activity.** See the text for abbreviations and further details.

Malonyl-CoA-dependent and malonyl-CoA-independent control of CPT-I activity - Together with previous observations (10-12), data in this paper allow a model to explain the OA-induced malonyl-CoA-independent control of hepatic CPT-I. As shown in Scheme I, OA may activate Ca^{2+}/CM-PKII by increasing its degree of phosphorylation upon inhibition of protein phosphatases 1 and 2A; this effect would be prevented by KN-62, an inhibitor of

Ca^{2+}/CM-PKII autophosphorylation. Activated Ca^{2+}/CM-PKII would phosphorylate cytoskeletal components, perhaps cytokeratins 8 and 18, thereby disrupting putative inhibitory interactions between the cytoskeleton and CPT-I. Stimulation of CPT-I upon disruption of the cytoskeleton would be also achieved by challenge of intact hepatocytes to IDPN or by treatment of permeabilized hepatocytes with trypsin in mild conditions. Stabilization of the cytoskeleton with taxol may prevent the malonyl-CoA-independent acute stimulation of CPT-I.

It is obvious that the notion that fatty acid translocation into mitochondria may be controlled by modulation of the interactions between CPT-I and cytoskeletal components (i.e. by a malonyl-CoA-independent mechanism) does not diminish the importance of malonyl-CoA as a physiological modulator of CPT-I activity (5,8). On one hand, since the pioneering work of McGarry and coworkers (8,43), changes in long-chain fatty acid oxidation under many different patho-physiological situations have been shown to be linked to changes in intracellular malonyl-CoA concentration and/or changes in the sensitivity of CPT-I to malonyl-CoA (1,2,5). On the other hand, several observations suggest that malonyl-CoA-dependent and malonyl-CoA-independent acute control of hepatic CPT-I activity might operate in concert. First, we have recently shown that stimulation of the AMP-activated protein kinase -a major protein kinase involved in the control of hepatic lipid metabolism- leads to an activation of hepatic CPT-I by malonyl-CoA-dependent and malonyl-CoA-independent mechanisms (44). Second, a fraction of hepatic acetyl-CoA carboxylase, the enzyme responsible for the synthesis of malonyl-CoA, has been recently suggested to be bound to the cytoskeleton (45). Third, it has been put forward that the 280-KDa isoform of acetyl-CoA carboxylase might interact with the outer leaflet of the mitochondrial outer membrane in order to channel malonyl-CoA for CPT-I inhibition (46). Fourth, the recent observation that the bulk of the CPT-I protein seems to face the cytoplasmic side of the mitochondrial outer membrane (29) makes more likely that interactions between CPT-I and cytoskeletal components might occur. In the context of the emerging role of cytoskeletal filamentous networks in intracellular signaling (47), current research in our laboratories is focussed on the possible existence of a coordinate control of CPT-I and acetyl-CoA carboxylase activities by modulation of interactions between the cytoskeleton and the mitochondrial outer membrane.

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DISCUSIÓN

Los resultados experimentales expuestos en el presente capítulo permiten identificar algunos de los elementos que parecen estar implicados en la regulación a corto plazo de la CPT-I:

i) Diferentes pruebas indican que el citoesqueleto desempeña un papel importante en la regulación de la CPT-I y que, podría constituir el elemento extramitocondrial necesario para que se verifique la regulación de la CPT-I que se pierde tras el aislamiento de mitocondrias. De entre los diferentes componentes del citoesqueleto, los filamentos intermedios (y sus principales proteínas constituyentes en hígado, las citoqueratinas) surgen como los principales candidatos a estar directamente implicados en la regulación de la CPT-I. A pesar de que resulta tentador especular con una interacción entre citoqueratinas y CPT-I (o alguna otra proteína mitocondrial que pudiera participar en la regulación a corto plazo de la enzima) lo cierto es que no existen pruebas directas, por lo que de momento dicha hipótesis permanece en el plano especulativo.

ii) La Ca^{2+}/CMPKII ha sido identificada como un factor soluble que se perdería tras la permeabilización de los hepatocitos y que sería una diana de la acción de las proteína fosfatasas 1 y 2A en la activación de la CPT-I. Así, la inhibición de estas proteína fosfatasas por ácido okadaico o por tautomicina, impediría que pueda llevarse a cabo la desfosforilación de la quinasa, que al aumentar su nivel de fosforilación pasa a encontrarse mayoritariamente en el estado autónomo. La quinasa activada fosforilaría diferentes sustratos en la célula y entre ellos diferentes citoqueratinas. Con ello podría estar induciendo la despolimerización del citoesqueleto (o al menos de los filamentos intermedios) y con ello un aumento en la actividad CPT-I.

A la vista de los datos expuestos en este capítulo es posible presentar un modelo de regulación de la CPT-I independiente de malonil-CoA que liga activación de Ca^{2+}/CMPKII, fosforilación y ruptura del citoesqueleto y activación de la CPT-I. Sin embargo, las modificaciones que el ácido okadaico induce en nuestro sistema experimental, probablemente difieran notablemente de las que ocurren habitualmente en la célula, ya que la alteración del equilibrio de fosforilación/desfosforilación que genera es probablemente de un orden mayor que el que resulta de la activación fisiológica de las diferentes quinasas. Por ello, a pesar de la utilidad que el ácido okadaico posee como instrumento para el estudio de la regulación a corto plazo de la CPT-I, es necesario verificar el posible papel fisiológico que este mecanismo pudiera tener en la regulación de la oxidación de ácidos grasos.

2.3. Posible papel fisiológico de la regulación a corto plazo (independiente de malonil-CoA) de la CPT-I

INTRODUCCIÓN

Una vez determinada la importancia que el citoesqueleto y la regulación de su estado de organización pueden tener en el control de la CPT-I, se hizo necesario buscar las implicaciones que este mecanismo pudiera tener en el contexto de la regulación fisiológica de la oxidación de ácidos grasos.

Por una parte, estudiamos la regulación de la actividad de la CPT-I en células de hepatoma (Fao y HepG2). Puesto que el ácido okadaico es un conocido agente carcinogénico, que produce entre otros efectos la desorganización del citoesqueleto, nos planteamos determinar qué tipo de regulación presenta la CPT-I en unas células, como las de hepatoma, en las que (dada su condición de células tumorales) el citoesqueleto también se encuentra desorganizado.

Por otra parte, teniendo en cuenta que la AMPK es quizá la proteína quinasa más importante en la regulación del metabolismo hepático de ácidos grasos (Hardie y Carling, 1997), nos planteamos estudiar si esta enzima podría estar implicada también en la regulción de la CPT-I no solo a través del control de la actividad ACC (y con ello de los niveles de malonil-CoA), sino también a través de un mecanismo independiente de malonil-CoA.

Por último, una serie de estudios acerca de la posible localización subcelular de la ACC indicaron que ambos mecanismos de regulación de la CPT-I (dependiente e independiente de malonil-CoA), podrían estar estrechamente relacionados.

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LOSS OF RESPONSE OF CARNITINE PALMITOYLTRANSFERASE I TO OKADAIC ACID IN TRANSFORMED HEPATIC CELLS

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ABSTRACT. The specific activity of carnitine palmitoyltransferase I (CPT-I) was similar in mitochondria isolated from rat Fao and human HepG2 hepatoma cells and from rat hepatocytes, but almost 2-fold higher in permeabilized hepatoma cells than in permeabilized hepatocytes. Short-term exposure to okadaic acid induced a ca. 80% stimulation of CPT-I in hepatocytes, whereas no significant response of the enzyme from hepatoma cells was evident. Thus, the high CPT-I activity displayed by hepatoma cells may be reached by hepatocytes upon challenge to okadaic acid. The present data may be explained by a disruption of interactions between CPT-I and cytoskeletal components in tumor cells which may be involved in the okadaic acid-induced activation of hepatic CPT-I as previously suggested [*Biochem Biophys Res Commun* **224:** 754-759, 1996].

Mitochondrial fatty acid oxidation provides a major source of energy in heart, skeletal muscle and liver (reviewed in [1-3]). Hepatic fatty acid oxidation also supplies extrahepatic tissues with ketone bodies as a glucose-replacing fuel [1-3]. Carnitine palmitoyltransferase I (CPT-I), the outer-mitochondrial-membrane carnitine palmitoyltransferase, catalyzes the pace-setting step of long-chain fatty acid translocation into

Abbreviations: CPT-I, carnitine palmitoyltransferase I; GDH, glutamate dehydrogenase

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the mitochondrial matrix [1-3]. Recent determination of flux control coefficients of the enzymes involved in hepatic long-chain fatty acid oxidation shows that CPT-I plays a pivotal role in controlling the flux through this pathway under different substrate concentrations and patho-physiological states [4,5]. It is well established that long-term changes in hepatic CPT-I activity occur in response to alterations in the nutritional and hormonal status of the animal [1-3]. In addition, CPT-I is subjected to allosteric inhibition by malonyl-CoA [1-3].

During the last years, a novel mechanism of control of hepatic CPT-I activity has been put forward. 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 (reviewed in [1]). Thus, cAMP analogues (e.g. dibutyryl-cAMP) [6], effectors which increase intracellular cAMP levels (e.g. glucagon, forskolin) [6] and protein phosphatase inhibitors (e.g. okadaic acid) [6,7] are able to stimulate hepatic CPT-I. This short-term activation of CPT-I is assumed to be mediated by a malonyl-CoA-independent mechanism [8] that may involve the phosphorylation of cytoskeletal component(s) and the subsequent disruption of interactions between CPT-I and the cytoskeleton [9,10]. However, the significance of this putative mechanism of control of CPT-I activity is as yet unknown. In the context of the aforementioned hypothesis, it is conceivable that the regulatory properties of CPT-I may change under patho-physiological situations in which the organization of the cytoskeleton is altered. Since it is well established that the cytoskeleton of transformed cells is disorganized, the present work was undertaken to study the regulation of CPT-I activity by okadaic acid in hepatoma cells compared to hepatocytes in primary cultures.

MATERIALS AND METHODS

Cell culture

The rat hepatoma cell line Fao and the human hepatoma cell line HepG2 were cultured as previously described [11]. They were transferred to their respective serum-free cultured media [11] supplemented with 1% (w/v) defatted and dialyzed bovine serum albumin 24 h prior to the experiments. Hepatocytes were isolated from male Wistar rats (250-300 g) which had free access to food and water by the collagenase perfusion method described in [7]. They were inoculated in DMEM containing 10% (v/v) fetal calf serum. After cell attachment (ca. 6 h), the medium was replaced with serum-free DMEM containing 10 nM dexamethasone and 1% (w/v) defatted and dialyzed bovine serum albumin, and the hepatocytes were cultured for 14-18 h before the experiments were performed.

CPT-I assay

CPT-I activity was determined in digitonin-permeabilized cells as the tetradecylglycidate-sensitive incorporation of radiolabelled L-carnitine into palmitoylcarnitine. Briefly, attached cells (plated in P6 plates) or cells in suspension (scraped from F75 flasks), as indicated in every case, were preincubated for 45 min in the absence or in the presence of 20 μM tetradecylglycidate (kindly donated by Dr. J.M.

Lowenstein, Brandeis University, Waltham, MA, USA), a specific irreversible inhibitor of CPT-I (cf. [7]). Incubations were continued for an additional 45-min period in the presence or the absence of varying concentrations of okadaic acid. Subsequently, carnitine palmitoyltransferase activity was monitored in hepatocyte monolayers [5] or suspensions [7].

For the determination of CPT-I activity in isolated mitochondria, the culture medium from 10-15 F75 flasks was aspirated, cells were washed in NaCl/P_i, scraped from the flasks, and homogenized in a medium containing 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose and 1 mM EDTA. The resulting crude homogenates were directly used for isolation of mitochondria and determination of CPT-I and glutamate dehydrogenase (GDH) activities exactly as described in [8].

Statistical analysis

Results shown represent the means ± S.D. of the number of experiments indicated in each case. Each experimental condition was always carried out at least in quadruplicate. Statistical analysis was performed by the Student's *t* test.

RESULTS AND DISCUSSION

The properties of CPT-I were studied in two hepatoma cell lines, namely human HepG2 cells and rat Fao cells, which are commonly used as a model to study liver lipid metabolism (cf. [12-14]). As shown in Table 1, CPT-I specific activity was not significantly different in mitochondria isolated from hepatoma cells than in mitochondria isolated from hepatocytes in primary culture. Similar qualitative results were obtained when values of enzyme activity were referred to mass of mitochondrial protein and to activity units of GDH, a mitochondrial marker (Table 1). This indicates that no differences in the mass of mitochondria are evident among the three types of cells.

CPT-I activity was subsequently monitored in a permeabilized-cell system. The use of this assay allows measurement of hepatocellular CPT-I activity in its physiological environment [7]. As shown in Fig. 1, CPT-I activity in primary hepatocytes was about half of that in hepatoma cells. This clearly indicates that CPT-I activity inside the hepatocyte is subjected to certain constrictions that do not operate in hepatoma cells or in preparations of purified hepatic mitochondria. This is in line with the recent idea that interactions between the mitochondrial outer membrane and extra-mitochondrial cell components, most likely localized in the cytoskeleton, might be involved in the control of hepatic CPT-I activity [8,9]. These interactions could be readily lost in preparations of purified mitochondria.

To test this hypothesis, the effect of the phosphatase inhibitor okadaic acid on CPT-I activity was determined in the three cells tested. One of the most remarkable effects elicited by okadaic acid in hepatocytes and other cell types is the hyperphosphorylation and subsequent disruption of the cytoskeleton (e.g. [15,16]). Fig. 1 shows that a remarkable 80% stimulation of CPT-I ensued upon exposure of primary hepatocytes to okadaic acid. Fifty percent activation of CPT-I occurred at ca. 10 nM okadaic acid,

TABLE 1. CPT-I activity in mitochondria isolated from hepatoma cells and primary hepatocytes

Cells	CPT-I activity (nmol/min per mg protein)	CPT-I activity (nmol/min per unit GDH activity)
Fao	1.87±0.25	2.56±0.79
HepG2	2.19±0.34	1.87±0.41
Hepatocytes	1.81±0.22	2.34±0.68

Values correspond to 3 separate experiments. See the Materials and Methods for further details.

indicating that CPT-I stimulation is mediated by the inhibition of protein phosphatase 1 [9,16]. This is in agreement with the observation that protein phosphatase 1 seems to be the main phosphatase involved in the regulation of the phosphorylation state of the cytoskeleton, and in turn in the control of cytoskeletal integrity [16]. In contrast to hepatocytes, a slight but not statistically significant stimulation of CPT-I to okadaic acid was evident in hepatoma cells (Fig. 1). Therefore, the high CPT-I activity displayed by hepatoma cells may be reached by hepatocytes upon challenge to okadaic acid.

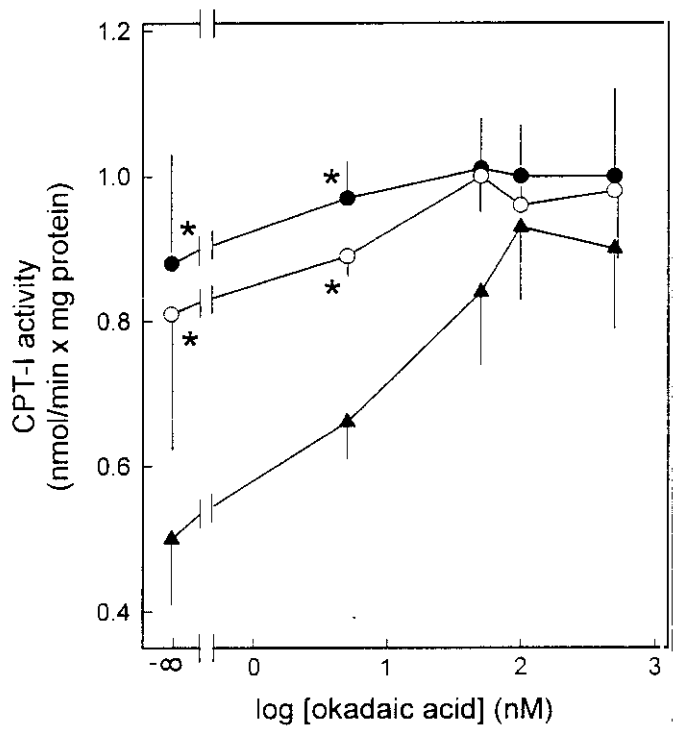

FIG. 1. Effect of okadaic acid on CPT-I activity in attached hepatoma cells and primary hepatocytes. Fao cells (●), HepG2 cells (o) and primary hepatocytes (▲) were incubated with varying concentrations of okadaic acid (0, 5, 50, 100 and 500 nM) for 45 min and CPT-I activity was subsequently determined with the permeabilized-cell assay. See the Materials and Methods for further details. Values correspond to 4 separate experiments. *Significantly different ($P < 0.01$) from primary hepatocytes.

TABLE 2. Comparative effect of okadaic acid on CPT-I activity in hepatoma cells and hepatocytes, both in suspension and in attached state

Cells	Okadaic acid (500 nM)	CPT-I relative activity (%)	
		Attached cells	Cells in suspension
Fao	No	100±17	100±22
	Yes	112±11 (5)	119±10 (3)
HepG2	No	100±23	n.d.
	Yes	120±7 (4)	n.d.
Hepatocytes	No	100±18	100±12
	Yes	180±12* (5)	171±18* (4)

Values correspond to the number of experiments indicated in parentheses. See the Materials and Methods for further details. n.d.: not determined. *Significantly different ($P<0.01$) from the corresponding values with no okadaic acid.

It might be argued that the distinct behaviour of primary hepatocytes and hepatoma cells may be a reflection of differences in their attachment to the substrate, that may in turn involve different configurations of the cytoskeleton. Hence, CPT-I activity was monitored in cell suspensions after cell scraping from the flasks. As shown in Table 2, okadaic acid produced a significant stimulation of CPT-I in hepatocyte suspensions but not in Fao-cell suspensions.

In conclusion, the present data support the notion that disruption of interactions between mitochondria and inhibitory cytoskeletal components may be involved in the okadaic acid-induced activation of hepatic CPT-I [9]. We have recently observed (authors' unpublished data) that intermediate filaments are the components of the cytoskeleton most likely involved in the control of CPT-I activity. Interestingly, the interactions between intermediate filaments and mitochondria become disrupted in a stress situation such as heat shock [17]. Our results indicate that this might be the reason for the enhanced CPT-I activity in hepatoma cells.

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Control of Hepatic Fatty Acid Oxidation by 5′-AMP-Activated Protein Kinase Involves a Malonyl-CoA-Dependent and a Malonyl-CoA-Independent Mechanism

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Incubation of rat hepatocytes with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), an activator of the 5′-AMP-activated protein kinase (AMPK), produced a twofold stimulation of palmitate oxidation and of the activity of carnitine palmitoyltransferase I (CPT-I), together with a profound decrease of the activity of acetyl-CoA carboxylase and of the intracellular level of malonyl-CoA. AICAR-induced CPT-I stimulation progressively blunted with time after cell permeabilization, pointing to reversal of conformational constraints of the enzyme in control cells due to the permeabilization-triggered dilution of intracellular malonyl-CoA. The stimulation stabilized at a steady 20–25%. This 20–25% increase in CPT-I activity survived upon complete removal of malonyl-CoA from the permeabilized cells, indicating that it was not dependent on the malonyl-CoA concentration of the cell. This malonyl-CoA-independent activation of CPT-I was not evident when mitochondria were isolated for assay of enzyme activity or when cells were disrupted by vigorous sonication. In addition, the microtubule stabilizer taxol prevented the malonyl-CoA-independent stimulation of CPT-I induced by AICAR. Hence, stimulation of hepatic fatty acid oxidation by AMPK seems to rely on the activation of CPT-I by two different mechanisms: deinhibition of CPT-I induced by depletion of intracellular malonyl-CoA levels and malonyl-CoA-independent stimulation of CPT-I, which might involve modulation of interactions between CPT-I and cytoskeletal components. © 1997 Academic Press

The 5′-AMP-activated protein kinase (AMPK)[2] plays a major role in the regulation of lipid metabolism in mammals. Thus, AMPK phosphorylates and inactivates key regulatory enzymes of lipid metabolism such as acetyl-CoA carboxylase (fatty acid synthesis), 3-hydroxy-3-methylglutaryl-CoA reductase (sterol/isoprenoid synthesis), and hormone-sensitive lipase (triacylglycerol/cholesteryl ester breakdown) [reviewed in Ref. (1)]. Although several protein kinases can phosphorylate purified acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA reductase *in vitro*, it is currently accepted that in intact hepatocytes and in the liver *in vivo* this phosphorylation is mainly performed by AMPK [cf. Refs. (1–3)].

Several studies have been performed on the potential involvement of AMPK in the control of fatty acid oxidation in the ischemic heart (4, 5) and the working muscle (6). However, the possible role of this kinase in the control of hepatic fatty acid oxidation has not been studied to date. Unlike the heart and the skeletal muscle, the liver is capable of expressing either high rates of lipogenesis or high rates of fatty acid oxidation depending on the hormonal and nutritional status of the animal, and hence regulation of fatty acid oxidation seems to be more complex in liver [reviewed in Ref. (7)] than in heart [reviewed in Ref. (8)] and skeletal muscle [reviewed in Ref. (9)]. Carnitine palmitoyltransferase I (CPT-I) is the key regulatory enzyme in the transport of long-chain fatty acids into the mitochondrial matrix (7, 10, 11). This enzyme is subject to allosteric inhibition by malonyl-CoA, the product of the reaction cata-

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[2] Abbreviations used: AMPK, 5′-AMP-activated protein kinase; CoA, coenzyme A; CPT-I, carnitine palmitoyltransferase I; AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; ZMP, 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate.

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lyzed by acetyl-CoA carboxylase (7, 10, 11). During the past few years, a novel mechanism of short-term control of hepatic CPT-I has been put forward [reviewed in Ref. (7)]. 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. Thus, cAMP analogs (e.g., dibutyryl-cAMP) (12), effectors that increase intracellular cAMP levels (e.g., glucagon and forskolin) (12), and protein phosphatase inhibitors (e.g., okadaic acid) (13) are able to stimulate hepatic CPT-I, whereas Ca^{2+}-mobilizing agents (e.g., vasopressin, α_1-adrenergic agonists, and extracellular ATP) inhibit hepatic CPT-I (14). Since these short-term changes in CPT-I activity are very stable and survive complete removal of malonyl-CoA from the medium, they are assumed to be mediated by a malonyl-CoA-independent mechanism which might involve phosphorylation of putative mediator protein(s) (7, 16).

Identification of physiological substrates of AMPK has been hampered by the lack of specific methods for activating the kinase in intact cells. AMPK was routinely activated in intact hepatocytes by incubation with fructose or by heat shock or arsenite [cf. Ref. (1)]. These treatments all deplete intracellular ATP and, therefore, have many nonspecific side effects. However, a more specific method for activating AMPK in intact cells has been recently reported. Incubation of intact hepatocytes with 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) causes uptake of this compound and subsequent accumulation within the cell of its monophosphorylated form, 5-aminoimidazole-4-carboxamide ribonucleoside monophosphate (ZMP) (16). The latter has been shown to mimic the effect of 5′-AMP on allosteric activation of rat liver AMPK without changing the levels of ATP, ADP, and AMP within the hepatocyte (16, 17). Thus, exposure to AICAR has been shown to inactivate acetyl-CoA carboxylase and 3-hydroxy-3-methylglutaryl-CoA reductase in isolated hepatocytes (3, 17) as well as hormone-sensitive lipase in isolated adipocytes (18). In the present report we show that activation of AMPK produces a stimulation of hepatic long-chain fatty acid oxidation, which relies in turn on activation of CPT-I by malonyl-CoA-dependent and independent mechanisms.

MATERIALS AND METHODS

Materials. [1-^{14}C]Palmitic acid, [1-^{14}C]octanoic acid, L-[*Me*-^{14}C]-carnitine, [1-^{14}C]acetyl-CoA, and ^3H_2O were supplied by Amersham International (Amersham, Bucks, UK). Digitonin, collagenase (type I), AICAR, ZMP, 5′-AMP, and taxol were purchased from Sigma Chemical Co. (St. Louis, MO). Okadaic acid was supplied by Calbiochem (San Diego, CA). Tetradecylglycidic acid was a kind gift from Dr. J. M. Lowenstein (Brandeis University, Waltham, MA).

Hepatocyte isolation and incubation. Male Wistar rats (250–300 g) which had free access to food and water were used throughout in this study. Hepatocytes were isolated by the collagenase perfusion method described in Ref. (19). Because lipogenesis is markedly depressed just after hepatocyte isolation, cells were incubated for 15 min at 37°C in a gyratory metabolic shaker and subsequently filtered through nylon mesh prior to use (20). Cell viability, as determined by trypan blue exclusion, always exceeded 95% in the final hepatocyte suspension.

Hepatocytes were incubated in Krebs–Henseleit bicarbonate buffer supplemented with 10 mM glucose and 1% (w/v) defatted and dialyzed bovine serum albumin. Incubations (4–6 mg of cellular protein/ml) were performed in a total volume of 2 ml at 37°C, with constant shaking (85 oscillations/min) and under an atmosphere of O_2/CO_2 (19:1). Stock solutions of AICAR, okadaic acid, and taxol were prepared in Me_2SO. Therefore, control incubations had the corresponding Me_2SO content. No significant influence of Me_2SO on any of the experimentally determined parameters was observed at the final concentration used (0.1%, v/v).

Rate of fatty acid oxidation. For determination of the rate of fatty acid oxidation, hepatocytes were incubated for 15 min with the cellular effectors indicated in every case. Reactions were subsequently started by the addition to cell incubations of [1-^{14}C]fatty acid (either palmitate or octanoate, 0.05 Ci/mol, 0.5 mM final concentration) bound to albumin. After 10 min, reactions were stopped with 0.5 ml of 2 M perchloric acid and oxidation products were extracted and quantified exactly as described before (13). Total oxidation products were calculated as the sum of acid-soluble products and CO_2. Acid-soluble products (mostly ketone bodies) routinely accounted for 90–95% of total oxidation products (13).

CPT-I assay. The activity of CPT-I was determined as the tetradecylglycidate-sensitive incorporation of radiolabeled L-carnitine into palmitoylcarnitine by four different methods (A, B, C, and D). In brief, hepatocytes were preincubated for 20 min in the absence or in the presence of 10 μM tetradecylglycidate, a specific irreversible inhibitor of CPT-I (13, 21). Incubations were continued for an additional 15-min period in the presence of the cellular effectors indicated in every case. Subsequently, aliquots were removed from the incubations to monitor CPT activity with the four different types of assay.

In methods A and B, CPT activity was measured in digitonin-permeabilized hepatocytes. Both methods were performed using the same detergent/cell protein ratio (about 40 μg digitonin/mg cell protein). Enzyme activity was routinely determined by method A ("one-step assay"). In this method, 100 μl of hepatocyte suspension is directly added to 100 μl of prewarmed digitonin-containing assay medium exactly as described in Ref. (13). Hence, monitoring of enzyme activity is performed immediately upon permeabilization of the cells (13, 22). In method B ("two-step assay"), hepatocytes are permeabilized and thoroughly washed prior to determination of enzyme activity. Thus, 1.0 ml of hepatocyte suspension was permeabilized with 0.20 mg of digitonin dissolved in 1.0 ml of 5 mM Tris–HCl (pH 7.4), 50 mM KF, 100 mM KCl, 2.5 mM EDTA, and 2.5 mM EGTA (F^- medium). The resulting mix was gently shaken for 5 s and rapidly diluted by transfer to tubes containing 40 ml of ice-cold F^- medium. Cell ghosts were sedimented by centrifugation at 350*g* for 15 s, and pellets were taken up in 1.0 ml of prewarmed F^- medium. The resulting suspensions of cell ghosts were incubated at 37°C for 5–15 min and then CPT activity was monitored (15).

In method C, CPT activity was measured in sonicated hepatocytes. Cells were sedimented by low-speed centrifugation (50*g*, 2 min). The cell pellet was resuspended in F^- medium and sonicated for two periods of 10 s while being cooled in ice/water. Samples of sonicated preparations were used for determination of CPT activity (15).

In method D, CPT activity was measured in mitochondria isolated from hepatocyte suspensions exactly as described before (15). Preparations of mitochondria were practically devoid of peroxisomes, as

judged from measurements of recovery of catalase activity (always less than 10% of total cellular activity).

Other analytical methods. Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid cell extracts by a radioenzymatic method as described in Ref. (19). Rates of *de novo* fatty acid and cholesterol synthesis were monitored as the incorporation of ^3H_2O into total fatty acids and digitonin-precipitable sterols, respectively (23). Acetyl-CoA carboxylase activity was measured in digitonin-permeabilized hepatocytes as the incorporation of radiolabeled acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction (23). Fatty acid synthase activity was determined in digitonin-permeabilized hepatocytes as the malonyl-CoA-dependent incorporation of radiolabeled acetyl-CoA into fatty acids (23). Protein was determined by the method of Lowry *et al.* (24), with bovine serum albumin as a standard.

Statistical analysis. Results shown represent the means ± SD of the number of hepatocyte preparations indicated in every case. Cell incubations and/or enzyme assays were always carried out in triplicate. Statistical analysis was performed by Student's *t* test.

RESULTS AND DISCUSSION

The effect of AICAR on fatty acid oxidation was studied in incubations of rat hepatocytes. Pilot experiments were performed to test the validity of our experimental system. As previously described (3, 16), addition of 0.5 mM AICAR to the hepatocyte incubation medium strongly depressed acetyl-CoA carboxylase activity (94 ± 5% inhibition, $n = 4$) and *de novo* fatty acid synthesis (92 ± 16% inhibition, $n = 4$). *De novo* cholesterol synthesis was similarly depressed (90 ± 8% inhibition, $n = 3$). In addition, 0.5 mM AICAR had no effect on the activity of fatty acid synthase (results not shown), a lipogenic enzyme which is not believed to be a substrate for AMPK.

AICAR stimulates in parallel CPT-I and long-chain fatty acid oxidation. Figure 1 shows the effect of AICAR on hepatic fatty acid oxidation. AICAR produced a dose-dependent increase in the rate of [^{14}C]-palmitate oxidation. Similar results were obtained when [^{14}C]oleate was used as substrate (results not shown). In contrast, [^{14}C]octanoate oxidation was not affected by AICAR. It is well established that long-chain fatty acids like palmitate and oleate are transported into mitochondria by a carnitine-dependent process, whereas medium-chain fatty acids like octanoate enter mitochondria independently of carnitine (7, 10, 11). Therefore, this observation suggests that the target for AICAR action might be CPT-I, the enzyme that catalyzes the pace-setting step of long-chain fatty acid translocation into the mitochondrial matrix (7, 10, 11). The other components of the fatty acid-translocating system, namely acyl-CoA synthetase, CPT-II, and carnitine:acylcarnitine translocase, are generally not considered to play a significant regulatory role in the transport of long-chain fatty acids into the mitochondrial matrix (7, 10, 11).

As shown in Fig. 1, CPT-I activity was enhanced by exposure of hepatocytes to AICAR. When CPT-I activity was determined at a very short assay time (20 s in Fig. 1), a good correlation was observed between maximal stimulation of enzyme activity (210 ± 16%) and palmitate oxidation (217 ± 22%). Furthermore, 0.5 mM AICAR was found to depress intracellular malonyl-CoA concentration from 87 ± 12 pmol/mg cell protein (incubations with no additions) to levels hardly detectable by the radioenzymatic assay (3 ± 3 pmol/mg cell protein, $n = 4$, $P < 0.01$ vs incubations with no additions). It is thus conceivable that the AICAR-induced stimulation of long-chain fatty acid oxidation may be mediated by the AMPK-dependent phosphorylation (inactivation) of acetyl-CoA carboxylase, thereby decreasing intracellular malonyl-CoA levels.

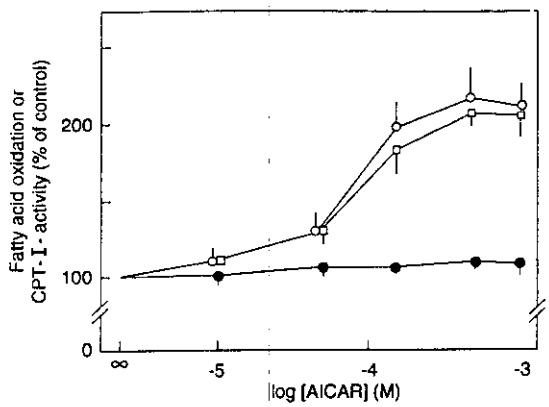

FIG. 1. Dose-dependent effect of AICAR on fatty acid oxidation and on CPT-I activity. Hepatocytes were preincubated for 15 min in the presence of different concentrations of AICAR. Subsequently, aliquots were removed to determine the rate of [^{14}C]palmitate (○) and [^{14}C]octanoate (●) oxidation, as well as CPT-I activity by method A (□) (see Materials and Methods). Reaction time of the CPT-I assay was 20 s. The 100% values of oxidation for [^{14}C]palmitate and [^{14}C]-octanoate were 58.7 ± 6.4 and 122.0 ± 16.5 nmol fatty acid converted into total oxidation products/h/mg cellular protein, respectively. The 100% value of CPT-I activity was 1.37 ± 0.36 nmol/min/mg cellular protein. Results correspond to four different hepatocyte preparations.

Hepatocytes take up AICAR, which is subsequently phosphorylated to produce ZMP (16). In addition, AICAR is not removed from the medium before enzyme activity is determined by method A. Therefore, to rule out the possibility that either AICAR or ZMP may have a direct effect on CPT-I activity, enzyme activity was determined in isolated liver mitochondria incubated in the presence of 0.5 mM AICAR or 10 mM ZMP [cf. Ref. (16)]. Assays on mitochondria incubated with 1 mM 5'-AMP were also carried out as a control. CPT-I activity was also determined in the permeabilized-cell assay (method A) after hepatocyte preincubation with no additions but adding 1 mM AICAR, 20 mM ZMP, or 2 mM 5'-AMP to the digitonin-containing assay mixture.

However, no effect of AICAR, ZMP, or 5′-AMP on CPT-I activity in either of the two systems could be detected (results not shown).

Malonyl-CoA-dependent and -independent mechanisms are involved in the AICAR-induced stimulation of CPT-I. It has been shown that CPT-I is reversibly sensitized to malonyl-CoA inhibition by malonyl-CoA itself [reviewed in Ref. (11)]. Thus, when mitochondria are incubated in the absence or presence of malonyl-CoA, the enzyme becomes less or more sensitive to inhibition, respectively (11). This effect is also observed in whole-cell systems: incubation of isolated hepatocytes with compounds that decrease intracellular malonyl-CoA levels makes the enzyme acquire a relaxed conformation which exhibits high activity and low sensitivity to inhibition by malonyl-CoA in a subsequent assay (11). The opposite ensues when hepatocytes are exposed to compounds that increase malonyl-CoA concentration, i.e., the enzyme acquires a tight conformation with low activity and enhanced sensitivity to inhibition by malonyl-CoA (11). Because permeabilization of the plasma membrane of the hepatocyte leads to a large dilution of cytosolic components including malonyl-CoA (22), and the transition between the two different sensitivity states is very fast at 37°C (11), the conformational state of CPT-I within the intact hepatocyte is only retained for very short periods of time in permeabilized cells [cf. Ref. (15)]. The permeabilized-hepatocyte system thus allows monitoring the conformational state of CPT-I when the assay of enzyme activity is performed at different times after permeabilization.

We used this experimental approach to test whether the stimulation of CPT-I observed in AICAR-treated hepatocytes actually involves a decrease of intracellular malonyl-CoA levels. As stated above, the AICAR-induced stimulation of [^{14}C]palmitate oxidation correlated well with the AICAR-induced stimulation of CPT-I when enzyme activity was determined 20 s after permeabilization of the hepatocyte plasma membrane (Fig. 1). In control incubations, a lag phase in the CPT-I assay was observed at very short reaction times (Fig. 2). This lag phase seems to be due to the conformational constraints of the CPT-I protein induced by intracellular malonyl-CoA [cf. Ref. (15)], and it rapidly disappears after complete leakage of malonyl-CoA from the permeabilized cells, allowing relaxation of the enzyme (Fig. 2). In contrast, by depleting intracellular malonyl-CoA, AICAR was indeed able to eliminate the lag phase inherent to the permeabilized-cell assay of CPT-I activity (Fig. 2). The magnitude of the AICAR-induced stimulation of CPT-I was reduced to 45 ± 14% at 40 s after permeabilization and to 30 ± 12% at 60 s after permeabilization (Fig. 2), indicating that differences in the conformational state of the CPT-I protein between control and AICAR-treated cells are disappearing. However, a constant 20–25% stimulation of CPT-I activity was observed in AICAR-treated hepatocytes up to 140 s after hepatocyte permeabilization (Fig. 2). In our system, this time is believed to be more than enough to allow reversal of conformational constraints of CPT-I induced by changes in intracellular malonyl-CoA concentration [cf. Ref. (11)].

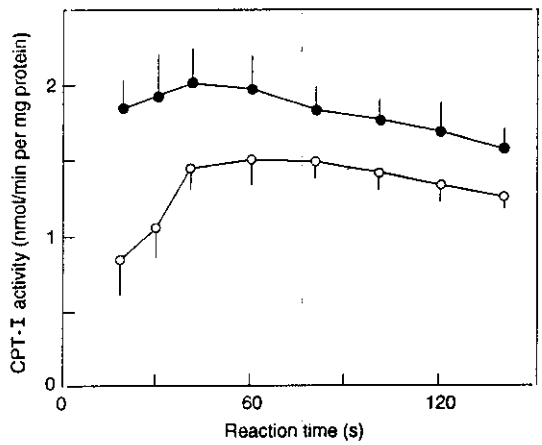

FIG. 2. Effect of AICAR on CPT-I activity at different reaction times. Hepatocytes were preincubated for 15 min in the absence (○) or in the presence (●) of 0.5 mM AICAR. Subsequently, aliquots were removed to determine CPT-I activity at different reaction times by method A. Note that enzyme velocity (and not product accumulation) is represented on the *y*-axis. Results correspond to six different hepatocyte preparations.

To obtain more direct evidence for the involvement of a malonyl-CoA-independent component in the AICAR-induced stimulation of CPT-I, enzyme activity was determined by a more complex procedure which eliminates any possible interference of malonyl-CoA. In such an assay (method B), hepatocytes are permeabilized with digitonin, followed by rapid and extensive washing of permeabilized cells to allow complete removal of malonyl-CoA. Then, permeabilized cells are incubated at 37°C for up to 15 min prior to determination of enzyme activity, so that any conformational constraint of the CPT-I enzyme will disappear (15). The use of this procedure corroborated that a 20–25% stimulation of CPT-I by AICAR is exerted by a malonyl-CoA-independent mechanism (Fig. 3). Moreover, the magnitude of the stimulation of CPT-I as determined by method B was identical with reaction times of 20, 60, and 120 s (results not shown). Note that for determination of CPT-I activity by method B cytosolic protein that leaked from the permeabilized cells is removed prior to determination of enzyme activity, and so enzyme specific activity (referred to as milligrams of protein) was always higher in method B than in method A (note to Table I).

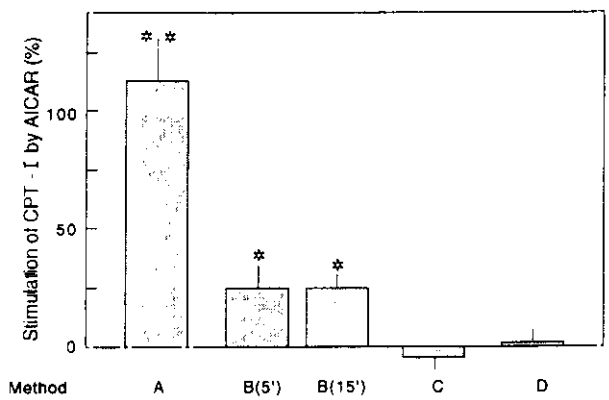

FIG. 3. Differential effect of AICAR on CPT-I activity as determined by methods A, B, C, and D. Hepatocytes were preincubated for 15 min in the absence or in the presence of 0.5 mM AICAR. Subsequently, aliquots were removed to determine CPT-I activity by the various methods. In the case of method B, the ghosts of permeabilized cells were incubated at 37°C for 5 or 15 min prior to determination of enzyme activity. Reaction time was 20 s for method A and 60 s for methods B, C, and D. Results correspond to eight different hepatocyte preparations. Significantly different versus incubations with no additions: $^{*}P < 0.05$; $^{**}P < 0.01$.

Malonyl-CoA-independent stimulation of CPT-I by AICAR may involve interaction of CPT-I with cytoskeletal components. The observation that the phosphatase inhibitor okadaic acid stimulates hepatic CPT-I by a stable mechanism led to the suggestion that inhibition of the phosphatases might have resulted in increased phosphorylation of CPT-I, with a consequent increase of enzyme activity (7). However, incubation of purified mitochondrial outer membranes or isolated mitochondria with different protein kinases (including AMPK) and protein phosphatases did not lead to any change in the kinetic and regulatory properties of CPT-I (15). It has also been shown that the malonyl-CoA-independent increase of CPT-I activity observed in okadaic acid-treated hepatocytes is not due to the direct phosphorylation of the enzyme, but may involve modulation of interactions between CPT-I and nondiffusible extramitochondrial components (15). Three observations indicate that this may also be the case of the malonyl-CoA-independent component of the AICAR-induced stimulation of CPT-I:

(i) Stimulation of CPT-I was not apparent when enzyme activity was measured in intact mitochondria isolated from AICAR-treated hepatocytes (method D), despite the presence of 50 mM fluoride in all the isolation buffers (Fig. 3). Likewise, when hepatocytes were vigorously disrupted by sonication after AICAR treatment (method C), the stimulatory effect of AICAR on CPT-I was not preserved (Fig. 3). Thus, malonyl-CoA-independent stimulation of CPT-I by AICAR is only evident when mitochondria are still associated with other cellular fractions, i.e., in the ghosts of the permeabilized cells, but not after separation of mitochondria from other cell components. Therefore, it appears that the malonyl-CoA-independent stimulation of CPT-I by AICAR requires components of the extramitochondrial compartment of the cell.

(ii) The possibility that cytoskeletal components may be involved in the malonyl-CoA-independent stimulation of CPT-I by AICAR was tested by using taxol. This complex diterpenoid binds to tubulin and stabilizes microtubules, preventing the disassembly of microtubules in a very efficient fashion (25). Interestingly, malonyl-CoA-independent activation of CPT-I induced

TABLE I

Combined Effects of AICAR, Okadaic Acid, and Taxol on the Activities of CPT-I and Acetyl-CoA Carboxylase

Additions	CPT-I activity (nmol/min/mg protein)		Acetyl-CoA carboxylase activity (nmol/min/mg protein)
	Method A	Method B	
None (n = 10)	1.44 ± 0.36	2.37 ± 0.37	0.26 ± 0.14
AICAR (n = 8)	3.07 ± 0.86*	2.94 ± 0.31**	0.01 ± 0.01*
Okadaic acid (n = 4)	2.38 ± 0.46*	3.93 ± 0.52*	0.03 ± 0.01*
Taxol (n = 4)	1.43 ± 0.23	2.35 ± 0.26	0.26 ± 0.02
AICAR + okadaic acid (n = 6)	4.51 ± 1.15*	3.93 ± 0.88*	0.01 ± 0.01*
AICAR + taxol (n = 6)	2.82 ± 0.40*	2.30 ± 0.52	001 ± 0.01*
Okadaic acid + taxol (n = 4)	1.50 ± 0.26	2.44 ± 0.33	0.06 ± 0.03*

Note. Hepatocytes were preincubated for 20 min with or without 10 μM taxol. Incubations were continued for an additional 15 min with or without 0.5 mM AICAR and/or 0.5 μM okadaic acid. Subsequently, aliquots were removed to determine the activities of acetyl-CoA carboxylase and CPT-I. The activity of CPT-I was assayed by methods A and B. In method A, assay time was 20 s. In method B, the ghosts of the permeabilized cells were incubated at 37°C for 15 min prior to determination of enzyme activity in a 60-s assay. Results correspond to the number of hepatocyte preparations shown in parentheses.

Significantly different versus incubations with no additions: $^{*}P < 0.01$; $^{**}P < 0.05$.

by AICAR was completely abolished by pretreatment of hepatocytes with taxol (Table I, method B). Likewise, taxol antagonized the okadaic acid-induced stimulation of CPT-I (Table I). It is also noteworthy that the inhibition of acetyl-CoA carboxylase by okadaic acid or AICAR was not prevented by taxol (Table I), indicating that the antagonistic effect of taxol on okadaic acid- and AICAR-induced CPT-I stimulation is independent of changes in intracellular malonyl-CoA concentration. Hence, it seems that blockade of microtubule dynamics prevents malonyl-CoA-independent stimulation of CPT-I by AICAR.

(iii) Stimulation of CPT-I by AICAR and okadaic acid was nearly additive when enzyme activity was determined by method A, but not when enzyme activity was determined by method B (Table I). This indicates that the effect of AICAR observed in method A is mostly due to a malonyl-CoA-dependent mechanism, whereas AICAR and okadaic acid seem to share a common malonyl-CoA-independent pathway to stimulate CPT-I as evidenced by method B.

CONCLUDING COMMENTS

In the present report we show that exposure of intact hepatocytes to AICAR produces a strong (twofold) stimulation of long-chain fatty acid oxidation. This effect seems to rely on the activation of CPT-I by two different mechanisms. On the one hand, AMPK-induced acetyl-CoA carboxylase inactivation would lead to the depletion of intracellular malonyl-CoA, with concomitant deinhibition of CPT-I. This mechanism, which has been proposed to operate in the ischemic heart (4, 5) and the working muscle (6), seems to make a major contribution (ca. 80%) to the AICAR-induced stimulation of hepatic long-chain fatty acid oxidation. On the other hand, AICAR induces a 20–25% stimulation of CPT-I by a stable, malonyl-CoA-independent mechanism. We have previously reported that incubation of purified mitochondrial outer membranes or isolated mitochondria with AMPK under phosphorylation conditions does not lead either to the phosphorylation of CPT-I or to any change in the kinetic and regulatory properties of CPT-I (15). Instead, data in this paper suggest that malonyl-CoA-independent modulation of CPT-I activity might rest on the modulation of interactions between CPT-I and cytoskeletal components. It has been established that the dynamics of mitochondria in living cells (shape changes, dislocation, and fusion and fission) may result from specific interactions of mitochondria with components of the cytoskeleton (26). In addition, it has been shown that porin and hexokinase are localized in domains of the mitochondrial outer membrane which interact specifically with microtubule- and microfilament-associated proteins, as well as with other still unidentified cytoskeletal or cytoplasmic elements (27, 28). In liver cells, these interactions may in turn determine the permeability of the mitochondrial outer membrane to ADP (29). Mitochondria have also been shown to contain specific binding sites for phalloidin-stabilized F-actin (30) as well as for fodrin, an actin-binding protein (27). In hepatocytes and in several cell lines, hyperphosphorylation of microtubules, actin microfilaments, and intermediate filaments leads to disruption of the cytoskeleton (31–33). In fact, we have recently obtained data indicating that cytoskeletal components might be involved in the okadaic acid-induced stimulation of CPT-I (34). Whether AMPK may phosphorylate cytoskeletal components related to the control of CPT-I activity is an intriguing possibility which is currently under study in our laboratory.

The results shown in this report must be interpreted with caution, since AICAR may have effects apart from activation of AMPK. Thus, for example, ZMP mimics the inhibition of fructose 1,6-*bis*-phosphatase by AMP, and thus treatment of hepatocytes with AICAR inhibits gluconeogenesis (17). In other cell types such as Chinese hamster fibroblasts and ovary cells, exposure to AICAR has been shown to alter the levels of different purine and pyrimidine nucleotides (35–37). However, as discussed by Corton *et al.* (16), none of these ancillary changes in nucleotides are evident in rat hepatocytes. In the latter, AICAR seems to be a rather specific activator of AMPK or at least the most specific compound for activation of AMPK available to date (16).

The AICAR-induced malonyl-CoA-dependent deinhibition of CPT-I is quantitatively more important than the malonyl-CoA-independent deinhibition of enzyme activity. Nonetheless, the latter data are actually highly reproducible and statistically significant (Table I). It is remarkable that the two mechanisms operate simultaneously in the short-term control of hepatic CPT-I activity, especially since AICAR is not expected to produce any stable (covalent) modification of CPT-I or the putative regulatory proteins of the cytoskeleton. It is also worth noting that when CPT-I activity is measured by method B to determine the malonyl-CoA-independent component of the AICAR effect, cells are extensively washed and further incubated for up to 15 min at 37°C. Hence, if the malonyl-CoA-independent deinhibition of CPT-I activity relies on protein–protein interactions, these may partially disappear after the potential modifications of the enzyme microenvironment. Therefore, it is conceivable that malonyl-CoA-independent control of CPT-I activity may be quantitatively more important within the cell than is evident under the conditions of our assay.

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Studies on the Intracellular Localization of Acetyl-CoA Carboxylase

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The present work was performed to identify the subcellular localization of hepatic acetyl-CoA carboxylase (ACC). Cellular organelles involved in fatty acid oxidation that contain a malonyl-CoA sensitive carnitine palmitoyltransferase (CPT) activity or that are linked to the control of this activity were analysed for the presence of ACC. No significant amount of ACC was observed in the mitochondrial fraction prepared from isolated rat hepatocytes. Furthermore, no association of ACC activity and mass with isolated hepatic peroxisomes could be detected. In cubation of isolated hepatocytes with compounds known to affect the integrity of the cytoskeleton like okadaic acid or taxol indicates that ACC is associated with this subcellular structure of the hepatocyte. Such association may allow for efficient regulation of CPT activity and thus of fatty acid oxidation. © 1997 Academic Press

Depending on the physiological state of the animal, the liver is a tissue that can either exhibit high rates of fatty acid biosynthesis or high rates of fatty acid oxidation. Control of the activity of acetyl-CoA carboxylase (ACC) is of special interest in this respect because the product of ACC, malonyl-CoA, is not only a substrate for the cytosolic process of long-chain fatty acid synthesis but is also an inhibitor of the activity of carnitine palmitoyltransferase (CPT), an important pacesetting enzyme of long-chain fatty acid oxidation (1). As a matter of fact, malonyl-CoA sensitive CPT activity is present both in mitochondrial outer membranes (CPT-I) (2) and in the peroxisomal matrix (3).

Given the functions of malonyl-CoA in or on different organelles of the cell, the enzyme responsible for its production, ACC, may be compartmentalized as well. Since its original discovery as a soluble enzyme (4) AAC has been assumed to be located in the cytoplasm. Earlier work concluded that the activity of the enzyme was not associated with subcellular particles (5), but later reports indicated that activity of ACC could be detected in high speed precipitates of rat-liver homogenates (6-8) and in a so-called mitochondrial fraction of such homogenates (9,10). Furthermore, permeabilization of isolated hepatocytes with digitonin also suggested association of the enzyme with some kind of intracellular structure (11).

These conclusions have relied on assays of enzyme activity in cellular fractions (6-11) and on measurement of ACC mass by a technique employing the binding of radiolabelled avidin (9,10). Both procedures are prone to error. The measurement of the activity of ACC is subject to modifi cation of that activity by several factors (12-15), and the avidin binding method is sensitive to interference by other biotin-containing proteins competing for labelled avidin (16). Therefore, the determination of subcellular distribution on the basis of avidin binding or enzyme activity is difficult to interpret.

The availability of ACC antibodies (17) and the technique of permeabilizing isolated hepa tocytes with digitonin (15) has permitted to re-address the issue of localization of ACC and to cir cumvent some of the methodological problems.

MATERIALS AND METHODS

Male Wistar rats (250-300 g) which had free access to food and water were used throughout in this study. Hepatocytes were isolated and incubated as described in (18). Acetyl-CoA carboxylase activity, isoform distribution and mass were determined in isolated hepatic mitochondria, isolated hepatic peroxisomes or in digitonin-permeabilized hepatocytes. For isolation of mitochondria from liver tissue, the procedure described by Roman-Lopez et al. (19) was followed. Mitochondria from hepatocytes were isolated by homogenizing cells (4-6 mg of protein) with a loose-fitting Dounce homogenizer in a low

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ionic strength, iso-osmotic medium consisting of 0.3 M mannitol and 2 mM Hepes pH 7.4 in the presence of a proteinase inhibitor mixture (17). The homogenate was centrifuged at 1500 × g for 2 min. The pellet was discarded and the supernatant centrifuged for 2 min at 16.000 × g. The resulting supernatant was termed the cytosolic fraction. The pellet was washed once by resuspension in the low ionic strength medium and recentrifugation at 16.000 × g. The resuspended final pellet was termed the mitochondrial fraction. Peroxisomes were isolated from animals fed a standard pelleted diet supplemented with 1% di(2-ethylhexyl)phtalate in order to proliferate the peroxisomal compartment (20). Treatment of animals and isolation of peroxisomes was performed as in (20). All homogenizing steps and subsequent procedures were performed at 4° C. The isolated fractions were snap frozen in liquid nitrogen and stored at −20° C. To determine the amount of ACC retained in the cell ghosts following incubation of the intact hepatocytes with different cellular effectors, the isolated cells were permeabilized and thoroughly washed prior to harvesting the cell ghosts. Thus, 1.0 ml of hepatocyte suspension was permeabilized with digitonin (ca. 60 μg per mg cell protein) dissolved in a medium containing 50 mM Hepes (pH 7.5), 0.25 M mannitol, 5 mM 2-mercaptoethanol and a proteinase inhibitor mixture as in (17). The resulting mix of cells and permeabilizing medium was gently shaken for 5 s. and rapidly diluted by transfer to tubes containing 40 ml of ice-cold medium without digitonin. Cell ghosts were sedimented by centrifugation at 350 *g* for 15 s, and pellets were taken up in 1.0 ml ice-cold medium containing 50 μl of the proteinase inhibitor mixture. The resulting suspensions of cell ghosts were kept at −80° C till mass measurements were performed. Determination of total ACC mass retained in cell ghosts following digitonin treatment or associated either with isolated mitochondria or peroxisomes, was performed with an ELISA assay essentially as described by Iverson *et al.* (21) employing a primary antiserum against rat-liver ACC (17). The distribution of isoforms was determined using immunoprecipitation, SDS/PAGE, immunoblotting and auto radiography as in (17). Activities of ACC and fatty acid synthase were monitored exactly as described before (15,17). Sources of chemicals as in (17).

RESULTS

The present study was undertaken to identify the subcellular localization of ACC. In order to investigate a possible association of ACC with mitochondria—as suggested by Allred and co-workers (9,10)—mitochondria were prepared from isolated hepatocytes. With such a preparation less mechanical force is required to liberate mitochondria as compared to whole tissue as the star ting material. Mechanical interference with an association of ACC and mitochondria was further kept to a minimum by using a loose-fitting Dounce homogenizer. To interfere as little as possible with potential electrostatic interactions between enzyme and organelle, a low ionic strength medium was chosen for isolation of the subcellular fractions. In addition, the whole isolation procedure—including subcellular fractionation by centrifugation—was aimed at speed rather than at recovery. Activity measurements for ACC of the resulting subcellular fractions could not be performed because of the presence of malonyl-CoA decarboxylase in mitochondria. The latter enzyme interferes with the assay for ACC activity (15). The subcellular fractions were analysed by SDS gel electrophoresis for the presence of the 265-kDa and 280-kDa isoforms of ACC

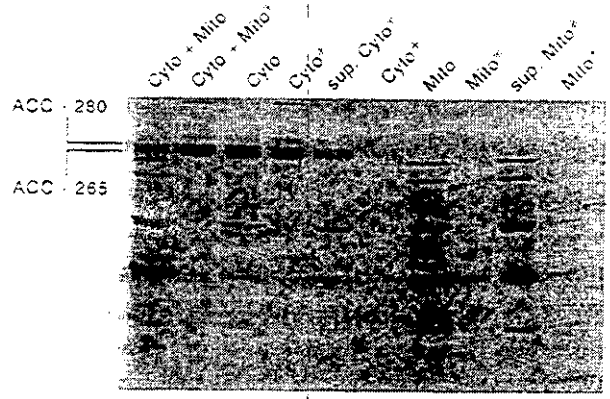

FIG. 1. Acetyl-CoA carboxylase (ACC) analysis of subcellular fractions of hepatocytes from normally fed rats by SDS gel electrophoresis. Cytosolic (Cyto) and mitochondrial (Mito) fractions obtained from isolated hepatocytes (see Materials and Methods) were analysed for the presence of the ACC-265 and ACC-280. A sample equivalent to 100 μg of protein of each fraction was loaded to the lanes as indicated. Following labeling with [^{35}S]streptavidin, SDS/PAGE and transfer to nitrocellulose, the biotin-containing proteins were visualized by autoradiography. *Indicates that the immunoprecipitate with a primary antiserum against rat-liver ACC was loaded in that lane; ⁻indicates that an immunoprecipitate with a pre-immune serum was loaded; the prefix *sup.* indicates that the supernatant of the immunoprecipitate was loaded.

(ACC-265 and ACC-280, respectively). The two isoforms have been suggested to play different biological roles. In particular, ACC-280 is of interest in this respect because it might be involved in the synthesis of malonyl-CoA for inhibition of CPT-I (22,23). Since the latter enzyme has a mitochondrial localization (24), an association of ACC-280 with mitochondria would be feasible. Fig. 1 shows the analysis of the presence of ACC isoforms in the subcellular fractions from isolated hepatocytes. The results do not reveal a mitochondrial localization of either of the two ACC isoforms. In an attempt to spare potential hydrophobic interactions between ACC and mitochondria, the latter organelles were also prepared from isolated hepatocytes in a high ionic strength medium (0.9% NaCl and 20 mM Tris pH 7.4) exactly as described for the low ionic strength medium. Also in this case no association of any of the ACC-isoforms with the mitochondrial fraction was apparent (data not shown). The analysis of the presence of ACC-isoforms in the mitochondrial fraction was repeated with two different primary antisera also raised against rat-liver ACC. The results were identical to those presented in Fig. 1. (data not shown). The analysis of the presence of ACC isoforms was also performed on subcellular fractions of rat-liver cells from animals in different nutritional states, *i.e.* starved for 48 h and starved for 48 h fol lowed by refeeding a carbohydrate-rich, fat-poor diet for 48 h. No significant mass of either ACC isoform was observed in the mitochondrial fraction in any of the nutritional states (data not shown).

TABLE 1

Activities of Phosphoglucoisomerase (PGI), Catalase, Fatty Acid Synthase (FAS), and Acetyl-CoA Carbozylase (ACC) in Post-Nuclear Supernatant (PNS) and in Isolated Peroxisomes from Liver of Control and Di(2-ethylhexyl)phtalate (DEHP)-Treated Rats

	PNS		Peroxisomes	
Enzyme	Control	DEHP	Control	DEHP
	(μmol/min/mg protein)			
Catalase	0.83	0.97	10.6	6.64
PGI	0.55	0.39	0.013	0.023
	(nmol/min/mg protein)			
FAS	1.88 ± 0.11	1.14 ± 0.03	n.d.	n.d.
ACC	0.34 ± 0.03	0.42 ± 0.01	n.d.	n.d.

Note. PGI and catalase are marker enzymes for cytosol and peroxisomes, respectively. Data represent means of two control and two DEHP-treated animals. n.d., nondetectable.

Hepatic peroxisomes exhibit CPT activity that is sensitive to inhibition by malonyl-CoA (3, 25). To study a possible association of ACC with this organelle, peroxisomes were isolated from liver tissue obtained from rats treated with di(2-ethylhexyl)phtalate (DEHP), a proliferator of the peroxisomal compartment (20). The data in Table 1 indicate that there is no association of ACC activity with peroxisomes from both control and DEHP-treated animals. Likewise, no significant association of ACC to peroxisomes was observed on the basis of measurements of enzyme mass (data not shown).

Our recent observation that cytoskeletal components are most likely involved in the control of the activity of CPT-I (26) prompted us to consider a cytoskeletal localization of ACC, since the latter enzyme is pivotal in the control of CPT-I activity (1). To analyse a potential association of ACC with the cytoskeleton, isolated hepatocytes were incubated with various compounds known to affect cytoskeletal integrity. Subsequently, cells were permeabilized by short-term treatment with digitonin followed by rapid removal of released cytosolic proteins including ACC. In this approach, ACC associated with a subcellular structure will be sedimented. The resulting pellet was used to determine ACC mass. The possibility of an association between ACC and cytoskeletal components was tested by the use of okadaic acid (OA), taxol and colchicine. OA has been shown to disrupt the cytoskeleton of hepatocytes (27). Taxol binds to tubulin and stabilizes microtubules, preventing the disassembly of microtubules in a very efficient fashion (28). The polymeric state of microtubules can be dissociated by colchicine (29). The data of Table 2 show that incubation of hepatocytes with OA resulted in the release of substantially more ACC than in control cells. Interestingly, the OA-induced effect was completely abolished by pretreating the hepatocytes with taxol (Table 2). Likewise, pretreatment of the cells with KN-62, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II, also prevented the OA-induced release of hepatic ACC. 5-Aminoimidazole-4-carboxamide ribonucleoside (AICAR), a specific activator of 5′-AMP-activated protein kinase, was without effect on the retention of ACC mass in cell ghosts (Table 2). Incubation of hepatocytes with colchicine did not affect the release of ACC from digitonin-permeabilized cells either. The data obtained with amyloglucosidase (Table 2) suggest that glycogen granules represent a subcellular structure capable of binding ACC. To determine whether the two ACC isoforms will differentially release upon permeabilization, the cell ghosts were also analysed for the presence of ACC-265 and ACC-280. However, irrespective of the incubation condition, the ratio of ACC-265/ACC-280 was identical, *i.e.* ACC-280 was always about one third of the total ACC mass (data not shown).

DISCUSSION

Several studies performed by others (6-10) as well as by our group (11) led to the suggestion of association of

TABLE 2

Mass Measurements of Acetyl-CoA Carboxylase Retained in Digitonin-Permeabilized Rat Hepatocytes

Additions	Percentage of total ACC mass retained in cell ghosts
Control	53.2 ± 18.0
OA	20.6 ± 8.0*
Taxol + OA	55.5 ± 6.6
KN-62 + OA	48.5 ± 10.5
AICAR	46.0 ± 19.5
Colchicine	64.5 ± 8.7
Taxol + colchicine	67.9 ± 1.0
Control[a]	15.8 ± 2.8
Control + amyloglucosidase[a]	4.6 ± 1.0*

Note. Hepatocytes were preincubated for 20 min with or without 10 μM taxol or 30 μM KN-62. Incubations were continued for 15 additional min with or without 0.5 mM 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), 0.5 μM okadaic acid (OA) or 0.1 mM colchicine. Subsequently, cell ghosts were prepared as described in Materials and Methods. ACC retained in the cell ghosts was quantified by avidin-based ELISA analysis using as the probing antibody a primary antiserum against rat-liver ACC (17).

[a] These results are from two sets of cell ghosts of control cells resuspended and incubated for 15 additional min with or without 50 U amyloglucosidase. Results represent the mean ± S.D. of 3 different hepatocyte preparations. The amount of ACC present in intact hepatocytes was set at 100%. Values of OA are significantly different ($P<0.01$) versus its control using the Student *t* test. This also applies to amyloglucosidase ($P<0.01$) versus its control.

ACC to a subcellular organelle. However, after careful isolation, no association of ACC with either mitochondria or peroxisomes could be observed in the present study. Nonetheless, in the intact cell an association may exist that is not firm so that the enzyme can escape into the supernatant during the biochemical preparation procedure. The present approach of using the technique of permeabilizing isolated hepatocytes rather than homogenizing the cells revealed an association of ACC with the cytoskeleton. The implication of this cytoskeletal connection of both ACC (this study) and CPT-I (26) is that it allows for an efficient regulation of fatty acid oxidation through malonyl-CoA-induced changes in CPT-I activity. Furthermore, the different digitonin-release-pattern of ACC following incubation of hepatocytes with OA as compared to the control situation raises the possibility that ACC may translocate from one compartment to another, depending on the situation of the cell, and thus efficiently control the activity of CPT-I.

It could be argued that the release pattern of ACC following digitonin permeabilization is a reflection of its state of aggregation and that OA by favoring the monomeric state of the enzyme would cause less ACC to be retained by the cell ghosts. However, the identical ACC release pattern following incubation of hepatocytes with insulin or glucagon—putative affectors of the aggregation state of ACC (30)—is not in line with this reasoning (unpublished data of the authors). The inability of AICAR to alter ACC retention by cell ghosts may also indicate that there is no relation between phosphorylation—at least by 5′-AMP-activated protein kinase—and retention of the enzyme by cell ghosts.

The antagonistic effect of KN-62 and taxol on OA-induced release of ACC is quite similar to the antagonistic effect of the two former compounds on the OA-induced stimulation of CPT-I activity (26,31). Data of KN-62 are of special interest since this compound also prevents quite effectively the OA-induced inhibition of ACC activity as measured in a permeabilized-cell assay (unpublished data of the authors). This implicates Ca^{2+}/calmodulin-dependent protein kinase II in the control of both ACC and CPT-I and is at odds with the opinion that 5′-AMP-activated protein kinase is the most important—if not unique—protein kinase involved in the control of ACC in intact hepatocytes (32). In addition, phosphorylation of cytoskeletal components—as shown by the effects of KN-62 and OA—may be required for ACC to be released from its anchoring place on the cytoskeleton. Interestingly, colchicine was without effect just like in the case of CPT-I activity (26) suggesting that specific protein-protein interactions between the two enzymes and cytoskeletal components and not the mere disruption of the cytoskeleton may be involved in the intracellular behavior of the two enzymes and their control.

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DISCUSIÓN

Los datos expuestos en el presente capítulo permiten sugerir que el mecanismo de regulación de la CPT-I independiente de malonil-CoA podría desempeñar un papel significativo en el control del metabolismo de ácidos grasos.

En primer lugar, los resultados obtenidos con células de hepatoma (capítulo 3.1), aunque todavía preliminares, son acordes con la idea de que las interacciones con el citoesqueleto están implicadas en el control de la actividad CPT-I. La capacidad carcinogénica del ácido okadaico, cuyas acciones sobre la célula incluyen la ruptura del citoesqueleto y la alteración de la forma celular, podría conllevar alteraciones debidas a la la pérdida de diversas interacciones del citoesqueleto con diferentes enzimas tales como la CPT-I. Así, los efectos que el ácido okadaico ejerce sobre la CPT-I en hepatocitos podrían mimetizar a los que tienen lugar durante la transformación de una célula en tumoral, en la que diversas proteínas sufren alteraciones de su funcionalidad. Durante estos procesos, numerosas quinasas ven alterada su regulación y sufren un aumento de su actividad por encima de loslímites fisiológicos. Así, la activación de la Ca^{2+}/CMPKII que el ácido okadaico trae consigo en hepatocitos aislados, podría no ser equiparable a la que tendría lugar tras la activación mediante mediadores fisiológicos en una célula no transformada, pero pudiera ser del mismo orden que la que tiene lugar en una célula tumoral.

Por otra parte, la AMPK también parece estar implicada en el control a corto plazo de la CPT-I. Los efectos del AICAR sobre la actividad de esta enzima pueden dividirse en dependientes e independientes de malonil-CoA (capítulo 2.3.2). Estos datos, junto a la posible localización de parte de las moléculas de ACC en el citoesqueleto, indican que ambos mecanismos pudieran estar interrelacionados.

La regulación de la CPT-I dependiente de malonil-CoA ha venido siendo considerada como el principal mecanismo de regulación a corto plazo de la enzima. En el interior de la célula, donde no es esperable que ningún agonista ejerza alteraciones tan drásticas como las producidas por el ácido okadaico, la contribución del mecanismo independiente de malonil-CoA podría ser complementaria a la del dependiente de los niveles de este metabolito. En este sentido, los datos obtenidos trás la activación de la AMPK por AICAR podrían acercarse más a lo que ocurre *in vivo*. Además, la posible localización de la ACC en el citoesqueleto sugiere asimismo que ambos mecanismos actúan de forma coordinada, ya que si la ACC y la CPT-I se localizasen próximas entre si en el interior de la célula, la concentración efectiva de malonil-CoA en esos puntos podría ser mucho mayor produciendo así una inhibición mucho más efectiva de la CPT-I. Así, la desorganización del citoesqueleto traería consigo no solo la pérdida de las posibles interacciones reguladoras con la CPT-I sino un alejamiento físico de la ACC y con ella de la fuente de malonil-CoA.

3. Discusión General y Conclusiones

Desde que hace ahora 20 años, McGarry y colaboradores descubrieran que la CPT-I es inhibida por malonil-CoA dicha inhibición (que permite el establecimiento de un control coordinado de síntesis y oxidación de ácidos grasos) ha venido siendo considerada como el principal mecanismo de regulación de esta enzima (McGarry y Brown, 1997). Sin embargo, a lo largo de los últimos años se ha puesto de manifiesto que la regulación de la CPT-I también depende de un mecanismo independiente de malonil-CoA (Guzmán y Geelen, 1993; la presente memoria), que se basaría en la existencia de interacciones entre ciertos componentes del citoesqueleto (probablemente los filamentos intermedios) y las mitocondrias. Así, el control de la actividad CPT-I podría ejercerse a tres niveles (Esquema 1):

i) Actividad de la ACC/niveles de malonil-CoA.
ii) Interacciones citoesqueleto-mitocondrias.
iii) Localización subcelular de la ACC.

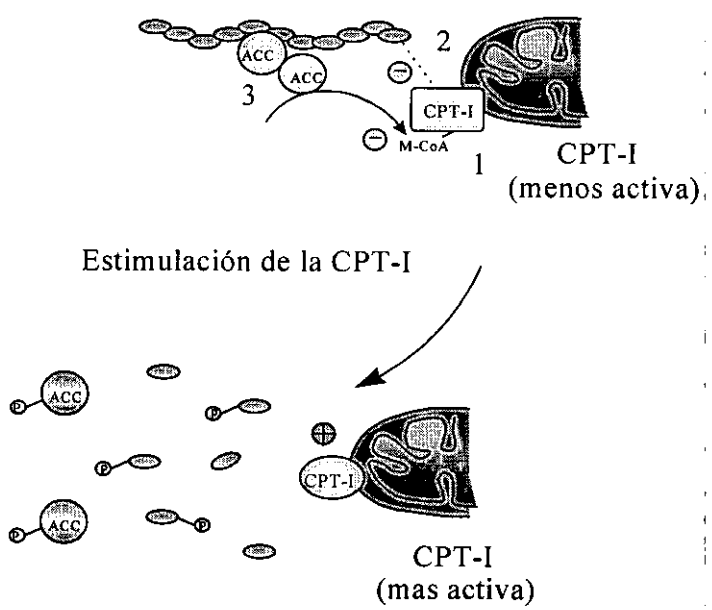
1. Regulación a corto plazo de la CPT-I

i) Actividad ACC.
El primer punto de control de la actividad CPT-I sería el de los niveles de malonil-CoA, que vienen determinados por la actividad de la ACC. El control de esta actividad enzimática en hígado depende de diferentes factores, pero fundamentalmente del grado de fosforilación de la enzima y por tanto de la actividad de las quinasas implicadas en su fosforilación (Hillgartner *et al.*, 1995).

ii) Interacciones citoesqueleto-mitocondrias.
En el interior de la célula podrían establecerse una serie de interacciones entre diferentes componentes del citoesqueleto (probablemente las citoqueratinas) y la CPT-I (o alguna otra proteína que pudiera estar implicada en regularla). Estas interacciones podrían constituir un mecanismo regulador de la CPT-I, de tal manera que su ruptura produjese un aumento de la actividad de la enzima (Capítulo 2.2).

La naturaleza de estas interacciones aún no ha sido determinada, pero el hecho de que la CPT-I pueda adoptar diferentes conformaciones en función de la concentración de malonil-CoA a la que ha sido expuesta (Zammit, 1994), junto a que la mayor parte de la enzima (incluyendo los dominios catalítico y regulador parecen encontrarse localizados hacia la cara citoplásmica de la membrana mitocondrial externa (Fraser *et al.*, 1997), posibilitaría la existencia de contactos con proteí-

Esquema 1



Esquema 1. Modelo de regulación acorto plazo de la actividad CPT-I. La actividad CPT-I podría verse regulada acorto plazo a tres niveles: 1) Actividad ACC/niveles de malonil-CoA. 2) Interacciones entre citoesqueleto y mitocondrias. 3) Localización subcelular de la ACC. Estos tres niveles de regulación podrían actuar de manera coordinada para determinar la actividad de la CPT-I en las diferentes situaciones fisiopatológicas. Abreviaturas: ACC, acetil-CoA carboxilasa; CPT-I, carnitina palmitoiltransferasa I; M-CoA, malonil-CoA

nas localizadas fuera de la mitocondria (como por ejemplo las citoqueratinas) que pudieran favorecer la conformación menos activa (tensa) de la enzima. En este contexto resulta interesante la sugerencia de que una proteína de la membrana externa mitocondrial (bcl-2) interacciona con la CPT-I en lo que podría constituir también una forma de regulación de la enzima (Paumen *et al.*, 1997b). La regulación de esas interacciones podría depender de la actividad de una serie de proteína quinasas que controlan el grado de organización del citoesqueleto.

iii) *Localización subcelular de la ACC.*

Se ha propuesto que la isoenzima de 280 KDa de la ACC podría tener una localización subcelular próxima a la CPT-I, asociándose a la propia membrana mitocondrial externa (Ha *et al.*, 1996). En la memoria se sugiere que una fracción de la ACC hepatocelular se encontraría asociada al citoesqueleto (Capítulo 2.3.3). Así, la concentración efectiva de malonil-CoA para inhibir a la CPT-I podría ser mucho mayor cuando la ACC se encontrase en las proximidades de la enzima. Los mismos factores que están implicados en el control del grado de polimerización del citoesqueleto podrían controlar el porcentaje de ACC asociada al mismo, y con ello la proximidad de ésta a la CPT-I.

2. Principales proteína quinasas implicadas en el control a corto plazo de la CPT-I

Puesto que el control de esas tres vías de regulación de la CPT-I depende en gran parte del grado de fosforilación de las diferentes proteínas implicadas, el control de la actividad de ciertas quinasas es el que determina la actividad a corto plazo de la CPT-I.

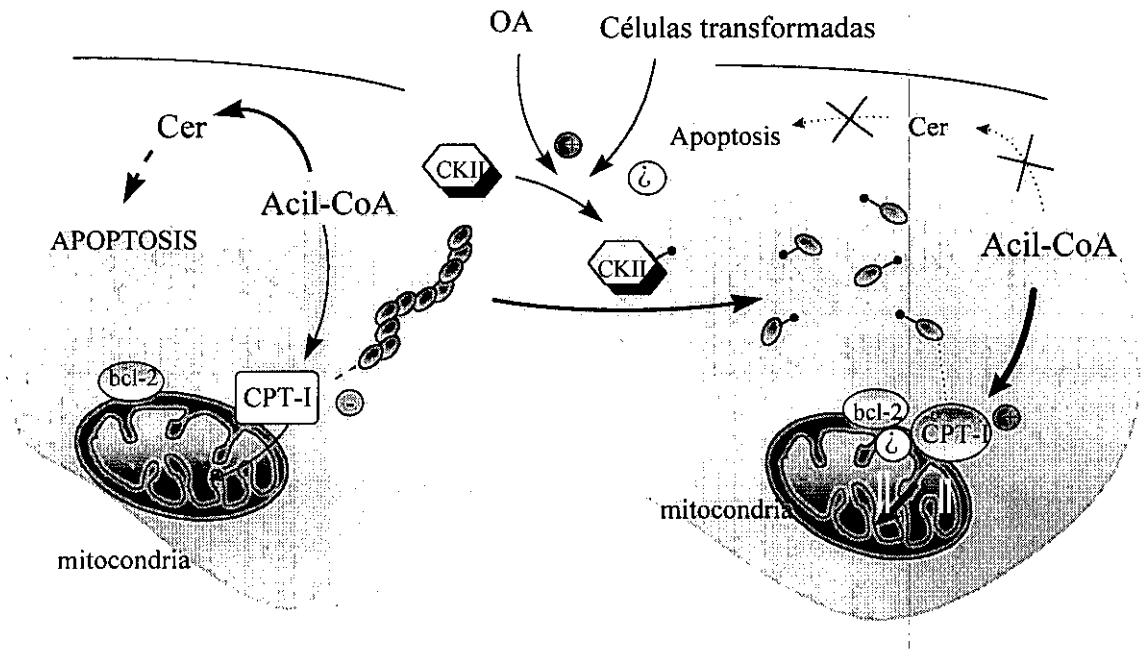
i) Ca^{2+}/CMPKII.

La activación de esta quinasa que induce el ácido okadaico conlleva: 1) la fosforilación y ruptura de los filamentos intermedios (Capítulo 2.2; Toivola *et al.*, 1997) y 2) la fosforilación e inactivación de la ACC (Haystead *et al.*, 1988). Ambos procesos traen consigo una fuerte estimulación de la actividad CPT-I. Sin embargo, la incubación con diferentes compuestos que producen un incremento en la concentración de Ca^{2+} intracelular no modifica la actividad de la CPT-I, lo que -como ya se ha discutido anteriormente (Capítulo 2.2)- podría ser el reflejo de un diferente patrón de activación de la Ca^{2+}/CMPKII de hígado con respecto a la de otros tejidos más estudiados como el cerebro. Por ello nos planteamos si el papel fisiológico que desempeña esta quinasa en la regulación de la CPT-I podría depender no tanto de un aumento transitorio de la concentración de Ca^{2+} intracelular como de otros factores que hicieran que la Ca^{2+}/CMPKII se viera estimulada de una forma más prolongada.

Puesto que el ácido okadaico es un agente promotor de tumores (Wera y Hemmings, 1995) y en células de hepatoma tiene lugar la sobreexpresión de diferentes proteína quinasas (Yang *et al.*, 1996; Tanaka y Wands, 1996), el nivel de activación de la Ca^{2+}/CMPKII que induce el ácido okadaico podría ser más parecido al que puede encontrarse en el interior de las células transformadas que al debido a una activación mediada por un incremento de la concentración de Ca^{2+} intracelular. Dado que una de las características de dichas células transformadas es que se producen grandes alteraciones en la morfología celular mediadas por una desorganización del citoesqueleto, los estudios llevados a cabo en células de hepatoma (Capítulo 2.3.1) son de gran interés. Estos datos indican que, en células Fao y HepG2 el ácido okadaico no modula la actividad de CPT-I, que estaría aumentada con respecto a la de hepatocitos. La posibilidad de que esa falta de respuesta sea debida a una pérdida (que podría ser constitutiva en esas células) de las interacciones con el citoesqueleto aparece como atractiva, puesto que estaría en consonancia con los datos anteriormente expuestos.

Por otra parte, como ya se ha mencionado, recientemente se ha establecido una relación entre actividad CPT-I y apoptosis (Paumen *et al.*, 1997a), de tal manera que una inhibición de la CPT-I aumentaría la disponibilidad de palmitoil-CoA para la síntesis de ceramidas que actúan como mediadores en dichos procesos de apoptosis (Hannun, 1996). Es interesante que: 1) las células de hepatoma cuya actividad CPT-I es más alta que la de los hepatocitos normales (Capítulo 2.3.1), son resistentes a la inducción de apoptosis por ácido palmítico (Paumen *et al.*, 1997a); 2) parte de los efectos de las ceramidas están mediados por una proteína fosfatasa activada por ceramida (CAPP) que es inhibible por ácido okadaico (Hannun, 1996); 3) bcl-2, una proteína que desempeña un papel importante en la regulación de la apoptosis interacciona con la CPT-I en la membrana mitocondrial externa (Paumen *et al.*, 1997b). Todos estos datos considerados conjuntamente apuntan a que la regulación de la actividad CPT-I podría ser importante en el control de los procesos apoptóticos. En suma, la activación de la CPT-I observada en células de hepatoma podría ser una forma de favorecer que la célula no entrase en apoptosis. En ese sentido resulta muy atractivo considerar que la desorganización del citoesqueleto (característica de las células tumorales y que ha sido descrita en este trabajo como un mecanismo de regulación de la CPT-I) pudiera contribuir a dicho bloqueo de la apoptosis (Esquema 2).

Esquema 2



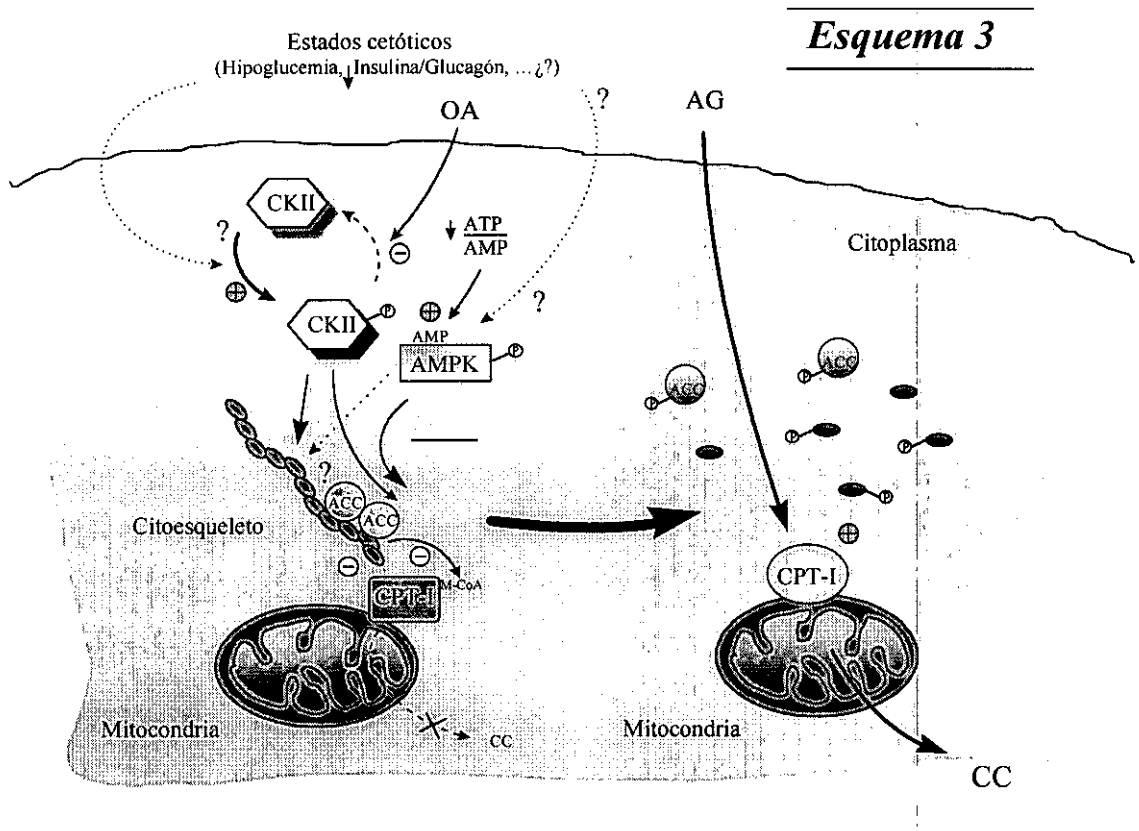
Esquema 2. La pérdida de las interacciones con el citoesqueleto podría contribuir a la inhibición de la apoptosis en células de hepatoma. La actividad CPT-I podría estar aumentada en células de hepatoma debido a la pérdida de de las interacciones con proteínas del citoesqueleto. Este aumento de actividad podría favorecer la metabolización del palmitoil-CoA (precursor de la síntesis de ceramidas) contribuyendo así al bloqueo de la apoptosis. Abreviaturas: Cer, ceramida; CKII, Ca^{2+}/calmodulina proteína quinasa II; OA, ácido okadaico.

ii) AMPK.

Diferentes datos aportados en esta memoria apuntan a que esta quinasa participaría en la regulación de la actividad CPT-I a través de los mecanismos dependiente e independiente de malonil-CoA. Así, el AICAR, un activador de la AMPK (Vincent *et al.*, 1996) estimula la CPT-I (Capítulo 2.3.2). En hígado, la AMPK es la principal quinasa responsable de la fosforilación e inactivación de la ACC, con lo que los niveles de malonil-CoA dependen en gran medida de la actividad de la AMPK (Hardie y Carling, 1997). Sin embargo, parte del efecto activador del AICAR no es debido al descenso en los niveles de malonil-CoA y puede ser prevenido por el taxol. Recientemente, hemos observado que la adición de AMPK a células permeabilizadas produce un fuerte incremento en la actividad CPT-I (datos no publicados), lo que sugiere que esta quinasa (al igual que la Ca^{2+}/CMPKII) podría afectar al estado de polimerización del citoesqueleto y estimular así a la CPT-I. Aunque por lo que sabemos aún no se ha descrito que la AMPK fosforile a elementos del citoesqueleto, esta hipótesis resulta muy atractiva , pues implicaría que la principal quinasa encargada de la regulación del metabolismo lipídico podría establecer un control coordinado de la síntesis y oxidación de ácidos grasos incidiendo en los tres mecanismos descritos al comienzo de la presente discusión: actividad ACC/niveles de malonil-CoA, interacciones citoesqueleto-mitocondrias y por tanto localización subcelular de la ACC (Esquema 3).

3. Efectos de otros mediadores

Numerosos mediadores extracelulares ejercen sus efectos sobre la CPT-I y muchos de ellos lo hacen a través de un mecanismo independiente de malonil-CoA (Guzmán y Geelen, 1993; la presente memoria). El principal factor regulador del metabolismo de ácidos grasos es el cociente insulina/glucagón (Zammit, 1996). Sin embargo el mecanismo a través del cual las variaciones en dicho cociente pueden inducir las modificaciones en la actividad CPT-I no ha sido completamente aclarado. La incubación de hepatocitos con glucagón, forskolina o db-cAMP produce una activación de la CPT-I (Guzmán y Geelen, 1993), pero la adición de PKA a células permeabilizadas no produce ningún cambio en dicha actividad enzimática (Capítulo 2.2.2). Además, esta quinasa no parece



Esquema 3. La Ca^{2+}/CMPKII y la AMPK son las dos primcipales proteínas quinasas responsables del control de la actividad CPT-I. La fosforilación y desorganización de los filamentos intermedios podría ser llevada a cabo por la Ca^{2+}/CMPKII y quizás también por la AMPK. Abreviaturas: ACC, acetil-CoA carboxilasa; AMPK, proteína quinasa activada por AMP; CKII, Ca^{2+}/calmodulina proteína quinasa II; CC, cuerpos cetónicos; CPT-I, carnitina palmitoil-transferasa I; M-CoA, malonil-Co; OA, ácido okadaico.

ser responsable de la fosforilación *in vivo* de las citoqueratinas (Toivola *et al.*, 1997) por lo que su implicación en la regulación de las interacciones entre citoesqueleto y mitocondrias parece poco probable. Por otra parte aunque se ha descrito que la PKA puede fosforilar a la ACC (Hillgartner *et al.*, 1995) y que los niveles de malonil-CoA disminuyen tras la incubación de los hepatocitos con glucagón (Guzmán y Geelen, 1993), actualmente se considera que la principal proteína quinasa responsable de la inactivación de la ACC en hígado es la AMPK (Hardie y Carling, 1997), cuya activación no se ha podido demostrar aún que dependa de la acción de mediadores extracelulares.

Tanto la insulina (Guzmán y Geelen, 1993) como los agentes que movilizan Ca^{2+} (Capítulo 2.1.1) o el hinchamiento celular (Capítulo 2.1.2) inhiben a la CPT-I utilizando un mecanismo desconocido. Si bien en todos esos casos se ha descrito la activación de proteína quinasas o fosfatasas, las posibles dianas de su acción no han sido determinadas . Por ejemplo, el ATP extracelular parece mediar sus efectos inhibitorios sobre la CPT-I a través de la activación de la PKC (capítulo 2.1.1). Sin embargo, la adición de esta quinasa a células permeabilizadas no tiene ningún efecto sobre la actividad CPT-I (Capítulo 2.2.3), y aunque se ha descrito que la PKC puede estar implicada en la fosforilación de citoqueratinas, no parece afectar a la fosforilación de los filamentos intermedios (Cadrin *et al.*, 1992).

Así., aunque permanece abierta la posibilidad de que otras proteína quinasas que se vean activadas en respuesta a las diferentes situaciones fisiopatológicas que regulan la oxidación de ácidos grasos y la cetogénesis puedan fosforilar a diferentes elementos del citoesqueleto y modular así la actividad de la CPT-I, hasta el momento solo hay pruebas de la implicación de la Ca^{2+}/CMPKII en la fosforilación y reorganización del citoesqueleto lo que junto a la modulación de los niveles de malonil-CoA, podría mediar la activación de la CPT-I.

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