Turnover of carnitine palmitoyltransferase mRNA and protein in H4IIE cells

Effect of cyclic AMP and insulin

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Regulation of the 68 kDa carnitine palmitoyltransferase (CPT) synthesis by (chlorophenylthio) cyclic AMP (cAMP) and insulin was studied in H4IIE cells in culture. Addition of 0.1 mM- or 1.0 mM-(chlorophenylthio) cAMP induced CPT mRNA and rate of transcription 2–4-fold by 15 min, reaching a plateau at 4–6-fold by 30 min. Addition of 5–15 nM-insulin plus 1.0 mM-cAMP suppressed the increases in transcription rate and mRNA levels occurring with cAMP alone. The $t_{1/2}$ for CPT mRNA was 70–80 min and was not affected by cAMP. The $t_{1/2}$ for CPT protein was 70 min, and was increased to 240 min in the presence of cAMP. The rate of CPT synthesis was also increased in the presence of cAMP. The data indicate that CPT synthesis is increased by cAMP via induction of transcription and subsequent increase in the CPT mRNA. Insulin acts to depress transcription and CPT mRNA. In addition, cAMP prolongs the $t_{1/2}$ of CPT.

INTRODUCTION

Data obtained *in vivo* have established that hepatic carnitine palmitoyltransferase (CPT total, outer and inner) activity increases in states characterized by low insulin/glucagon ratios (Brady *et al.*, 1985, 1986; Bremer, 1981; Cook, 1984; Cook *et al.*, 1984; Harano *et al.*, 1972; Saggerson *et al.*, 1982, 1984; Saggerson & Carpenter, 1981). By 24 h of starvation, the translation rate, CPT mRNA and the transcription rate of the 68 kDa mitochondrial CPT are also increased 2–3-fold (Brady *et al.*, 1988). In BB Wistar spontaneously diabetic rats, hepatic CPT activity, CPT mRNA and transcription rates are also increased 2–6-fold, and return to control values on insulin administration (Brady & Brady, 1989a). When glucagon or cyclic AMP is administered *in vivo*, there is a 3–5-fold increase in hepatic CPT transcription rate and CPT mRNA within 1 h, but no increase in CPT activity or immunoreactive protein (Brady & Brady, 1989b). These results suggest that *in vivo* the half-life ($t_{1/2}$) of CPT mRNA and of CPT protein may be quite different. Estimates of CPT half-life *in vivo* were from 2 days (Brady & Brady, 1987) to 6.5–7.5 days (Ozasa *et al.*, 1983). Preliminary estimates of CPT mRNA turnover *in vivo* were 6–8 h (L. J. Brady & P. S. Brady, unpublished work). The counter-regulatory response of hormones *in vivo* may contribute to the complexities of data interpretation.

Thus we turned to a cell model *in vitro*, the H4IIE hepatoma line, where hormonal additions can be controlled. The H4IIE line has been shown to be responsive to cAMP, dexamethasone and insulin, and Granner and colleagues have characterized the response of phosphoenolpyruvate carboxykinase to these hormones (Granner *et al.*, 1983; Sasaki *et al.*, 1984; Magnuson *et al.*, 1987; Sasaki & Granner, 1988; Chu *et al.*, 1988). Previous studies in primary hepatocytes have shown that

glucagon addition increased CPT activity by 30–50% within 30 min, and by 85% by 24 h (Harano *et al.*, 1982). Insulin depressed CPT activity by 34% (Harano *et al.*, 1982). CPT phosphorylation (68 kDa mitochondrial CPT) and activity increased with glucagon administration within 30 min (Harano *et al.*, 1985). These data indicate that increased CPT activity in response to glucagon can occur rapidly via covalent modification. The present experiments examine the effect of hormones on CPT synthesis and degradation in H4IIE cells.

EXPERIMENTAL

Cell culture

H4IIE cells were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and cultured in RPMI 1640 medium containing 2.5% (v/v) fetal bovine serum and 2.5% (v/v) newborn-calf serum in equilibrium with 5% CO₂ in air at 37 °C. The cells were plated at 5 × 10⁶ cells per 25 cm² flask. Before experiments, the medium was replaced with serum-free medium for 12 h; then the medium was replaced with serum-free medium of the same composition containing the various hormone additions (noted in the Figure legends). At the times indicated, the cells were scraped from the flask with a rubber policeman, washed once with phosphate-buffered saline (per litre: 8.0 g of NaCl, 0.2 g of KCl, 1.5 g of Na₂HPO₄, 0.2 g of KH₂PO₄, pH 7.4), and pelleted by centrifugation in an Eppendorf Microfuge for 15 s. The cells were then suspended in 45 µl of cold 10 mM-Tris/HCl (pH 7.0)/1.0 mM-EDTA (TE buffer). They were lysed by adding 5 µl of 5% Nonidet P-40 and incubating on ice for 5 min. Another 5 µl of Nonidet P-40 was then added. Samples were removed for SDS/polyacrylamide-gel electrophoresis and Western blotting at this point. This procedure has

Abbreviations used: CPT, carnitine palmitoyltransferase; cAMP, chloro(phenylthio) cyclic AMP.

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been described in detail (Brady *et al.*, 1988). The nuclei were then pelleted by centrifuging for 2.5 min in the Eppendorf Microfuge, and 50 μ l of supernatant was transferred to an Eppendorf tube containing 30 μ l of 20 $\times$ SSC (0.3 M-trisodium citrate/3 M-NaCl) plus 20 μ l of 37% (w/v) formaldehyde. The mixture was incubated at 60 °C for 15 min and dotted on to nitrocellulose at 5 and 10 μ g.

CPT mRNA and protein turnover

Turnover of a cellular component (C) is given by:

$$dC/dt = K_s - K_d C$$

where K_s = synthesis rate; K_d = first-order rate constant.

When insulin (synthesis inhibitor for CPT) is added, $K_s = 0$ and $\ln C = \ln C_0 - K_d t$, where C_0 = concn. at zero time.

Since half-life ($t_{1/2}$) = $\ln 2/K_d$, we can estimate $t_{1/2}$ as the time necessary for the log value of mRNA or protein to decay by 50%. mRNA turnover was determined as described by Hod & Hanson (1988).

In an initial experiment CPT mRNA was determined by probing a dot-blot of cytoplasmic RNA recovered from approx. 0.5×10^6 cells with the CPT cDNA probe. CPT mRNA was quantified by densitometric scanning of the autoradiogram developed from this blot. Transcription rates were determined as described below. These data are presented as Fig. 1 and serve as the basis for subsequent studies. Fig. 1 shows that CPT mRNA reaches a plateau at about 1 h after cAMP administration, and stayed at this value for 12–24 h (results not shown).

For the [3 H]uridine incorporations, 5×10^6 cells were incubated with serum-free medium for 12 h, then medium containing either cAMP or no hormone was added and incubation proceeded for 3 h. At this point, 0.2 mCi of [3 H]uridine was added to each flask. Incubation continued for 1 h. During the labelling time, controls showed no change in total CPT mRNA (dot-blot) or protein (Western blot). At the end of the labelling period, cells were rinsed with medium containing 20 mM-glucosamine, 5 mM-uridine and 5 mM-cytidine. The radioactive label was then chased with 5 mM-uridine and 5 mM-cytidine for the various times indicated to minimize re-incorporation of label. Cells were harvested at the various times shown on the graphs and prepared as described above for mRNA and transcription determinations.

Protein turnover and synthesis were determined as described by Culpepper & Liu (1983). For determination of the effect of cAMP on CPT synthesis, cells were grown in serum-free media for 12 h. Then cAMP was added to cells and the cells were incubated at 37 °C for the times noted in Fig. 6. As described by Culpepper & Liu (1983), 20 min before harvest the cells were labelled with [35 S]-methionine (100 μ Ci/ml). At the end of 20 min, cells were rinsed twice with cold phosphate-buffered saline and 1 ml of trypsin (1:250 in Ca^{2+} / Mg^{2+} -free Hanks buffered saline solution; Sigma Chemical Co., St. Louis, MO, U.S.A.). Cells were then washed once with 2 ml of serum-free RPMI 1640 and once with 3 ml of the same medium, followed by a final wash in cold phosphate-buffered saline. Cells were finally suspended in 45 μ l of TE buffer and 5 μ l of 5% (v/v) Nonidet P-40. CPT was made to react with rabbit anti-(rat CPT) antibody in excess for 2 h at 37 °C, then overnight at 4 °C. Protein A-agarose (20 μ l) was added and the cells were incubated

overnight at 4 °C. The suspension was then centrifuged in an Eppendorf microfuge and re-dissolved in 0.0625 M-Tris/HCl (pH 6.8)/2% SDS/10% glycerol/5% 2-mercaptoethanol, and prepared for electrophoresis. CPT turnover was determined as described by Culpepper & Liu (1983). Cells that had been grown for 12 h in serum-free medium were labelled with 50–100 μ Ci of [35 S]methionine/ml; controls were labelled for 4 h, and cAMP-treated cells for 2.5 h. At the end of this period, cells were rinsed with 2 $\times$ 3 ml of RPMI 1640 containing 2 mM unlabelled methionine. Cells were then incubated for 30 min at 37 °C with 3 ml of 2 mM-methionine, then washed and the same media were replaced; cells were harvested at various times afterwards, as noted in Fig. 6. Precipitation and determination of labelled CPT were done as described above for the synthesis experiments.

Determination of protein, mRNA and transcription rate

CPT mRNA was quantified by Northern and dot blots. CPT protein was assessed by Western blot after SDS/polyacrylamide-gel electrophoresis. We have previously described the application of these techniques to CPT (Brady *et al.*, 1988; Brady & Brady, 1989a). Transcription rates were determined by nuclear run-on assays using [α - 32 P]UTP as described by Morris *et al.* (1987), as previously described (Brady *et al.*, 1988).

Materials

[α - 32 P]UTP, [γ - 32 P]ATP, [5,6- 3 H]uridine and [35 S]methionine were obtained from DuPont/New England Nuclear (Boston, MA, U.S.A.). RPMI 1640, fetal-bovine serum and newborn-calf serum were obtained from Sigma. Culture flasks were from Corning. (Chlorophenylthio) cyclic AMP (hereafter referred to as cAMP) was obtained from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). pBluescript was obtained from Stratagene (La Jolla, CA, U.S.A.). Pig insulin was kindly given by Dr. Thomas Jeatran, Lilly Co. (Indianapolis, IN, U.S.A.). For most purposes, water was diethyl pyrocarbonate-treated.

RESULTS

Verification of inhibition of mRNA synthesis by insulin

Fig. 1 shows the response of CPT transcription rate and CPT mRNA to additions of cAMP and insulin + cAMP. Both transcription rate and CPT mRNA were increased within 15 min of cAMP addition. Insulin addition blocked these increases in mRNA over the time of the experiment (1 h as shown here; and up to 8 h, not shown). Thus we assumed that the K_s for CPT mRNA was 0 in the experiments where insulin was added under these conditions. Fig. 2 also shows the long-term response of CPT mRNA and CPT protein to cAMP. Fig. 3 shows the increase in immunoreactive CPT as measured by a representative Western blot. Although only semi-quantitative, densitometric scanning of Western blots from three experiments indicated a statistically significant (relative to the initial value) 2-fold increase at 12–24 h. To assess this apparent effect of cAMP more accurately, the synthesis and degradation of CPT protein were investigated (see below).

CPT-mRNA turnover

This was estimated in two ways, as described by Hod & Hanson (1988). First, we measured the decay of CPT

Fig. 1. Effect of cAMP and insulin on CPT mRNA and transcription rates

Cells were treated as described in the Experimental section. Cells were preincubated in serum-free medium for 12 h, then serum-free medium containing the various hormones was added, and transcription rate and mRNA were measured at the times indicated (Brady *et al.*, 1988; Brady & Brady, 1989a). In brief, values are expressed relative to the mean initial value. mRNA values were determined by densitometric scanning of autoradiograms developed by probing RNA from 5×10^6 cells with a CPT probe. Initial transcription was 339 p.p.m. cAMP was added at 1 mM; insulin + cAMP were added together at zero time (insulin at 15 nM). Each point represents the mean of three experiments $\pm$ S.E.M. Symbols: CPT mRNA: ■, cAMP; □, insulin. Transcription rate: ●, cAMP; ○, insulin.

mRNA after initial cAMP induction (3 h), followed by insulin addition to block synthesis of CPT mRNA. Secondly, we measured the decay of [5,6- 3 H]uridine that had been incorporated into CPT mRNA. Fig. 4 presents the data showing the decay of CPT mRNA after cAMP stimulation for 3 h, followed by insulin addition, in the presence and absence of cAMP. The $t_{1/2}$ for CPT mRNA in the presence of insulin alone was about 70 min (determined by linear-regression analysis). When no hormone additions were made, the decay was similar to that when insulin was added. However, where insulin was not present (cAMP added alone), K_s was not 0, so interpretation of the results is more complicated. Addition of cAMP or cAMP + insulin greatly attenuated the decay of CPT mRNA seen in the presence of insulin alone. From these data and the data showing cAMP stimulation of transcription rate, it can be concluded that cAMP alone increased transcription rates over this time, but, in addition, may have potentially stabilized CPT mRNA. The results with cAMP + insulin addition are confusing, since Fig. 1 shows insulin inhibition of transcription rate

Fig. 2. Long-term relationship of CPT immunoreactive protein and CPT mRNA

CPT mRNA and immunoreactive protein were measured as described by Brady *et al.* (1988).

and mRNA induction in the presence of cAMP. The specific protocol used for the experimental additions is probably responsible for the difference in data. To obtain the data for Fig. 1, both insulin and cAMP were added at zero time, whereas for Fig. 4 the cells were first induced for 3 h with cAMP, and then cAMP + insulin together were added. Insulin was not effective in depressing the effect of previous cAMP addition under these conditions.

The second set of studies examined the decay of [5,6-

Fig. 3. CPT immunoreactive protein

CPT immunoreactive protein at the various time points was detected by Western blot and quantified as described by Brady *et al.* (1988).

Fig. 4. Effect of cAMP and insulin on CPT mRNA decay

Cells were cultured as described in the Experimental section and incubated in serum-free medium for 12 h before the experiment began. Cells were then induced with serum-free medium containing 0.1 mM-cAMP for 3 h; then the medium was changed to serum-free medium with the additions shown, and CPT mRNA was isolated as described in the Experimental section and quantified by dot-blot (Brady *et al.*, 1988). Each point is the mean for three experiments. The S.E.M., determined from analysis of variance, was 0.28. Additions: $\square$, none; $\blacksquare$, 5 nM-insulin; $\triangle$, 1 mM-cAMP; $\blacktriangle$, 1 mM-cAMP + 5 nM-insulin.

^3H]uridine incorporated into CPT mRNA after a pulse-chase under steady-state conditions. Steady-state conditions were confirmed as described by Hod & Hanson (1988). Control cells received no hormone addition for 3 h before the addition of ^3H]uridine; cAMP-treated cells had cAMP present for 3 h before addition of ^3H]uridine to reach a steady-state induction of CPT mRNA before labelling. That the cells were in steady state is confirmed by the relatively stable amount of CPT mRNA in control and cAMP-treated cells over time (determined by dot-blot; results not shown), even though the absolute level of CPT mRNA was higher in the cAMP-treated cells. The uridine-labelling method does not use inhibitors of RNA synthesis; the $t_{1/2}$ is determined by measuring the decay of label from CPT mRNA over time. The incorporation of labelled uridine into total cellular RNA was equivalent in control and cAMP-treated cells (21×10^6 and 23×10^6 d.p.m.). Re-incorporation of label was minimized by the continued presence of cytidine and uridine to dilute out the labelled uridine. Fig. 5 (inset) shows that decay of total labelled RNA also occurs, but the decay of CPT mRNA is expressed relative to total RNA radioactivity (d.p.m.). These experiments show that the $t_{1/2}$ of CPT mRNA is about 70–80 min under control conditions or when cAMP is present. The data in Fig. 5 suggest that the effect of cAMP is strictly on CPT mRNA transcription; it does not affect RNA degradation.

CPT protein turnover

The $t_{1/2}$ for the CPT protein was estimated from the loss of ^{35}S]methionine label over time after a pulse of

Fig. 5. CPT mRNA turnover

Cells were grown in serum-free medium for 12 h, then the medium was changed to one with no cAMP addition (control) or with 1 mM-cAMP in serum-free medium for 3 h, and $[5,6-^3\text{H}]$ uridine was added during the last 1 h. The medium was then replaced with serum-free medium containing no additions or 1 mM-cAMP, and the decay of label was monitored over time. RNA was extracted and quantified by dot-blot as described in the Experimental section and by Brady *et al.* (1988). Values are expressed relative to the initial value (407 ± 65 p.p.m. for the control, 867 ± 114 p.p.m. for the cAMP-treated). Each point is the mean for three experiments. The S.E.M., determined from the analysis of variance, was 0.15. Curves: $\blacksquare$, cAMP; $\bullet$, control. Inset shows d.p.m. in total cellular RNA.

^{35}S]methionine, followed by a chase of unlabelled methionine. The protein $t_{1/2}$ in the absence of cAMP is about 70 min; in the presence of cAMP, the $t_{1/2}$ is extended to 240 min (Fig. 6). Synthesis rate (K_s) was also estimated by examining the incorporation of ^{35}S]methionine over time in the presence and absence of cAMP (Fig. 7). This Figure shows that cAMP increases CPT synthesis about 2-fold under these conditions.

DISCUSSION

The present studies show that the transcription of CPT mRNA from DNA in H4IIE cells is induced by cAMP and inhibited by insulin. There is a good correlation between the induction of transcription and the increase in CPT mRNA, but some temporal lag in the increase in immunoreactive protein under the conditions used. Taken as a whole, the data suggest that increases in transcription rate are probably the major factor in the increased CPT mRNA and protein levels seen in response to cAMP. Further, CPT mRNA degradation is not affected by cAMP.

The present experiments were done in response to the observations that (1) *in vivo*, CPT activity does not rise within a few hours in starvation, but that injection of

Fig. 6. CPT protein turnover

Cells were cultured and the studies done as described in the Experimental section. In addition, total trichloroacetic acid-precipitable radioactivity was measured and found to be constant over time (approx. 2.1×10^6 d.p.m.). CPT protein was precipitated initially with anti-CPT antibody for 2 h at 37 °C, then overnight at 4 °C. Protein A-agarose was added and the antibody precipitated overnight at 4 °C. The protein A-agarose/antibody was precipitated by centrifugation in an Eppendorf centrifuge, then washed five times with 0.1 M-NaCl/1 mM-EDTA/10 mM-Tris/1 % Nonidet P-40, pH 8.0, and finally washed in 0.0625 M-Tris (pH 6.8)/2 % SDS/10 % glycerol/5 % mercaptoethanol, and centrifuged. The supernatant was applied to a 10 %-acrylamide/SDS gel and electrophoresed. The CPT was detected by autoradiography and the band cut out and counted for radioactivity by liquid scintillation. Values are expressed relative to the initial value (p.p.m.): 214 for control and 350 for cAMP-treated. Each point is the mean for three experiments. The S.E.M., determined from the analysis of variance, was 0.07. Curves: ■, cAMP; ●, control.

Fig. 7. CPT synthesis

Cells were labelled with [35 S]methionine in the presence or absence of cAMP, as noted in the Experimental section. Incorporation of the label was noted over the times indicated by the methods described for Fig. 6. Each point is the mean $\pm$ S.E.M. for three experiments. Curves: ■, cAMP ($y = 33.71 \pm 78.19x$; $r = 0.99$). ●, Control ($y = 25.06 \pm 32.28x$; $r = 0.97$).

cAMP or glucagon increased CPT-mRNA transcription and amounts within 15–30 min, without increases in CPT activity or immunoreactive protein (Brady & Brady, 1989b), and (2) CPT-protein $t_{1/2}$ was 2–7.5 days as measured *in vivo* (Ozasa *et al.*, 1983; Brady & Brady, 1987), but CPT-mRNA $t_{1/2}$ *in vivo* was 6–8 h. Our main question, then, was ‘what is the relationship between CPT-mRNA turnover and CPT-protein turnover?’. Since CPT is a membrane protein, this question is even more complicated, as targeting and insertion of nascent protein into the mitochondrial inner membrane might play a role in stability. The H4IIE hepatoma cell line was chosen since it has been shown to be responsive to insulin and cAMP by Granner’s group, as well as because it allowed us to determine the effects of each hormone separately over time, without a counter-regulatory response (Granner *et al.*, 1983; Sasaki *et al.*, 1984; Magnuson *et al.*, 1987).

The rate of turnover of eukaryotic mRNA varies greatly, from minutes to hours and days (Ragow, 1987). mRNAs with short half-lives (approx. 30 min) include those for interferon, granulocyte/macrophage-stimulat-

ing factor and oncogenes *c-myc* and *c-fos*. The turnover of many eukaryotic mRNAs is influenced by the cell cycle and/or hormones. Vitellogenin mRNA is stabilized by oestrogen with a $t_{1/2}$ of 16 h in control and 480 h in oestrogen-treated cells, and Type I procollagen mRNA is destabilized by glucocorticoids and stabilized by transforming growth factor β (Ragow, 1987). The $t_{1/2}$ of CPT mRNA of 70 min is within the range reported for other regulatory proteins where synthesis is induced by glucagon/cAMP and is depressed by insulin. One such regulatory protein is phosphoenolpyruvate carboxykinase. One estimate of $t_{1/2}$ of its mRNA in FTO-2B cells is 40 min in the presence of insulin; however, cAMP extended this to about 250 min when present, in contrast with the lack of effect of cAMP on CPT-mRNA $t_{1/2}$ presented here. We have previously shown that glucagon and cAMP administered *in vivo* increase CPT transcription rate and mRNA within 15–30 min (Brady & Brady, 1989b). The reasonably short CPT-mRNA $t_{1/2}$ should have physiological relevance in that it would allow a rapid change in fatty acid oxidation via changing

synthetic capacity under conditions such as feeding of a high-fat diet, starvation or diabetes. However, under conditions *in vivo* this was not reflected in an increase in CPT activity within 5 h.

We used two independent means to measure CPT-mRNA turnover, and both methods gave similar results, a $t_{1/2}$ of about 70–80 min. This $t_{1/2}$ was equivalent in cells that were cAMP-treated (Fig. 5). This suggests that the major effect of cAMP on CPT mRNA is via transcriptional increases, not stabilization of mRNA. CPT immunoreactive protein began to increase within 1–2 h in these studies, but did not attain statistical significance until 12 h. The lack of increase in immunoreactive CPT in the studies *in vivo* might indicate that a counter-regulatory response to glucagon or cAMP was elicited to depress the effects of hyperglycaemic response to glucagon or cAMP. *In vitro*, this lag suggests that translational or post-translational (such as membrane processing) events may limit the rate of appearance of active CPT. Since the CPT is synthesized as a larger-molecular-mass precursor for targeting to and through the mitochondrial inner membrane, it is tempting to propose that the membrane processing is the limiting event. This lag in the appearance of CPT protein behind CPT mRNA will certainly require further investigation.

Fig. 7 shows that CPT synthesis was increased over control by cAMP addition. These data complement the data showing increased CPT-mRNA transcription rate and mRNA amounts when cAMP was added to the media. However, in the presence of cAMP, the decay of labelled methionine from CPT was attenuated such that protein $t_{1/2}$ was extended from 70 min to 240 min. This is in contrast with the data for CPT-mRNA turnover that show that cAMP does not affect the CPT-mRNA $t_{1/2}$. Post-translational modifications and protein processing are also steps at which the regulation of the number of molecules of active enzyme occurs. Harano *et al.* (1985) have proposed that the glucagon-mediated increase in CPT activity results from phosphorylation of the CPT. Although we have not examined this proposed mechanism, it is also possible that phosphorylation of CPT enhances stability.

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