EFFECT OF CORDYCEPIN AND CYCLOHEXIMIDE ON THE INDUCTION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE BY DEXAMETHASONE OR N^6, $O^{2'}$-DIBUTYRYL CYCLIC AMP IN THE ISOLATED PERFUSED RAT LIVER

W. KRONE, W. MARQUARDT, H.J. SEITZ and W. TARNOWSKI

Physiologisch-Chemisches Institut, Universität-Krankenhaus Eppendorf, 2 Hamburg 20, Martinistrasse 52, Bundesrepublik Deutschland

Received 4 July 1975

1. Introduction

In a previous communication we have reported [1] that injection of N^6, $O^{2'}$-dibutyryl cyclic adenosine-3', 5'-monophosphate (DBcAMP) into adrenalectomized rats initially stimulated hepatic phosphoenolpyruvate carboxykinase (PEP-CK, EC 4.1.1.32) to the same degree as in intact animals. However, despite of repeated DBcAMP administration, the raise in enzyme activity ceased after 2 hr, but could be restored when hydrocortisone had been replaced at zero time. Essentially the same results were obtained with the isolated perfused rat liver [2], indicating the apparent interaction between glucocorticoids and cyclic AMP in PEP-CK induction at the tissue level. From these findings it was concluded that cyclic AMP elevates PEP-CK activity by a mechanism which in principle works independently from glucocorticoids, while glucocorticoids stimulate a different process which is required for the maintenance of cAMP-mediated enzyme induction.

As to the mode of the interaction between glucocorticoids and cyclic AMP it was assumed that glucocorticoids promote an increase of translatable PEP-CK messenger-RNA (mRNA) while cyclic AMP stimulates the translation of preexisting mRNA templates. If this hypothesis were correct inhibitors of translation should prevent the stimulation of PEP-CK activity by both glucocorticoids and cyclic AMP. In contrast, inhibitors of transcription would be expected to prevent the elevation of the enzyme provoked by glucocorticoids but not to block the action of DBcAMP on PEP-CK induction.

In the present work this assumption was tested in the isolated perfused rat liver using cordycepin or cycloheximide, respectively. The results obtained have confirmed our working hypothesis: Glucocorticoids appear to stimulate PEP-CK-induction at the level of transcription whereas cAMP promotes enzyme synthesis at a posttranscriptional step.

2. Materials and methods

Male Wistar rats (weighing 250 g each; obtained from E. Jautz, Kisslegg/Allgau, Germany) were used as liver donors. They were fed ad libitum on a low-protein diet (C 1004; Altromin GmbH, Lage/Lippe, Germany) for 3 days prior to perfusion. At the beginning of the experiments animals were anesthetized by intraperitoneal injection of hexobarbital (15 mg/100 g body wt). Livers were isolated and perfused by the technique of Schimassek [3], with a 10% (v/v) emulsion of fluorocarbon 43 made up in the nonionic detergent Pluronic F 68 by sonication and diluted with a saline buffer. Further details of the preparation of the fluorocarbon medium have been described [4]. The perfusate volume was 150 ml containing 135 ml of the fluorocarbon medium, 15 ml of pig plasma and 100 mg of glucose/100 ml.

At the beginning of perfusion one liver lobe was ligated at its base and excised. Cordycepin or cyclo-

North-Holland Publishing Company – Amsterdam

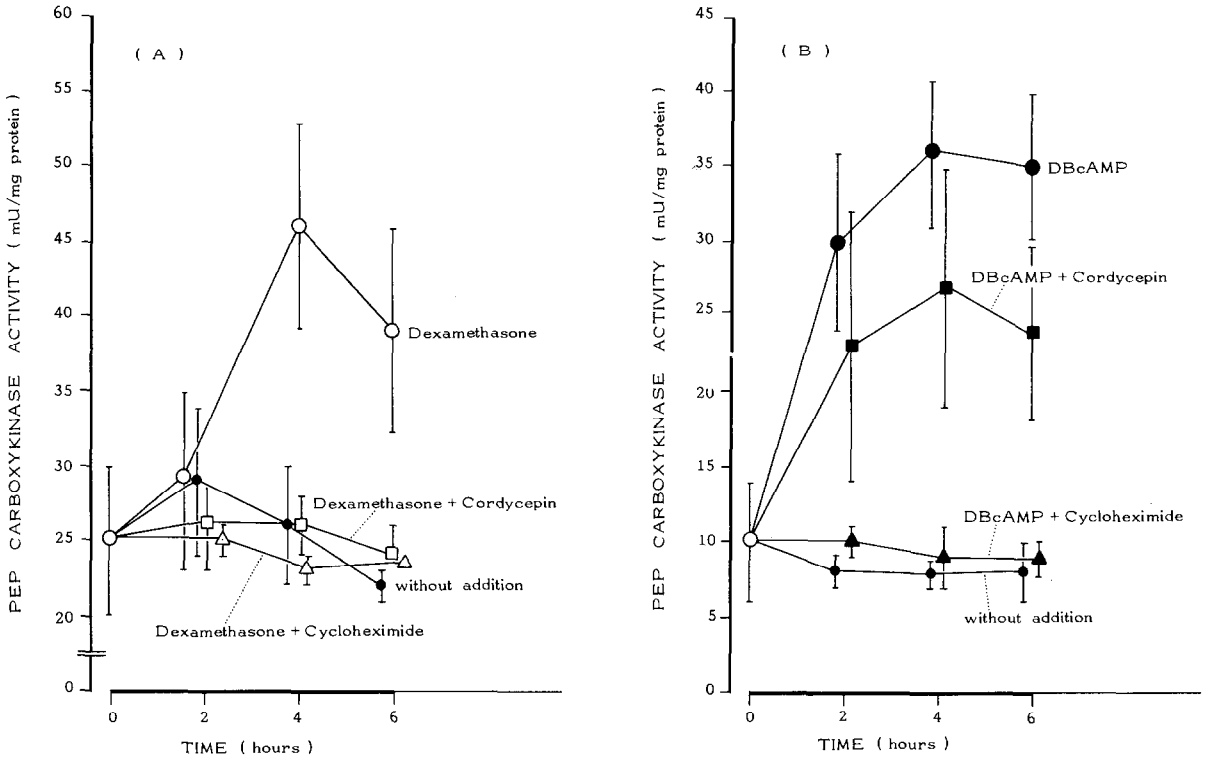

Fig.1. Effect of cordycepin and cycloheximide on the response of hepatic phosphoenolpyruvate carboxykinase activity to dexamethasone (A) or dibutyryl cyclic AMP (DBcAMP) (B) in the isolated perfused liver of intact fed rats. Perfusion was performed as described under Materials and methods. Dexamethasone (50 μM) or DBcAMP (0.2 mM) was added to the medium into the reservoir at zero time. Cordycepin (10 μg/ml) or cycloheximide (20 μg/ml) was added 20 min prior to the inducers.

Number of determinations in fig.1A: For 'without addition' n = 3; for dexamethasone, n = 4; for dexamethasone + cordycepin, n = 4; for dexamethasone + cycloheximide, n = 3.

Statistical analysis in fig.1A: Dexamethasone values, 2, 4 and 6 hr versus 'without addition': not significant (= n.s.), $p < 0.005$ and $p < 0.005$; dexamethasone values, 2, 4 and 6 hr versus dexamethasone + cordycepin values: n.s., $p < 0.0025$ and $p < 0.005$; dexamethasone values, 2, 4 and 6 hr versus dexamethasone + cycloheximide values: n.s., $p < 0.0025$ and $p < 0.01$

Number of determinations in fig.1B: For 'without addition', n = 4; for DBcAMP, n = 8; for DBcAMP + cordycepin, n = 7; for DBcAMP + cycloheximide, n = 3.

Statistical analysis in fig.1B: DBcAMP values, 2, 4 and 6 hr versus 'without addition': $p < 0.0005$; DBcAMP values, 2, 4 and 6 hr versus DBcAMP + cordycepin values: $p < 0.05$, $p < 0.01$ and $p < 0.0025$; DBcAMP values, 2, 4 and 6 hr versus DBcAMP + cycloheximide: $p < 0.0005$; DBcAMP + cordycepin values, 2, 4 and 6 hr versus cordycepin values (cf. table 1): $p < 0.005$, $p < 0.0005$ and $p < 0.0005$.

Values are given as means ± SD.

heximide was added immediately after start of perfusion, DBcAMP or dexamethasone 20 min later (= zero time) to the reservoir to give the final concentrations indicated in the results and in the legend to fig.1. At 2, 4 and 6 hr further liver lobes were removed.

Tissue was immediately homogenized in 7 vol of ice-cold 0.15 M KCl solution in a glass Potter–Elvehjem homogenizer with a Teflon pestle at 1000 rev/min for 30 sec. The homogenate was centrifuged at 150 000 *g* for 30 min at 0 °C and PEP-CK activity was immediately assayed in the supernatant by the method of Seubert and Huth [5]. Enzyme activity units (U) are expressed as μmoles of oxaloacetate converted into phosphoenolpyruvate per min at 37 °C under the conditions of the

assay. Protein was measured by the biuret method [6]. The significance of differences between means was established by Student's t-test.

Substrates, nucleotides (including DBcAMP) and test enzymes were purchased from C.F. Boehringer und Söhne GmbH (Mannheim, Germany). Dexamethasone (Fortecortin) was obtained from Hoechst AG (Frankfurt/Main-Hoechst, Germany). Cordycepin and cycloheximide were obtained from Sigma Chemical Company (St. Louis, USA).

3. Results

The effect of cordycepin or cycloheximide on the increase of PEP-CK activity, produced by either dexamethasone or DBcAMP in the isolated perfused rat liver is shown in fig.1.

As can be seen from fig.1A dexamethasone (50 μM), after a lag period of 2 hr, stimulated PEP-CK activity effectively during another 2-hr period, the elevated enzyme level remaining approximately constant up to the end of the perfusion. When cordycepin (10 μg/ml) or cycloheximide (20 μg/ml) were added into the perfusate 20 min prior to the inducer, the glucocorticoid-mediated rise of enzyme activity was completely prevented.

DBcAMP (0.2 mM) provoked an immediate and rapid increase of PEP-CK activity for at least 4 hr (fig.1B). Again, cycloheximide totally blocked the response of the enzyme to the cyclic nucleotide. However, in contrast to PEP-CK stimulation provoked by dexamethasone, the stimulation provoked by DBcAMP is not prevented by cordycepin, though the increase of the enzyme was somewhat less pronounced.

It is worth noticing that neither cordycepin nor cycloheximide led to a significant decrease in PEP-CK activity throughout the experimental period (table 1). Similar results have been obtained recently by Ballard and Hopgood [7]: While rat liver PEP-CK is normally degraded with a half-time of approximately 6 hr [8], these authors found that after injection of actinomycin D or cycloheximide into adult rats the turnover time of the enzyme protein was much longer.

4. Discussion

From our present findings three conclusions can be drawn:

(1) Since cycloheximide completely blocked the increase of PEP-CK activity, elicited by either dexamethasone or DBcAMP, the response of the enzyme to both these agents is apparently the consequence of de novo enzyme synthesis. This result, obtained with the isolated intact rat liver, is in accordance with recent findings, obtained with separate experimental systems: In cultured Reuber H_{35}-hepatoma cells cycloheximide prevented the increase of PEP-CK activity provoked by dexamethasone [9]. The antibiotic also suppressed the rise of enzyme activity produced by DBcAMP in adult rat liver [10], in fetal rat liver in

Table 1
Effect of cordycepin and cycloheximide on phosphoenolpyruvate carboxykinase activity in the isolated perfused rat liver of intact fed rats

Reference	Treatment	No.	Perfusion time (hr)			
			0	2	4	6
Fig.1A	None	3	25 ± 5	29 ± 5	26 ± 4	22 ± 1
	Cordycepin	4	26 ± 3	23 ± 2	21 ± 4	21 ± 2
	Cycloheximide	4	25 ± 4	25 ± 1	23 ± 1	24 ± 2
Fig.1B	None	4	10 ± 4	8 ± 1	8 ± 1	8 ± 2
	Cordycepin	4	12 ± 3	9 ± 2	8 ± 4	8 ± 2
	Cycloheximide	4	10 ± 2	10 ± 1	8 ± 1	9 ± 2

Perfusion was performed and cordycepin (10 μg/ml) or cycloheximide (20 μg/ml) was added immediately after start of perfusion as described under Materials and methods.
Values are given as means ± SD.

organ culture [11] and in cultured hepatoma cells [9]. As to the DBcAMP-mediated increase of PEP-CK activity in vivo [10] and in Reuber H_{35} tumor cells [12] enhanced enzyme synthesis has been proved directly: In both systems the cyclic nucleotide concomitantly stimulated the rate of [^{3}H]leucine incorporation into immuno-precipitated specific enzyme protein.

(2) Since cordycepin* completely blocked the increase of PEP-CK activity, provoked by dexamethasone, but did not prevent the elevation of the enzyme after DBcAMP administration, it is obvious, that the response of the enzyme to glucocorticoids requires new mRNA synthesis whereas its response to cyclic AMP does not. Similar results have been reported recently for Reuber H_{35} tumor cells: The early rise of PEP-CK due to DBcAMP was not prevented by the addition of actinomycin D, whereas the effect of dexamethasone was completely suppressed [12]. These findings confirm the hypothesis derived from our previous in vivo and in vitro experiments [1,2]: Glucocorticoids stimulate PEP-CK de novo synthesis by increasing the level of translatable mRNA whereas cyclic AMP enhances the expression of preexisting mRNA templates.

On the other hand there are data which seem to be contradictory to that hypothesis: (*i*) Actinomycin D largely prevented the induction of hepatic PEP-CK provoked by intraperitoneal injection of cAMP into rat fetuses in utero [14]. (*ii*) Actinomycin D strongly suppressed the 4-fold increase of PEP-CK activity after addition of DBcAMP to fetal rat liver cells in organ culture [11]. It should be noted, however, that the half-life of PEP-CK mRNA appears to be about 1 hr (cf. below) and that of the enzyme protein is about 6 hr [8]. One would expect, therefore, that the inability of actinomycin to prevent the cAMP-mediated induction of PEP-CK could be detected only for a short period after the addition of the inhibitor. Yet, in the studies cited above, enzyme activity was not monitored before 3.5 or 4 hr, respectively.

(3) From the time-course of cAMP-mediated PEP-CK induction in the presence of cordycepin (cf. fig.1B) the half-life of the PEP-CK mRNA template can be estimated to be approximately 1 hr. This value is identical with that obtained for the adult rat liver in vivo [15].

* Cordycepin is assumed to abolish mRNA biogenesis by inhibiting the posttranscriptional addition of a polyadenylic acid segment to heterogeneous nuclear RNA [13].

Acknowledgements

We wish to thank Miss B. Dunkelmann and Miss D. Luda for excellent technical assistance. This work was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich -34-Endokrinologie.

References

[1] Krone, W., Huttner, W. B., Seitz, H. J. and Tarnowski, W. (1974) FEBS Lett. 46, 158–161.
[2] Krone, W., Huttner, W. B., Seitz, H. J. and Tarnowski, W. (1975) FEBS Lett. 52, 85–89.
[3] Scimassek, H. (1963) Biochem. Z. 366, 460–467.
[4] Krone, W., Huttner, W. B., Kampf, S. C., Rittich, B., Seitz, H. J. and Tarnowski, W. (1974) Biochim. Biophys. Acta 372, 55–71.
[5] Seubert, W. and Huth, W. (1965) Biochem. Z. 343, 176–191.
[6] Layne, E. (1957) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds.) Vol. 3, pp. 450–451. Academic Press, Inc., New York.
[7] Ballard, F. J. and Hopgood, M. F. (1973) Biochem. J. 136, 259–264.
[8] Hopgood, M. F., Ballard, F. J., Reshef, L. and Hanson, R. W. (1973) Biochem. J. 134, 445–453.
[9] Barnett, C. A. and Wicks, W. D. (1971) J. Biol. Chem. 246, 7201–7206.
[10] Wicks, W. D., Lewis, W. and McKibbin, J. B. (1972) Biochim. Biophys. Acta 264, 177–185.
[11] Wicks, W. D. (1969) J. Biol. Chem. 244, 3941–3950.
[12] Wicks, W. D. and McKibbin, J. B. (1972) Biochem. Biophys. Res. Commun. 48, 205–211.
[13] Darnell, J. E., Philipson, L., Wall, R. and Adesnik, M. (1971) Science 174, 507–510.
[14] Yeung, D. and Oliver, I. T. (1968) Biochemistry 7, 3231–3239.
[15] Tilghman, S. M., Hanson, R. W., Reshef L., Hopgood, M. F. and Ballard, F. J. (1974) Proc. Natl. Acad. US 71, 1304–1308.