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REGULATION OF PHOSPHO*ENOL*PYRUVATE CARBOXYKINASE BY GLUCAGON AND GLUCOCORTICOIDS IN PRIMARY CULTURES OF RAT HEPATOCYTES

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Summary

Activity changes of phospho*enol*pyruvate carboxykinase (GTP:oxaloacetate carboxylase, transphosphorylating, EC 4.1.1.32) were followed in hepatocyte cultures under defined hormone conditions. In the absence of hormones a cellular activity of 10 U/g protein was established. In the continuous presence of glucagon (20 nM) or triamcinolone (0.1 μM) or glucagon and triamcinolone activity levels of 17, 23 and 60 U/g protein were found at the third day of culture. After 2 days culture in hormone-free medium, phospho*enol*pyruvate carboxykinase activity could be elevated by the respective hormones with initial rates of about 2.5, 4.5 or 10 U/g protein per h. Increase of enzyme activity was reduced by simultaneous addition of 50 nM insulin and was completely inhibited in the presence of 10 μg/ml cycloheximide. Addition of actinomycin D (100 ng/ml) inhibited the independent effects of glucagon and of glucocorticoids on phospho*enol*pyruvate carboxykinase activity. Glucagon however, did enhance phospho*enol*pyruvate carboxykinase activity even in the presence of 300 ng/ml actinomycin D provided that hepatocytes had been pretreated with a glucocorticoid. Dose-dependent stimulation of (1) phospho*enol*pyruvate carboxykinase activity, of (2) glucose formation from lactate and of (3) interconversion of glycogen phosphorylase by glucagon was studied, and half-maximal effects were obtained at 50 ± 30 pM glucagon.

The rate of enzyme activity decline was investigated after withdrawal of glucagon and triamcinolone from 2-day-old cultures. Phospho*enol*pyruvate carboxykinase activity decreased with a constant rate of about 2 U/g protein per h for about 16 h. No influence of insulin, of cycloheximide or of glucagon, triamcinolone and cycloheximide was observed.

Introduction

Phospho*enol*pyruvate carboxykinase in liver can be enhanced by starvation, by the action of glucagon or alternatively by cyclic AMP and glucocorticoids (for review see refs. 1 and 2). With regard to the mechanism of regulation it was demonstrated in Reuber H 35 hepatoma cells [3,4] and perfused rat liver [5] that cyclic AMP could increase phospho*enol*pyruvate carboxykinase activity although RNA synthesis was blocked [3—5]. For the action of glucocorticoids, however, RNA synthesis was essential [3,5—7]. Consequently, a posttranscriptional involvement of cyclic AMP and a 'permissive' effect of glucocorticoids on phospho*enol*pyruvate carboxykinase regulation was concluded [3—5,7]. At variance with this conclusion, cyclic AMP induction of phospho*enol*pyruvate carboxykinase in fetal rats, organ cultures and hepatocyte cultures has been shown to be sensitive to actinomycin D [8,9,12]. Furthermore, it was demonstrated recently that cyclic AMP increases the level of mRNA for phospho*enol*pyruvate carboxykinase in fetal and in adult rat liver [10,11]. These conflicting results were difficult to resolve since different model systems have been used. However, it is conceivable that both, transcriptional and post-transcriptional stimulation by cyclic AMP are involved in phospho*enol*pyruvate carboxykinase regulation.

Cultured hepatocytes provide a model system, which is tightly related to intact liver [12] and which allows discrimination between different hormone actions on the same enzyme target [13]. Therefore, experiments with cultured hepatocytes could show simultaneously, (1) the additive actions of glucagon and glucocorticoids on mRNA synthesis and (2) the permissive actions of glucocorticoids for glucagon stimulation of phospho*enol*pyruvate carboxykinase synthesis.

Materials and Methods

Hepatocyte cultures were prepared by liver perfusion using fed rats as described previously [14]. Isolated parenchymal cells were suspended in cold (4—8°C) culture medium containing 5% newborn calf serum at $2.5 \cdot 10^5$ cells/ml. $2.5 \cdot 10^6$ cells were added to one 10 cm plastic culture dish. 3 h after seeding, the serum containing culture-medium was removed and 10 ml of fresh medium were added. This medium consisted of salts, amino acids and vitamins according to Dulbecco's modification of minimal essential medium, plus 10 mM glucose, 5 mM lactate, 0.1% bovine serum albumin, 40 mM $NaHCO_3$, penicillin (50 units/ml), streptomycin (50 μg/ml), and hormones as indicated. Medium was exchanged daily.

For phospho*enol*pyruvate carboxykinase synthesis and degradation experiments, dishes were incubated for 30 min with 10 ml hormone-free medium in order to reduce previously added hormones to below 1 pM. Then fresh medium, containing inducers or inhibitors as indicated, was added. The incubation was stopped by washing the dishes twice with cold saline (0.9% NaCl/10 mM Tris, pH 7.4). Cultures were frozen on a block cooled by liquid N_2 and stored at —40°C for subsequent determinations of enzyme activities.

For the phospho*enol*pyruvate carboxykinase assay, 0.5 ml of 0.15 M KCl

were added to one dish and the cells were scraped into this solution. The suspension was sonicated 2 × 5 s and the enzyme activity was determined according to Seubert and Huth [15] with the modifications described by Krone et al. [7]. Determinations were done in the cell homogenates of each culture dish separately. Data derived from equally treated culture dishes were pooled from different experiments for statistical evaluation. Protein determination was performed fluorimetrically [16]. Phosphorylase *a* and *a* + *b* activity was determined according to Stalmans et al. [17].

For measurements of gluconeogenesis, cultures were incubated with 10 ml culture medium without hormones, glucose or phenol red, but with 5 mM lactate (as normal) and 1 mM pyruvate. After 1 h of preincubation, this medium was replaced by 6 ml of the same medium containing various glucagon concentrations. After 4, 8 and 20 h, glucose content in the medium and phospho*enol*pyruvate carboxykinase activity in the cell layer were determined in each dish. For glucose determination the media were freeze-dried and the sediments were again solubilized in 0.6 ml H_2O. Glucose content was determined as described earlier [14].

Results

Phosphoenolpyruvate carboxykinase activities in hepatocyte cultures

When hepatocytes were cultured in a hormone-free medium, specific activity of phospho*enol*pyruvate carboxykinase declined rapidly and stable activity levels in the range of 10 U/g protein were established (Fig. 1). When 20 nM glucagon or a synthetic glucocorticoid such as triamcinolone (0.1 μM) were added to the culture medium from 3 h after seeding, the initial activity decreased but the stable activity level was 2.5- or 1.8-fold higher than under control conditions. In the presence of both hormones, phospho*enol*pyruvate

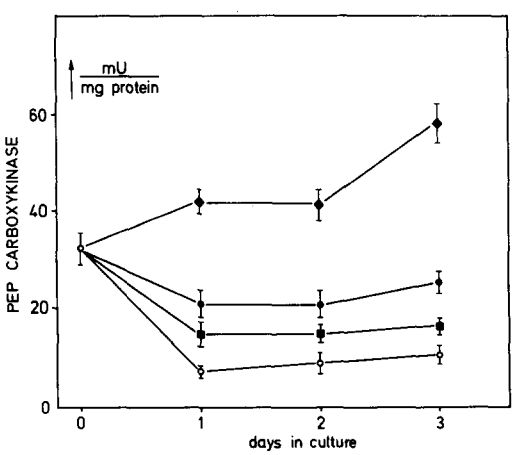
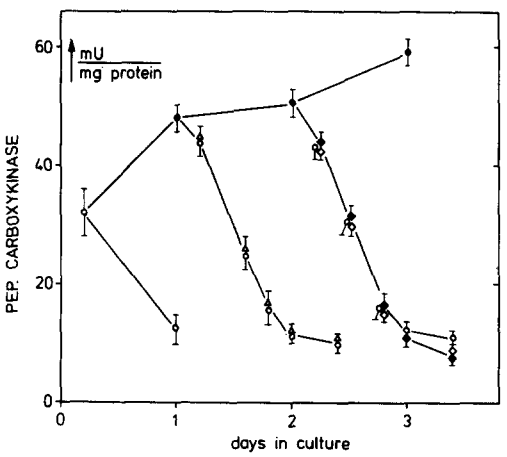


Fig. 1. Phospho*enol*pyruvate carboxykinase (PEP carboxykinase) activity in cultured hepatocytes. Hepatocytes were cultured under defined hormone concentrations from 3 h after seeding: ○, no addition (control); ■, 0.1 μM triamcinolone; ●, 20 nM glucagon; ◆, glucagon and triamcinolone. After 1, 2 and 3 days three dishes per point were harvested and stored at −40° C until enzyme determination. Enzyme activity was measured in each dish separately. Data are drawn from four experiments and are given as mean ± S.D. (n = 12).

carboxykinase activity rose and specific activities exceeded that of the initial suspension by about 2-fold after 3 days in culture (Fig. 1). The activity observed with the hormone combination was always higher than the sum of the activities found with glucagon or triamcinolone separately (Fig. 1).

When glucagon and triamcinolone were withdrawn from cultures previously kept in the presence of this hormone combination, phospho*enol*pyruvate carboxykinase declined as shown in Fig. 2. After an initial delay enzyme activity decreased with a constant rate of about 2 U/g protein per h. This rate was not influenced by either insulin (20 nM), or cycloheximide (10 μg/ml), or glucagon and triamcinolone in the absence of protein synthesis (Fig. 2). After 16 h the rate of decline slowed down and stable activity levels were approached (Fig. 2).

The low phospho*enol*pyruvate carboxykinase activity found in hepatocytes cultivated for 2 days in hormone free medium could be elevated by addition of either glucagon or triamcinolone, or the combination of both hormones (Fig. 3). Maximum activity levels were reached after 8 h and remained stable up to 24 h, except in the presence of glucagon. Fig. 3 demonstrates that the simultaneous presence of glucagon and triamcinolone evoked a 500% increase in specific activity. This rise exceeded the sum of increases observed in the presence of either glucagon (200%) or triamcinolone (100%) alone. Incubation



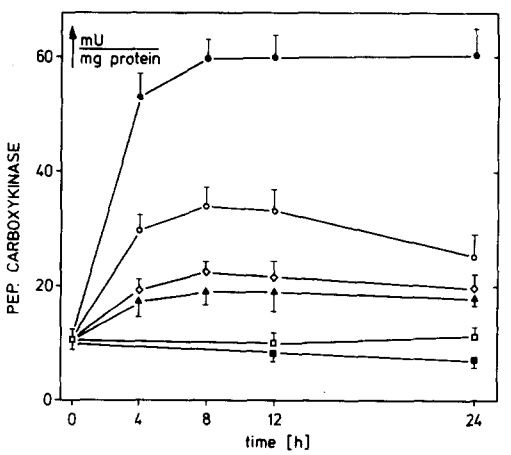


Fig. 2. Decline of phospho*enol*pyruvate (PEP) carboxykinase activity. Hepatocytes were cultured in the absence (○) or in the presence (●) of 20 nM glucagon and 0.1 μM triamcinolone from 3 h after seeding. At day 1 or day 2 cultures were washed for 30 min in hormone-free medium and were then incubated in media containing the indicated additives: (○) no hormone, (△) 20 nM insulin, (◇) 10 μg/ml cycloheximide, (◆) cycloheximide, glucagon and triamcinolone. At the indicated time points three dishes were harvested per experiment and enzyme activity was determined in each dish separately. Data are drawn from three experiments and are given as mean ± S.D. (n = 9).

Fig. 3. Increase of phospho*enol*pyruvate (PEP) carboxykinase activity in 2-day-old hepatocytes. Hepatocytes were cultivated for 2 days in hormone free media. Medium was then changed and cultures were incubated in the presence of various additives: □, no hormone; ◇, 1 μM triamcinolone; ○, 20 nM glucagon; ●, glucagon and triamcinolone; ▲, 50 nM insulin, triamcinolone and glucagon; ■, 10 μg/ml cycloheximide, triamcinolone and glucagon. At the indicated time points three dishes per experiment were harvested and stored at −40°C. Enzyme activity was determined in each dish separately. Data are drawn from four experiments and are given as mean ± S.D. (n = 12).

with the natural glucocorticoids cortisol (1 μM) or corticosterone (1 μM) revealed the same effect on phospho*enol*pyruvate carboxykinase activity during 4 h incubation as triamcinolone (1 μM) (data not shown).

Addition of insulin (50 nM) to the medium together with glucagon and triamcinolone reduced the inducing effect of the latter hormones from 500% to 80% (Fig. 3). If cycloheximide (10 μg/ml) was present in the medium, the basal activity of phospho*enol*pyruvate carboxykinase was slightly depressed and no elevation of phospho*enol*pyruvate carboxykinase activity could be observed in the presence of hormones (Fig. 3).

Dose-response relationship between glucagon and enzyme activities and gluconeogenesis

Fig. 4 shows parallel glucagon dependence in 2-day-old hepatocytes for (1) phospho*enol*pyruvate carboxykinase induction, (2) stimulation of gluconeogenesis and (3) phosphorylase interconversion (Fig. 4). Half-maximal effects were evoked for all at 50 ± 30 pM glucagon. The amount of gluconeogenesis as described in Fig. 4 was probably due to glucose formation from lactate and

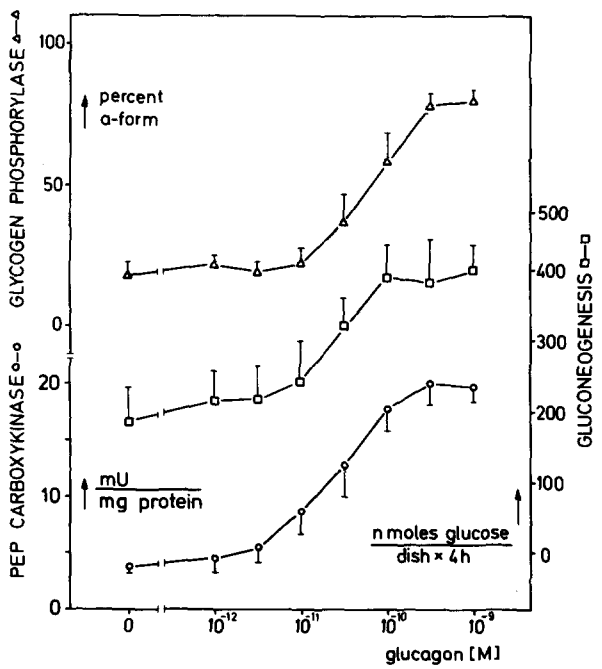


Fig. 4. Enhancement of phospho*enol*pyruvate (PEP) carboxykinase content, gluconeogenesis and glycogen phosphorylase *a* by glucagon. Hepatocytes were precultured for 2 days under control conditions. Phospho*enol*pyruvate carboxylase activity and glucose formation were determined in the same cultures after 4 h incubation in a glucose-free medium containing the indicated glucagon concentration (see Methods). For measurement of glycogen phosphorylase interconversion, cells were changed to 10 ml hormone-free medium containing 20 mM glucose. 30 min later the respective amount of glucagon was added in 100 μl medium. 20 min after glucagon addition, medium was removed and cultures were immediately frozen and stored at −40°C until the determination of the enzymatic activity. Each curve was repeated in four experiments with four dishes per point. Glycogen phosphorylase *a* and *a* + *b* was measured in each dish separately. Data are given as mean ± S.D. (n = 16).

pyruvate since the initial glycogen content of about 100 nM glucose/dish did not change during the experiment, whereas the glucose content of the medium increased linearly during 20 h (data not shown). Similar changes in phosphorylase *a* and of phospho*enol*pyruvate carboxykinase activity as observed with optimum glucagon concentrations could be induced by 0.5 mM cyclic AMP (data not shown).

Influence of glucocorticoid pretreatment of hepatocytes on phosphoenolpyruvate carboxykinase induction

1-Day-old cultures were incubated for a further 24 h various triamcinolone concentrations resulting in phospho*enol*pyruvate carboxykinase activities between 10 and 20 U/g protein (Table I, $t = 0$). The amount of protein was about 4 mg/dish regardless of the pretreatment. These 2-day-old hepatocytes pretreated with different triamcinolone concentrations were further incubated for 4 h at various hormone conditions. The following increases of phospho*enol*pyruvate carboxykinase activity were observed (Table I): (1) If triamcinolone (1 μM) was added to cultures without triamcinolone pretreatment, a net increase of 7.5 U/g protein was obtained. (2) The addition of glucagon (20 nM) evoked a phospho*enol*pyruvate carboxykinase increment of 20 U/g protein in untreated, but 35 U/g protein in triamcinolone pretreated (1 μM) cultures. (3) Addition of glucagon plus triamcinolone revealed a net increase of about 45 U/g protein in untreated as well as in pretreated hepatocytes. Thus, an extra additive enzyme activity of 15 U/g protein was observed in the simultaneous presence of both hormones or by glucagon separately, provided the cells had been pretreated by triamcinolone.

Hormone induced phospho*enol*pyruvate carboxykinase increases were inhibited by various concentrations of actinomycin D (Fig. 5). A concentration of 100 ng/ml actinomycin D was found to suppress completely activity elevation in control cultures exposed to either glucagon, triamcinolone or both hormones simultaneously. However, in cultures pretreated with triamcinolone,

TABLE I

INFLUENCE OF HEPATOCYTE PRETREATMENT WITH TRIAMCINOLONE ON PHOSPHO*ENOL*-PYRUVATE CARBOXYKINASE INDUCTION

24 h after seeding hepatocytes were incubated for another 24 h at the indicated triamcinolone concentrations. After this period of pretreatment four dishes per condition were collected and phospho*enol*pyruvate carboxykinase activity was determined ($t = 0$). The remaining dishes were incubated for 4 h at defined hormone concentrations. Results are drawn from three experiments and enzyme activity was determined in at least three dishes per experiment separately ($n = 9$). n.d., not determined.

Addition during 4 h incubation	Phospho*enol*pyruvate carboxykinase activity (U/g protein)			
	C *: 0	0.1 nM	10 nM	1 μM
$t = 0$	9.6 ± 1.2	12.5 ± 1.3	17.2 ± 1.7	19.4 ± 2.3
1 μM Triamcinolone	17.1 ± 1.1	n.d.	n.d.	n.d.
20 nM Glucagon	29.5 ± 1.6	34.1 ± 2.9	52.2 ± 3.3	55.8 ± 3.3
Triamcinolone + glucagon	53.2 ± 2.5	54.5 ± 3.0	64.1 ± 3.5	67.5 ± 3.1

* C, triamcinolone concentration during 24-h pretreatment.

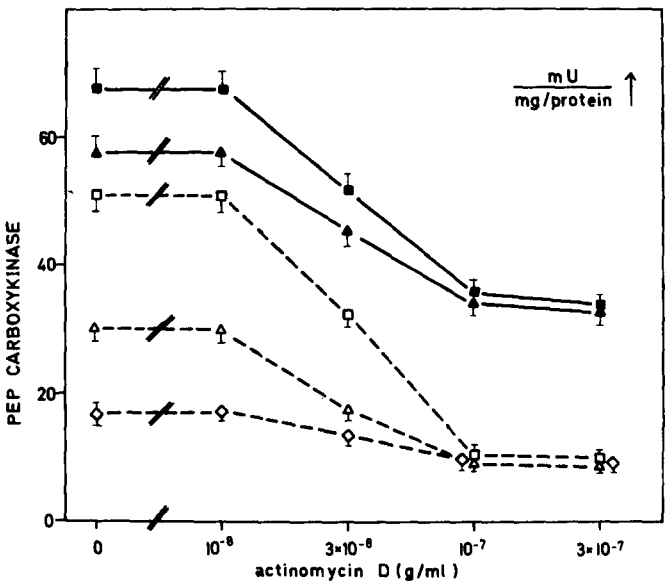


Fig. 5. Influence of phospho*enol*pyruvate (PEP) carboxykinase induction by actinomycin D in hepatocytes pretreated in the absence or presence of triamcinolone. 24 h after seeding cultures were incubated for a further 24 h either in the absence (open symbols) or the presence (filled symbols) of 1 μM triamcinolone. After this pretreatment cultures were incubated for 30 min in hormone-free medium and were then exposed for 4 h to the indicated concentrations of actinomycin D and hormones: ◇, 1 μM triamcinolone; △, 20 nM glucagon; □, glucagon and triamcinolone. Results were drawn from three experiments and enzyme activity was determined in at least three dishes per experiment separately ($n = 9$).

a net increase of 15 U/g protein was induced by either glucagon or glucagon and triamcinolone even though RNA synthesis was blocked by actinomycin D.

Discussion

Minimum and maximum phospho*enol*pyruvate carboxykinase activities found in cultured hepatocytes (Fig. 1) did not differ more than 2-fold from those measured in intact liver or in Reuber H 35 hepatoma cells under comparable conditions [3]. Elevations of phospho*enol*pyruvate carboxykinase activity after hormonal treatment depended on protein synthesis (Fig. 3) and therefore seem to be related to de novo synthesis of enzyme protein. Concerning the decrease of specific phospho*enol*pyruvate carboxykinase activity it has been shown by Hopgood et al. [18] that activity decline in liver corresponds to enzyme degradation. Therefore, we discuss changes in enzyme activity as alterations of enzyme concentration.

In the absence of hormones, cellular activities were low (Fig. 1) and breakdown of phospho*enol*pyruvate carboxykinase in the presence of cycloheximide was hardly detectable (Fig. 3). A fast degradation rate was observed if glucagon and triamcinolone were withdrawn from cultures previously treated by these hormones (Fig. 2). Initiation of breakdown was independent of protein synthesis, indicating that the degrading enzymes should have been present during the period of active enzyme synthesis. The degradation proceeded with a

constant rate of about 2 U/g protein per h and this rate decreased until reaching a basal level of activity (Fig. 2). This suggests that at phospho*enol*pyruvate carboxykinase activities above 15 U/g protein the maximal activity of degrading enzymes limited the degradation rates, while below 15 U/g protein the substrate concentration became rate limiting. Enzyme breakdown was not influenced by insulin or glucagon and triamcinolone in the absence of protein synthesis (Fig. 2). The observed rate of degradation was apparently slower as compared with intact liver [2], indicating that phospho*enol*pyruvate carboxykinase degradation in vivo may be accelerated by hormones which are not applied in this study.

Phospho*enol*pyruvate carboxykinase levels were increased and synthesis rates were accelerated independently by glucagon and triamcinolone (Figs. 1, 3). The combination of both hormones enhanced cellular activities and synthesis rate in a non-additive way (see below). Insulin inhibited hormone induced phospho*enol*pyruvate carboxykinase synthesis (Fig. 3) in accordance with results obtained in vivo [3].

The results presented in Figs. 1, 2 and 3 coincide with the data of phospho*enol*pyruvate carboxykinase regulation derived from different experimental systems [2,3,18]. Taken together, these results and those of the present investigation support the idea that actual phospho*enol*pyruvate carboxykinase activities are balanced by enzyme breakdown and synthesis, and that glucagon, glucocorticoids and insulin interfere primarily with enzyme synthesis.

Three different effects of glucagon revealed the same dose-response relationship in 2-day-old hepatocytes (Fig. 4). Half-maximal effects were evoked at glucagon concentrations of about one order of magnitude below those found in analogous experiments on hepatocyte suspensions [19—22], but were similar to those reported for perfused liver [23]. It is conceivable, that in cultured hepatocytes the intrinsic sensitivity of liver tissue towards glucagon was reestablished. This sensitivity is lowered in vivo by insulin [23,24] and in vitro possibly by enzymatic treatment during preparation of hepatocytes. In this respect it is of interest that sensitivity of glycogenesis towards insulin was reduced in hepatocyte suspensions [25], but was reestablished in hepatocyte cultures [14].

Glucagon and glucocorticoids both independently increased phospho*enol*pyruvate carboxykinase activity and synthesis (Figs. 1, 3). Since these separate effects were both suppressed by actinomycin D (Fig. 5) it may be assumed that both hormone actions depend on synthesis of new mRNA. This assumption is in accordance with results of Inedjian and Hanson [10] and Ruiz et al. [11] who demonstrated an increase of phospho*enol*pyruvate carboxykinase mRNA in liver due to the influence of external cyclic AMP. It may therefore be concluded that two different fractions of mRNA were provided by glucagon and triamcinolone in an additive fashion.

A more than additive increase of enzyme activity and of synthesis rate was observed if both hormones were present simultaneously (Figs. 1, 3). Thus, with regard to enzyme synthesis, three fractions of phospho*enol*pyruvate carboxykinase might be distinguished in Table I: the fraction due, (1) to corticoid (7.5 U/g protein), (2) to glucagon (20 U/g protein) and (3) an extra additive effect of glucagon and triamcinolone together (15 U/g protein). The same

amount of enzyme as in fraction 3 could be synthesized by glucagon even in the actual absence of triamcinolone in glucocorticoid pretreated cells, and furthermore, synthesis of this fraction did not depend on RNA synthesis (Fig. 5). Thus, it is likely that fraction 3 is synthesized in the presence of glucagon, using the mRNA produced during the precedent glucocorticoid treatment. This indicates an accelerating function of glucagon at the translational level. Similar effects have been observed in different experimental systems [4—7] and have been explained as 'permissive' effects of glucocorticoids for glucagon action. Thus, the present data of phospho*enol*pyruvate carboxykinase regulation in cultured hepatocytes may be explained by the simultaneous influence of hormones on transcription and translation: (1) additive increase of phospho*enol*pyruvate carboxykinase mRNA by glucagon and glucocorticoids and (2) the glucagon dependent acceleration of phospho*enol*pyruvate carboxykinase mRNA translation.

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