Effects of insulin, glucagon, dexamethasone, cyclic GMP and spermine on the stability of phosphatidate phosphohydrolase activity in cultured rat hepatocytes

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1. Hepatocytes were preincubated with 10 mM-glucagon and 100 μ M-corticosterone to increase phosphatidate phosphohydrolase activity. Addition of 10 nM-glucagon or 100 μ M-8-bromo cyclic GMP to a second incubation mixture that contained cycloheximide increased the half-life of the phosphohydrolase activity. Dexamethasone (100 nM) had no significant effect, but insulin (500 pM) or spermine (1 mM) decreased the half-life. None of these compounds altered the general rate of degradation of proteins labelled with [3 H]leucine. 2. There appears to be a specific control of the half-life of phosphatidate phosphohydrolase activity, which could contribute to its long-term regulation in the liver.

INTRODUCTION

The regulation of the activity of phosphatidate phosphohydrolase in the liver appears to be under complex hormonal control. The activity of this enzyme is increased over a period of hours by glucagon, cyclic AMP, glucocorticoids [1,2] and growth hormone [3]. These increases are antagonized by insulin [1–3] and spermine [4]. The physiological expression of the phosphohydrolase depends on the translocation of the cytosolic form of the enzyme, which is metabolically inactive, to its site of action on those membranes on which glycerolipid synthesis occurs [5,6].

The long-term control of phosphatidate phosphohydrolase activity resembles that of several important enzymes involved in gluconeogenesis, including tyrosine aminotransferase and phosphoenolpyruvate carboxykinase [1]. The synthesis of these latter enzymes is also increased by glucagon, cyclic AMP and glucocorticoids. In addition, they possess relatively short half-lives, about 2 and 4 h respectively [7]. No such information is yet available for the half-life of phosphatidate phosphohydrolase. We therefore decided to investigate if this enzyme activity had a similarly short half-life and whether this was under hormonal control.

Values for the half-lives of several enzymes have been estimated by a variety of methods, which each give similar results. These methods include determining the rate of disappearance of isotopically labelled enzymes after immunoprecipitation, and the decrease in enzyme activity in the presence or absence of protein-synthesis inhibitors [7,8], the former being the more definitive method. Since antibodies to phosphatidate phosphohydrolase are not yet available, the half-life was determined by measuring the decrease in phosphohydrolase activity in the presence of cycloheximide. These results could therefore reflect changes in the inactivation of the enzyme as well as its degradation. Rat hepatocytes in monolayer culture were used to provide a well-defined system for studying changes in the enzyme's activity [1,2,9,10].

EXPERIMENTAL

Animals and materials

The sources of the rats and most of the materials have been described [1,2]. General biochemicals were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. [3 H]Leucine was purchased from Amersham International, Amersham, Bucks., U.K.

Preparation and culture of hepatocytes

Hepatocytes were prepared and attached to Primaria tissue-culture dishes in Leibovitz L-15 medium containing 10% (v/v) newborn-calf serum for 6–8 h [1, 2]. They were then maintained in serum-free medium containing 0.2% (w/v) fatty-acid-poor bovine serum albumin for at least 24 h before beginning the experiments. This was to ensure stable rates of protein degradation, since it has been reported that freshly prepared hepatocytes and those in the first 24 h of culture have relatively high catabolic rates [11,12]. However, this rate decreases and stabilizes with increasing culture time to values similar to those found *in vivo*. The rate of protein degradation in monolayer cultures of rat hepatocytes in Leibovitz medium remains constant for 4–6 days, and it is not altered by replacing the medium with fresh medium [9]. This latter culture system has been shown to be suitable for studying protein turnover [9,10].

After the initial stabilization period, the hepatocytes were incubated in the presence or absence of hormones as indicated. The medium was removed, the cells were washed, and fresh medium containing 5 μ g of cycloheximide/ml in the presence or absence of hormones was added. The hepatocytes were then collected after two washes with protein-free medium [1].

Measurement of total protein degradation

Hepatocyte proteins were labelled by incubating the cultures after the initial stabilization period with Leibovitz medium which contained 1 μ Ci of

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[^3H]leucine/ml. The final concentration of leucine was 0.95 mM. The hepatocytes were then incubated for two 1 h periods with medium that did not contain hormones or [^3H]leucine, in order to allow the degradation of short-lived proteins and to wash out any remaining [^3H]leucine from the cells. This washing medium contained 10 mM-leucine, to prevent the reutilization of [^3H]leucine. The hepatocytes were then incubated with the same medium containing 5 μg of cycloheximide/ml and hormones as indicated. Samples (250 μl) of the medium were removed at intervals, and proteins were precipitated with trichloroacetic acid. The samples of medium that were removed were replaced with fresh medium, and the hepatocytes were collected after 12 h.

Analyses

The activities of phosphatidate phosphohydrolase [13] and lactate dehydrogenase [14] were measured as described. Protein concentrations were determined by the method of Bradford [15], but by using PAGE Blue G-90. The activities of the phosphohydrolase and dehydrogenase were expressed relative to protein, to compensate for variations in cell numbers between culture dishes. The radioactivity in acid-soluble and -insoluble material from the medium and cell pellets was determined [16] after precipitation with a final concentration of 10% (w/v) trichloroacetic acid containing 5 mM-leucine. Precipitates were dissolved in formic acid.

Fig. 1. Comparison of the half-lives of phosphatidate phosphohydrolase and lactate dehydrogenase in hepatocytes that had been preincubated in the presence or absence of glucagon and corticosterone

Hepatocytes were preincubated for 12 h in the presence (●, ○) or absence (▲, △) of 10 nM-glucagon and 100 μM -corticosterone. The medium was replaced without the further addition of hormones, and 5 μg of cycloheximide/ml was added. The percentage of the phosphatidate phosphohydrolase (△, ○) and lactate dehydrogenase (▲, ●) remaining over the subsequent 12 h is shown. Results are means $\pm$ S.D. of the averages from duplicate plates from three independent experiments. The absolute enzyme activities at the beginning of the second incubation were 2.34 ± 0.56 and 0.29 ± 0.089 nmol of diacylglycerol formed/min per mg of protein and 0.91 ± 0.29 and 0.85 ± 0.56 μmol of lactate formed/min per mg of protein for cells that were preincubated in the presence or absence of hormones respectively.

Fig. 2. Relationship between the half-life of phosphatidate phosphohydrolase activity and the increase in this activity that was produced by preincubation with glucagon and corticosterone

Hepatocytes were preincubated with 100 μM -corticosterone (▽), or with 10 nM-glucagon (◇) alone or in combination with 100 nM- (□), 1 μM - (■), 10 μM - (●) or 100 μM -corticosterone (△) for 12 h, and the activities of phosphatidate phosphohydrolase are expressed relative to incubations which did not contain these hormones (○). The half-life of the phosphohydrolase was then determined in a second incubation which contained 5 μg of cycloheximide/ml and no further additions of hormones (Fig. 1). The combined results were obtained from five independent experiments.

The ^3H was corrected for cell loss by measurement of lactate dehydrogenase.

RESULTS AND DISCUSSION

Hepatocytes were incubated for 12 h in the presence or absence of various combinations of glucagon (10–100 nM) and corticosterone (100 nM–100 μM), to increase the phosphatidate phosphohydrolase by 2–9.5-fold. Corticosterone was used rather than dexamethasone in this part of the experiment, since it is more readily degraded by the hepatocytes and it can be more easily removed from the cells by changing the medium. After the incubation, the culture dishes were washed and the medium was replaced by fresh medium containing 5 μg of cycloheximide/ml to block protein synthesis [17], and they were maintained for up to a further 12 h. The phosphohydrolase activity was found to decline exponentially. The activities were plotted on a semi-logarithmic scale as the percentage of phosphohydrolase activity remaining against time (Fig. 1). Linear-regression analysis was used to determine the slope of the line. From this the half-life of the phosphohydrolase activity was calculated. As the phosphohydrolase activity was increased relative to control incubations containing no hormones, the estimated half-lives decreased. However, if the increases in phosphohydrolase activity were greater than about 3–4-fold, then there was no further decrease in the half-life of the enzyme activity (Fig. 2). The estimated half-life of the phosphohydrolase for cells not stimulated by hormones was 12.9 ± 1.3 h (means $\pm$ S.D. for three independent experiments). The corresponding value for cells incubated with 10 nM-glucagon and

Table 1. Effects of various hormones and compounds on the half-life of phosphatidate phosphohydrolase activity and the rate of general protein degradation

Hepatocytes were preincubated for 12 h with 10 nM-glucagon and 100 μ M-corticosterone. The medium was replaced by fresh medium containing 5 μ g of cycloheximide/ml in the presence or absence of the compounds indicated. The half-life of phosphatidate phosphohydrolase activity and the rate of degradation of proteins labelled during the preincubation with [3 H]leucine were calculated by linear-regression analysis of values plotted as shown in Fig. 1. Results are means $\pm$ S.D. for four independent experiments for the phosphohydrolase and for three independent experiments for protein degradation. The significance of the difference between groups was calculated by using a paired *t* test, and it is shown by: **P* < 0.05; †*P* < 0.02.

	Additions	Estimated half-life (h)	Protein degradation rate (%/h)
I	None	6.9 $\pm$ 0.2	1.8 $\pm$ 0.2
II	Glucagon (10 nM)	12.1 $\pm$ 2.0 I versus II†	2.1 $\pm$ 0.3
III	Insulin (500 pM)	5.2 $\pm$ 0.8 I versus III†	1.9 $\pm$ 0.3
IV	Glucagon (10 nM) + insulin (500 pM)	6.4 $\pm$ 0.5 II versus IV†	1.9 $\pm$ 0.3
V	Dexamethasone (100 nM)	6.4 $\pm$ 0.9	1.9 $\pm$ 0.3
VI	Dexamethasone (100 nM) + glucagon (10 nM)	9.8 $\pm$ 2.2	2.0 $\pm$ 0.2
VII	8-Bromo cyclic GMP (100 μ M)	8.6 $\pm$ 1.0 I versus VII*	2.0 $\pm$ 0.03
VIII	Spermine (1 mM)	5.0 $\pm$ 1.1 I versus VIII	2.1 $\pm$ 0.1

100 μ M-corticosterone for 12 h was 7.0 $\pm$ 0.4 h, and this was significantly lower (*P* < 0.05). The equivalent rates of total protein degradation for cells in the presence and absence of the hormones were 1.81 $\pm$ 0.22%/h and 1.88 $\pm$ 0.19%/h respectively, which are similar to those found *in vivo* [10,17]. Only 5% of the lactate dehydrogenase activity was lost from the cells, irrespective of hormone treatment (Fig. 1). These latter results demonstrate that the hepatocytes were not being lysed over the 12 h incubation with cycloheximide.

Experiments on tyrosine aminotransferase and tryptophan oxygenase have also shown that their half-lives depend on the extent to which their activities have been increased. In these cases an overestimate of the half-life was obtained by using enzyme decay measurements from non-induced cells when comparing with immunoprecipitation studies [8]. The higher degradation rate of induced enzyme activity may be caused by the overall greater chance of the enzyme being metabolized by a proteinase [7]. Alternatively, the variation in half-life values of phosphatidate phosphohydrolase (Table 1) could have been a reflection of the effects of glucagon and corticosterone in enhancing general protein degradation. However, when this was measured under the conditions described in Fig. 2, there was no difference between the rate of disappearance of those proteins that had been labelled with [3 H]leucine. The hormones might also have altered the rate at which the phosphohydrolase was inactivated.

In other experiments, various hormones and compounds were added with the cycloheximide after the preincubation with 10 nM-glucagon and 100 μ M-corticosterone. This was done to determine whether the half-life of phosphatidate phosphohydrolase activity could be further modified.

The addition of 10 nM-glucagon with cycloheximide caused an apparent increase in the half-life of the

phosphohydrolase activity compared with that in the absence of hormones (Table 1). This was not due to a continued increase in enzyme synthesis as a result of incomplete inhibition by cycloheximide, since dexamethasone, which should have produced a marked increase in the phosphohydrolase activity [1,2], had no significant effect on the half-life of activity in the presence of cycloheximide. This lack of effect occurred in the presence or absence of glucagon (Table 1). However, it is not known how much corticosterone or glucagon from the preincubation remained in the hepatocytes after they were washed. The lack of an effect of dexamethasone on the half-life of the phosphohydrolase activity could have occurred because it was unable to augment the existing effects of residual corticosterone.

Insulin (500 pM) decreased the half-life of the phosphohydrolase activity, and it also antagonized the effects of glucagon (Table 1). Spermine (1 mM) acted like insulin, since it appeared to decrease the half-life of the phosphohydrolase activity. By contrast, cyclic GMP (0.1 mM) acted like glucagon and increased the half-life of phosphohydrolase activity (Table 1).

The effects of these hormones and compounds on the rate of general protein degradation were also determined under similar incubation conditions. None of the additions affected the overall degradation of those proteins that had become labelled with [3 H]leucine in the presence of glucagon and corticosterone (Table 1). It therefore appeared that cycloheximide may block the stimulation of protein degradation by glucagon and glucocorticoids (Table 1; references [16–18]) and the inhibition by insulin [19–21] or spermine [22]. However, as well as inhibiting protein synthesis, cycloheximide can also inhibit general protein degradation by preventing autophagy [16,17]. Similarly, the relatively high concentrations of amino acids in the Leibovitz L-15 medium [23] would also inhibit protein degradation by

autophagy [12,16,19]. This could contribute to the relatively low rates of protein degradation observed in our experiments, even when no hormones were added. These degradation rates are similar to those described by other workers, who added insulin and dexamethasone to their cultures [9,17]. Thus the changes in half-life of the phosphohydrolase activity in Table 1 occurred when autophagy was largely inhibited, which implies that the effects are fairly specific for the enzyme.

The decay of induced enzyme activity after removal of the inducer has been used to estimate the half-life of several hepatic enzymes. However, unless the inducer is completely removed, an overestimate of the half-life is obtained. Inhibition of protein synthesis with cycloheximide overcomes this problem. This methodology has been used in studies with tyrosine aminotransferase, tryptophan oxygenase, phosphoenolpyruvate carboxykinase, glucokinase, δ -aminolaevulinate synthase, glycerol-3-phosphate dehydrogenase and catalase [8]. The values obtained by using this technique were similar to those obtained by the combined pulse-chase and immunoprecipitation techniques.

Studies with tyrosine aminotransferase have shown that the decay in its activity was faster with cyclic AMP rather than with glucocorticoids [24,25]. Although insulin increased the half-life of the aminotransferase, there were similar changes in overall protein turnover [26]. These effects appear opposite to those on the phosphatidate phosphohydrolase activity. However, cyclic GMP increased the half-life of the activities of phosphatidate phosphohydrolase (Table 1) and tyrosine aminotransferase [27]. Lamb & McCue [28] demonstrated that incubating hepatocytes for 24 h with fatty acids produced increases in the activity of phosphatidate phosphohydrolase. Furthermore, they suggested this effect might be brought about by the action of cyclic GMP [28]. The decrease in half-life of the phosphohydrolase activity that was demonstrated in Table 1 in the presence of cyclic GMP could contribute to this effect.

The mechanisms by which the half-life of phosphatidate phosphohydrolase activity is regulated in the hepatocytes (Fig. 1; Table 1) are not known. It could involve inactivation as well as protein degradation [29], but the presence of cycloheximide and the relatively high concentrations of amino acids in the medium would largely prevent autophagy [12,16,17]. However, the latter process could also be important in controlling the phosphohydrolase activity in the liver *in vivo*. Further advances in this respect will have to await the availability of antibodies to the phosphohydrolase. However, the present results do demonstrate that the stability of the phosphohydrolase activity is modified by changing the hormonal environment to which the hepatocytes are exposed. These effects could contribute to the long-term control of the phosphohydrolase that has been described *in vivo* and *in vitro* [5]. For example, changes in the half-life of the phosphohydrolase activity could be involved in regulating: (a) the decline in the high phosphohydrolase activity that is observed in the liver after surgical stress and sub-total hepatectomy [30]; (b) the diurnal variation in the phosphohydrolase activity [31]; and (c) in the action of insulin in decreasing the high phosphohydrolase activities found in the liver of diabetic animals [32,33].

It has been demonstrated that glucagon, which increases the total phosphatidate phosphohydrolase

activity in rat hepatocytes, probably through a cyclic-AMP-mediated increase in protein synthesis [1,2], also increases the stability of the phosphohydrolase activity (Table 1). Conversely, insulin and spermine, which decrease the effects of glucagon [2], growth hormone [3] and dexamethasone [1,2,4] in increasing the phosphohydrolase activity, also decrease its half-life (Table 1). Thus, in these instances, there seems to be a reciprocal effect between the actions of insulin and glucagon on the increase in the total phosphatidate phosphohydrolase activity in the liver and on its removal.

We thank Professor R. J. Mayer for advice. The work was supported by a project grant from the Medical Research Council and an equipment grant from the Humane Research Trust. We also thank the Science and Engineering Research Council and Beecham Pharmaceuticals for providing a CASE award to R. A. P. during part of this work. We are grateful to the Boots Co. for its gift of insulin.

REFERENCES

- Pittner, R. A., Fears, R. & Brindley, D. N. (1985) *Biochem. J.* **225**, 455–462
- Pittner, R. A., Fears, R. & Brindley, D. N. (1985) *Biochem. J.* **230**, 525–534
- Pittner, R. A., Bracken, P., Fears, R. & Brindley, D. N. (1986) *FEBS Lett.* **202**, 133–136
- Pittner, R. A., Bracken, P., Fears, R. & Brindley, D. N. (1986) *FEBS Lett.*, in the press
- Brindley, D. N. (1984) *Prog. Lipid Res.* **23**, 115–133
- Martin, A., Hopewell, R., Martin-Sanz, P., Morgan, J. & Brindley, D. N. (1986) *Biochim. Biophys. Acta* **876**, 581–591
- Schimke, R. T. (1973) *Adv. Enzymol. Relat. Areas Mol. Biol.* **37**, 135–187
- Recheigl, M. (1971) in *Enzyme Synthesis and Degradation in Mammalian Systems* (Recheigl, M., ed.), pp. 236–310, Karger, Basel
- Evans, P. J. & Mayer, R. J. (1982) *Biochem. Biophys. Res. Commun.* **107**, 51–58
- Evans, P. J. & Mayer, R. J. (1983) *Biochem. J.* **216**, 151–161
- Seglen, P. O. (1977) *Biochim. Biophys. Acta* **496**, 182–191
- Schwarze, P. E. & Seglen, P. O. (1980) *Exp. Cell Res.* **130**, 185–190
- Pollard, A. D. & Brindley, D. N. (1984) *Biochem. J.* **217**, 464–469
- Saggerson, E. D. & Greenbaum, A. L. (1969) *Biochem. J.* **115**, 405–417
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Woodside, K. H. (1975) *Biochim. Biophys. Acta* **421**, 70–79
- Brookes, C., Wright, A., Evans, P. J. & Mayer, R. J. (1984) *Carcinogenesis* **5**, 759–765
- Woodside, K. H., Ward, W. F. & Mortimore, G. E. (1974) *J. Biol. Chem.* **249**, 5458–5463
- Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1977) *Biochem. J.* **164**, 399–407
- Ballard, F. J., Wong, S. C. C., Knowles, S. E., Partridge, W. C., Martin, T. J., Wood, L. M. & Gunn, J. M. (1980) *J. Cell. Physiol.* **105**, 335–346
- Draznin, B. & Trowbridge, M. (1982) *J. Biol. Chem.* **257**, 11988–11993
- Tabor, C. W. & Tabor, H. (1976) *Annu. Rev. Biochem.* **45**, 285–306
- Leibovitz, A. (1963) *Am. J. Hyg.* **78**, 173–180
- McNamara, D. J. & Webb, T. E. (1979) *Arch. Biochem. Biophys.* **163**, 777–783
- Lewis, E. J., Calle, P. & Wicks, W. D. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 5778–5782

26. Spencer, C. J., Heaton, J. H., Gelehster, T. D., Richardson, K. I. & Garwin, J. L. (1978) *J. Biol. Chem.* **253**, 7677–7682
27. Strinden, S. T. & Stellwagen, R. H. (1983) *J. Cell. Physiol.* **117**, 69–75
28. Lamb, R. G. & McCue, S. B. (1983) *Biochim. Biophys. Acta* **753**, 356–363
29. Mayer, R. J. & Doherty, F. (1986) *FEBS Lett.* **193**, 181–193
30. Mangiapane, E. H., Lloyd-Davies, K. A. & Brindley, D. N. (1973) *Biochem. J.* **134**, 103–112
31. Knox, A. M., Sturton, R. G., Cooling, J. & Brindley, D. N. (1979) *Biochem. J.* **180**, 441–443
32. Murthy, U. K. & Shipp, J. C. (1979) *Diabetes* **28**, 472–478
33. Woods, J. A., Knauer, T. E. & Lamb, R. G. (1982) *Biochim. Biophys. Acta* **666**, 482–492

Received 30 May 1986/29 August 1986; accepted 18 September 1986