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Inhibition of phosphatidylethanolamine synthesis by glucagon in isolated rat hepatocytes

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Exposure of isolated rat hepatocytes to glucagon or chlorophenylthio cyclic AMP led to an inhibition of the incorporation of [1,2-^{14}C]ethanolamine into phosphatidylethanolamine. Pulse–chase experiments and measurement of the activities of the enzymes involved in the CDP-ethanolamine pathway provided evidence that the inhibitory effect of glucagon on the synthesis *de novo* of phosphatidylethanolamine was not caused by a diminished conversion of ethanolamine phosphate into CDP-ethanolamine. The observations suggested that the glucagon-induced inhibition of the biosynthesis of phosphatidylethanolamine is probably due to a decreased supply of diacylglycerols, resulting in a decreased formation of phosphatidylethanolamine from CDP-ethanolamine and diacylglycerols.

INTRODUCTION

The effects of glucagon and insulin on hepatic lipogenesis have been reviewed extensively (Geelen *et al.*, 1980). Short-term effects of glucagon include stimulation of glycogenolysis, gluconeogenesis and fatty acid oxidation, whereas fatty acid synthesis is inhibited by glucagon. These effects of glucagon in liver are mediated through an increase in its second messenger cyclic AMP.

Recently it was demonstrated that short-term incubations of hepatocytes with glucagon resulted in an inhibition of the synthesis of phosphatidylcholine (PC) (Pelech *et al.*, 1984). However, this effect could not be correlated with a decrease of the activity of CTP:choline-phosphate cytidylyltransferase, the supposedly rate-regulatory enzyme of the CDP-choline pathway (Pelech & Vance, 1984). When hepatocytes were treated with chlorophenylthio cyclic AMP (CPT-cyclic AMP), a more potent and stable cyclic AMP analogue than cyclic AMP itself (Miller *et al.*, 1975), the inhibition of the biosynthesis of PC was indeed accompanied by a diminished activity of choline-phosphate cytidylyltransferase (Pelech *et al.*, 1981). The inhibition of this enzyme was probably due to a cyclic AMP-dependent change in the phosphorylation state of the enzyme protein (Pelech & Vance, 1982).

Although the regulation of PC synthesis by glucagon and cyclic AMP analogues has been investigated extensively in the past few years, the hormonal control of phosphatidylethanolamine (PE) synthesis *de novo* is not as well studied as that of PC. In a previous study we reported that treatment of cultured hepatocytes with glucagon for 4 h enhanced the incorporation of labelled ethanolamine and ^{32}P into PE (Geelen *et al.*, 1979). More recently, Pritchard *et al.* (1981) demonstrated that the incorporation of labelled ethanolamine into phospholipids was decreased in hepatocytes in monolayer culture incubated with CPT-cyclic AMP for 1 h.

In the present study we examined the short-term effects of glucagon and CPT-cyclic AMP on the biosynthesis of PE via the CDP-ethanolamine pathway in freshly isolated rat hepatocytes. The results of this study suggest that the inhibition of this pathway by glucagon is probably due to a diminished supply of diacylglycerols, resulting in a decreased rate of formation of PE from CDP-ethanolamine and diacylglycerols.

EXPERIMENTAL

Materials

CPT-cyclic AMP was obtained from Boehringer (Mannheim, Germany). Aminophylline was purchased from Sigma (St. Louis, MO, U.S.A.). Insulin and glucagon were given by Lilly Research Laboratories (Indianapolis, IN, U.S.A.). [1,2-^{14}C]Ethanolamine was obtained from New England Nuclear (Dreieichenhain, Germany). CDP-[1,2-^{14}C]ethanolamine was purchased from ICN Radiochemicals (Irvine Scotland, U.K.), and [1,2-^{14}C]ethanolamine phosphate was prepared from [1,2-^{14}C]ethanolamine by a modification of the procedure of Sundler (1975). Ethanolamine kinase that was required for this procedure was partially purified from rat liver by $(NH_4)_2SO_4$ precipitation (Ishidate *et al.*, 1985). The $(NH_4)_2SO_4$-precipitable fraction (30–45% satd.) was redissolved in 10 mM-Tris/HCl (pH 7.5)/2 mM-mercaptoethanol/1 mM-EDTA and dialysed for 2 h against the same buffer. The incubation mixture (4 ml) comprised 10 mM-Tris/HCl (pH 8.5), 10 mM-$MgCl_2$, 4 mM-ATP, 0.25 mM-[1,2-^{14}C]ethanolamine and partially purified ethanolamine kinase. The incubation was carried out for 120 min at 37 °C. Ethanolamine phosphate was separated from ethanolamine by anion-exchange chromatography (Sundler, 1973). The yield of the entire procedure was 85–90%. The sources of all other materials have been described previously (Tijburg *et al.*, 1987).

Isolation and incubation of hepatocytes

Male Wistar rats (200–250 g) were meal-fed on a standard pelleted diet and had free access to water. The

Abbreviations used: CPT-cyclic AMP, 8-(4-chlorophenylthio)adenosine 3',5'-monophosphate; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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regular diet contained (by wt.) 23% protein, 15% fat and 62% carbohydrate. The rats were subjected to a 12 h-light (06:00–18:00 h)/12 h-dark cycle. The animals were meal-fed between 04:00 and 07:00 h for at least 10 days. Hepatocytes were routinely prepared between 09:00 and 10:00 h by the collagenase-perfusion method essentially as described by Seglen (1976), except that the operational temperature was maintained at 37 °C throughout the entire isolation procedure. The isolated cells were resuspended in Krebs–Ringer bicarbonate buffer (pH 7.4) supplemented with 10 mM-glucose, 1% defatted and dialysed bovine serum albumin, 0.1 mM-choline and 0.1 mM-ethanolamine, at a concentration of 4–5 mg of cellular protein/ml. Incubations were carried out in 25 ml Erlenmeyer flasks, under an atmosphere of O_2/CO_2 (19:1). All incubations were performed in triplicate, except where indicated otherwise.

Pulse-label and pulse–chase studies

In pulse-label studies, hepatocytes were incubated with labelled ethanolamine for 60 min in the absence or presence of glucagon (0.1 μM), CPT-cyclic AMP (0.5 mM), aminophylline (1.0 mM) or insulin (85 nM). The incubations were terminated by the addition of 6.5 vol. of chloroform/methanol (1:1, v/v), and lipids were extracted (Sundler *et al.*, 1974). Phospholipids were separated by t.l.c. on silica gel G with chloroform/methanol/water (65:35:4, by vol.) as developing solvent.

In pulse–chase studies the cells were pulsed with 0.1 mM-[1,2-^{14}C]ethanolamine (4400 d.p.m./mol) for 30 min. The prelabelled hepatocytes were washed twice by centrifugation (100 *g*, 2 min) and resuspension, and subsequently chased in Krebs–Ringer buffer containing 0.1 mM unlabelled ethanolamine in the absence or presence of glucagon for the time periods indicated. Phospholipids and water-soluble ethanolamine metabolites were extracted as described previously (Tijburg *et al.*, 1987). Labelled ethanolamine, ethanolamine phosphate and CDP-ethanolamine were separated by t.l.c. on silica gel H with methanol/0.5% NaCl/NH_3 (10:10:1, by vol.) as the eluent. Spots of water-soluble ethanolamine metabolites were detected by spraying with ninhydrin, scraped from the plates and assayed for radioactivity.

Enzyme assays

At the end of an incubation period of 60 min, the cells were washed twice in a buffer that comprised 0.145 M-NaCl/10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/10 mM-NaF and homogenized in the same buffer with 50 strokes of a Dounce homogenizer. Subcellular fractionation was performed exactly as described previously (Tijburg *et al.*, 1987). The activities of ethanolamine kinase (EC 2.7.1.82) and CTP:ethanolamine-phosphate cytidylyltransferase (EC 2.7.7.14) were determined in the cytosolic fraction. The methods for these assays have been described (Weinhold & Rethy, 1974; Tijburg *et al.*, 1987). The enzyme activity of CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1) was determined in the microsomal fraction by measurement of the incorporation of CDP-[1,2-^{14}C]ethanolamine into PE in the presence of endogenous diacylglycerols (Tijburg *et al.*, 1987).

Measurement of the pool sizes of PE precursors

Water-soluble PE precursors were extracted as described previously (Tijburg *et al.*, 1988). Ethanolamine, ethanolamine phosphate and CDP-ethanolamine were separated by h.p.l.c. The details of this novel method have been described (Tijburg *et al.*, 1988). The pool sizes of the ethanolamine-containing PE precursors were determined as reported previously (Tijburg *et al.*, 1988; Sundler & Åkesson, 1975).

For measurement of the cellular amounts of diacylglycerols, the lipids were extracted as described above and isolated by t.l.c. on silica-gel G with light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (40:10:1, by vol.) as developing solvent. Diacylglycerols were extracted from the silica, hydrolysed (Tijburg *et al.*, 1987) and determined enzymically as free glycerol (Wieland, 1984).

Other methods

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Results shown represent means ± S.D. of the numbers of experiments indicated. Statistical analysis was performed by Student's *t* test.

RESULTS

Pulse-label and pulse–chase studies

The effects of glucagon and cyclic AMP analogues on PE biosynthesis were studied in freshly isolated rat hepatocytes. The incorporation of [1,2-^{14}C]ethanolamine into PE in glucagon-treated cells was decreased to 74.5 ± 5.0% of that measured in control incubations (Table 1). This inhibition was dose-dependent and reached maximum values at a glucagon concentration of 0.1 μM (results not shown). The inhibition of PE biosynthesis by CPT-cyclic AMP was comparable with that induced by glucagon (Table 1). In addition, aminophylline, an inhibitor of cyclic AMP phosphodiesterase, also decreased the formation of PE from labelled ethanolamine (Table 1). It can be inferred from the data in Table 1 that the incorporation of labelled ethanolamine into PC was also lower in hormone-treated cells than in control incubations. However, these effects were more

Table 1. Inhibition of the incorporation of [1,2-^{14}C]ethanolamine into phospholipids by glucagon and other compounds

Freshly isolated hepatocytes were incubated for 60 min with [1,2-^{14}C]ethanolamine in the absence or presence of glucagon (0.1 μM), CPT-cyclic AMP (0.5 mM) or aminophylline (1.0 mM). The incubations were terminated and phospholipids were isolated as described in the Experimental section. Values are expressed as means ± S.D. for the numbers of experiments indicated; for aminophylline the results are given as the mean ± ranges; * indicates significant differences from control ($P < 0.01$).

Addition	[1,2-^{14}C]Ethanolamine incorporated (nmol/h per mg of protein)	
	Phosphatidylethanolamine	Phosphatidylcholine
None	4.54 ± 0.57	0.47 ± 0.10 (11)
Glucagon	3.38 ± 0.46*	0.32 ± 0.07* (11)
CPT-cyclic AMP	3.44 ± 0.49*	0.16 ± 0.02* (6)
Aminophylline	3.59 ± 0.31	0.38 ± 0.07 (2)

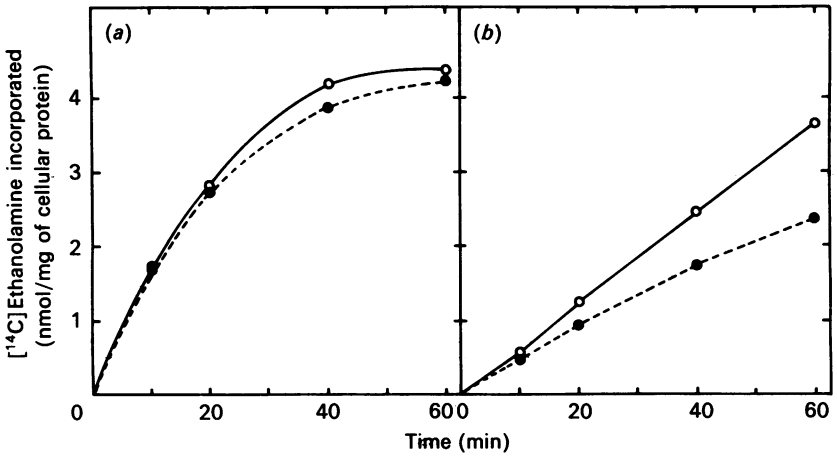

Fig. 1. Effects of glucagon on the incorporation of [1,2-^{14}C]ethanolamine into PE and its water-soluble precursors

Hepatocytes were incubated with [1,2-^{14}C]ethanolamine in the absence (○) or the presence (●) of 0.1 μM-glucagon. At various times up to 60 min, the cells were washed and the radioactivity in cellular PE and the ethanolamine metabolites was determined. Values are means of duplicate incubations of one representative experiment, which was repeated with similar results. (*a*) Water-soluble ethanolamine metabolites; (*b*) PE.

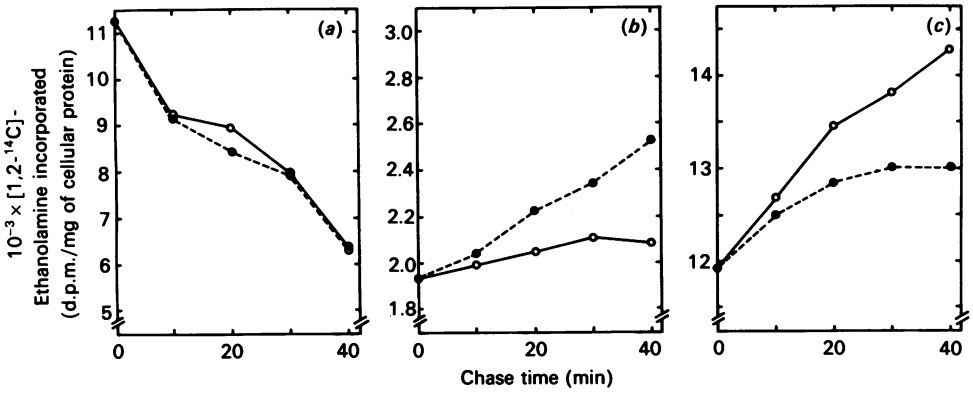

Fig. 2. Effects of glucagon on the labelling of PE and its precursors

Freshly isolated hepatocytes were pulsed with 0.1 mM-[1,2-^{14}C]ethanolamine (4400 d.p.m./nmol) for 30 min and then chased with 0.1 mM-ethanolamine up to 40 min in the absence (○) or presence (●) of glucagon. Incorporation of the label into (*a*) ethanolamine phosphate, (*b*) CDP-ethanolamine and (*c*) PE+PC is shown. Each point represents the mean of three different cell preparations.

pronounced in hepatocytes incubated with CPT-cyclic AMP than in glucagon-treated cells.

Insulin and glucagon displayed opposite effects on PE biosynthesis. Whereas glucagon decreased the incorporation of ethanolamine into PE, insulin slightly stimulated the formation of PE from labelled ethanolamine, to 110 ± 1% of the control value ($n = 3$; $P < 0.01$; results not shown).

Pelech *et al.* (1981) demonstrated that incubation with CPT-cyclic AMP diminished the uptake of choline in hepatocytes. Therefore we investigated whether glucagon had similar effects on ethanolamine uptake. As shown in Fig. 1(*a*), glucagon did not decrease the total amount of radioactivity in water-soluble ethanolamine metabolites in the cells. The amount of radioactivity of each of the cellular precursors of PE, i.e. ethanolamine, ethanolamine phosphate or CDP-ethanolamine, in glucagon-treated hepatocytes was unchanged as compared with the control incubations (results not shown). The time course of the effect of glucagon on PE biosynthesis (Fig. 1*b*) demonstrates that this hormone-induced inhibition is a rather slow effect. The incorporation of labelled ethanolamine into PE after treatment with glucagon for 10 min was only 14% lower than in control incubations. The glucagon-dependent inhibition gradually increased to 33% after incubation for 60 min in the presence of this hormone.

The effect of glucagon on the rate of PE synthesis was evaluated in pulse–chase experiments. When hepatocytes were pulsed with labelled ethanolamine for 30 min and subsequently chased in the absence or presence of glucagon, the appearance of label in PE in glucagon-treated cells was, indeed, decreased as compared with control cells (Fig. 2*c*). This effect was accompanied by an accumulation of labelled CDP-ethanolamine in hormone-treated cells (Fig. 2*b*). However, the rate of disappearance of label from ethanolamine phosphate was not affected by glucagon (Fig. 2*a*). This indicates that it is unlikely that glucagon exerts its effect at the level of CTP:ethanolamine-phosphate cytidylyl-

transferase, the supposedly rate-regulatory step in PE biosynthesis *de novo* (Sundler & Åkesson, 1975). It is interesting that the loss of label from ethanolamine phosphate is not completely recovered in the fraction containing PE+PC (Fig. 2). Actually, we found in the medium an additional labelled compound that could partly account for the observed discrepancy. Although this labelled compound has not yet been identified, it is not ethanolamine, ethanolamine phosphate or CDP-ethanolamine (L. B. M. Tijburg, unpublished work).

Effect of glucagon on the activities of the enzymes involved in the CDP-ethanolamine pathway

The activities of the PE-biosynthetic enzymes from control and glucagon-treated hepatocytes were measured, in an attempt to correlate the decreased flux through the pathway, as observed in pulse-label and pulse–chase experiments, with a possible inhibition of one of the enzymes. Incubation of hepatocytes with glucagon for 60 min did not affect the activity of ethanolamine kinase (Table 2), whereas the ethanolamine-phosphate cytidylyltransferase activity in hormone-treated cells was not significantly different from that in control hepatocytes either (Table 2). The latter observation is in line with those of pulse–chase experiments as presented in Fig. 2, which indicate that the glucagon-induced inhibition of PE synthesis is not accompanied by an inhibition of the conversion of ethanolamine phosphate into CDP-ethanolamine (Fig. 2*a*). However, incubation of hepatocytes with glucagon decreased the activity of ethanolaminephosphotransferase to 82% of the activity in control cells (Table 2). When the enzyme activity was determined in the presence of exogenous diacylglycerols, the glucagon-induced decrease in the activity of ethanolaminephosphotransferase disappeared (results not shown).

Determination of the pool sizes of PE precursors

The observations of pulse–chase experiments and measurement of the enzyme activities as described above indicated that the effect of glucagon might be exerted at the last step of PE biosynthesis, the formation of PE from CDP-ethanolamine and diacylglycerols. Hence we determined whether the supply of ethanolamine-containing PE precursors, on the one hand, and that of diacylglycerols on the other, could restrict PE synthesis *de novo*.

Treatment of hepatocytes with 0.1 μM-glucagon for 60 min induced a considerable decrease in the cellular amount of diacylglycerols to 71±5% of that in untreated cells (Table 3). The cellular concentration of diacylglycerols remained lower for at least 90 min in hormone-treated cells as compared with the controls (Table 3). Since ethanolaminephosphotransferase is supposed to be operating near its equilibrium (Infante, 1977), it is likely that the decreased activity of ethanolaminephosphotransferase in the presence of glucagon is the result of a decreased concentration of diacylglycerols.

Incubation of hepatocytes in the presence of glucagon slightly decreased the pool size of cellular ethanolamine (Table 4). The pool size of ethanolamine phosphate, on the other hand, was not affected by the presence of glucagon, whereas the amount of CDP-ethanolamine was slightly higher in glucagon-treated cells than in

Table 2. Effects of glucagon on the activities of the enzymes of the CDP-ethanolamine pathway

Hepatocytes were incubated in triplicate in the absence or presence of glucagon for 60 min. Subcellular fractionation and determination of the enzyme activities were carried out as outlined in the Experimental section. Results are presented as means±S.D. for the numbers of experiments indicated in parentheses; * indicates significant difference from control ($P < 0.01$).

Enzyme	Activity: Control (nmol/min per mg of protein)	Activity: + Glucagon (% of control)
Ethanolamine kinase	0.76±0.18	99.0±2.5 (5)
Ethanolamine-phosphate cytidylyltransferase	1.96±0.24	101.0±4.9 (5)
Ethanolamine phosphotransferase	0.12±0.03	82.1±6.9* (6)

Table 3. Effects of glucagon on hepatic diacylglycerol contents

Liver cells were incubated with or without glucagon for various time periods up to 90 min. The incubations were terminated by the addition of chloroform/methanol (1:1, v/v). Diacylglycerols were isolated and determined as free glycerol as described in the Experimental section. The results are expressed as means±ranges of two different cell preparations with triplicate incubations.

Time (min)	Diacylglycerol (nmol/mg of cellular protein): Control	+ Glucagon
0	0.54±0.02	–
20	0.65±0.05	0.46±0.01
40	0.68±0.15	0.47±0.00
60	0.66±0.07	0.47±0.02
90	0.61±0.05	0.47±0.02

Table 4. Effects of glucagon and CPT-cyclic AMP on the pool sizes of water-soluble PE precursors

Triplicate incubations were carried out for 60 min in the absence or presence of glucagon or CPT-cyclic AMP. The separation and determination of the ethanolamine-containing precursors of PE are outlined in the Experimental section. Values are means±ranges of two different cell preparations (n.d., not determined); * indicates significant difference from control ($P < 0.05$).

	Control (nmol/mg)	Pool size (% of control): + Glucagon	Pool size (% of control): + CPT-cyclic AMP
Ethanolamine	1.09±0.29	86±1*	n.d.
Ethanolamine phosphate	5.32±0.49	91±7	95±6
CDP-ethanolamine	0.25±0.02	111±11	140±13

control incubations (Table 4). Treatment of hepatocytes with CPT-cyclic AMP enhanced the pool size of CDP-ethanolamine, although this effect was not statistically significant.

DISCUSSION

The present study shows that glucagon inhibits the biosynthesis of PE via the CDP-ethanolamine pathway in freshly isolated rat hepatocytes. Since both aminophylline and the cyclic AMP derivative CPT-cyclic AMP showed the same ability to inhibit PE synthesis (Table 1), it is likely that the effect of glucagon is mediated by raising the intracellular concentration of cyclic AMP.

In a previous study from this laboratory (Geelen *et al.*, 1979) we reported that incubation of hepatocytes in monolayer culture with glucagon for 4 h enhanced the incorporation of labelled ethanolamine into PE and PC. The results presented in the present study from pulse-label as well as pulse–chase experiments clearly indicate that treatment of hepatocytes with glucagon for less than 1 h inhibits the formation of PE from labelled ethanolamine. Our observations with respect to the time-dependent effect of glucagon on the synthesis of PE are in line with those of Pelech *et al.* (1982) on the effects of cyclic AMP on PC synthesis. These investigators demonstrated that incubation of hepatocytes with CPT-cyclic AMP for less than 6 h decreased the incorporation of labelled choline into PC. However, on prolonged incubation, this effect was reversed and PC biosynthesis was stimulated by CPT-cyclic AMP (Pelech *et al.*, 1982). It was suggested by these authors that the cell can tolerate a diminished PC synthesis only for a limited period before its viability will be decreased. For the effects of glucagon on the biosynthesis of PE a similar reasoning may be used. The short-term inhibition of PE synthesis *de novo* by glucagon, as demonstrated in the present study, is consistent with the other effects of glucagon on lipid metabolism (Geelen *et al.*, 1980). However, these effects may be reversed after longer exposure, possibly in order to maintain cell viability.

It can be concluded from pulse–chase experiments with labelled ethanolamine (Fig. 2) that the decreased PE synthesis in the presence of glucagon is not accompanied by an inhibition of the conversion of ethanolamine phosphate into CDP-ethanolamine, the reaction catalysed by the putative rate-regulatory enzyme of the pathway, CTP:ethanolamine-phosphate cytidylyltransferase (Sundler & Åkesson, 1975). The observations of the pulse–chase experiments are corroborated by the measurement of the activity of this enzyme, which was not subject to alterations after treatment with glucagon (Table 2). Moreover, incubation of hepatocytes with glucagon did not affect the pool size of ethanolamine phosphate (Table 4). Comparable results were obtained with CPT-cyclic AMP. Incubation of cells with this cyclic AMP analogue did not affect the activity of ethanolamine-phosphate cytidylyltransferase (results not shown), nor the cellular concentration of ethanolamine phosphate (Table 4).

Vance and co-workers (Pelech *et al.*, 1981, 1982) observed that cyclic AMP analogues inhibit PC biosynthesis in the short term. This effect is possibly mediated by a decreased activity of choline-phosphate cytidylyltransferase. Studies with rat liver cytosol (Pelech & Vance, 1982) showed that choline-phosphate cytidylyltransferase can be inhibited by incubation with Mg^{2+} and ATP. Inhibitors of cyclic AMP-dependent protein kinase prevented this inhibition. These observations suggest that the control of the activity of choline-phosphate cytidylyltransferase is mediated through a cyclic AMP-dependent phosphorylation–dephosphorylation mechanism. There seems to be a clear distinction between the cyclic AMP-dependent regulation of PC and PE biosynthesis via the CDP-choline and CDP-ethanolamine pathways respectively. Although several observations indicate (Pelech *et al.*, 1981; Pelech & Vance, 1982) that the biosynthesis of PC is under cyclic AMP-dependent control, the present results with hepatocytes suggest that ethanolamine-phosphate cytidylyltransferase is not regulated by a cyclic AMP-dependent mechanism (Fig. 2, Table 2). Preliminary experiments with rat liver cytosol showed that incubation with Mg^{2+} and ATP decreased the activity of CTP:ethanolamine-phosphate cytidylyltransferase. However, this inhibition could not be prevented by the addition of a cyclic AMP-dependent protein kinase inhibitor from rabbit muscle (L. B. M. Tijburg, unpublished work).

Although a direct cyclic AMP-dependent regulation of ethanolamine-phosphate cytidylyltransferase cannot be excluded entirely at present, our observations in pulse–chase experiments with glucagon (Fig. 2) and measurement of the enzyme activities (Table 2) point to an indirect inhibition of the incorporation of ethanolamine into PE. It is strongly suggested that the inhibition of the PE synthesis is due to a decreased rate of the last step of this biosynthesis, the formation of PE from CDP-ethanolamine and diacylglycerols. The latter effect is probably not the result of a diminished supply of CDP-ethanolamine, as the pool size of this compound is even slightly enhanced in glucagon-treated cells (Table 4). However, the amount of cellular diacylglycerols was lower in hepatocytes incubated in the presence of glucagon as compared with the controls (Table 3). This is in line with the observation by Butterwith *et al.* (1984), who reported that incubation of hepatocytes with CPT-cyclic AMP for 1 h led to a translocation of phosphatidate phosphohydrolase from the microsomal compartment to the cytosol, indicating that this enzyme is less active in the presence of CPT-cyclic AMP. This might induce a lower concentration of diacylglycerols, which in turn results in a lower activity of ethanolamine-phosphotransferase in the presence of glucagon (Table 2). In conclusion, the observations in the present study suggest a possible role for the supply of diacylglycerols in the glucagon-determined rate of the biosynthesis of PE in rat hepatocytes.

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