Modulation of phosphoenolpyruvate carboxykinase mRNA levels by the hepatocellular hydration state

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Exposure of isolated perfused rat livers to hypo-osmotic (225 mosmol/l) perfusion media for 3 h led to a decrease of about 60 % in mRNA levels for phosphoenolpyruvate carboxykinase (PEPCK) compared with normo-osmotic (305 mosmol/l) perfusions. Conversely, PEPCK mRNA levels increased about 3-fold during hyperosmotic (385 mosmol/l) perfusions. The anisotonicity effects were not explained by changes in the intracellular cyclic AMP (cAMP) concentration or by changes of the extracellular Na^+ or Cl^- activity. Similar effects of aniso-osmolarity on PEPCK mRNA levels were found in cultured rat hepatoma H4IIE.C3 cells, the experimental system used for further characterization of the effect. Whereas during the first hour of anisotonic exposure no effects on PEPCK mRNA levels were detectable, near-maximal aniso-osmolarity effects were observed within the next 2–3 h. PEPCK mRNA levels increased sigmoidally with the osmolarity of the medium, and the anisotonicity

effects were most pronounced upon modulation of osmolarity between 250 and 350 mosmol/l. The aniso-osmolarity effects on PEPCK mRNA were not affected in presence of Gö 6850, a protein kinase C inhibitor. cAMP increased the PEPCK mRNA levels about 2.3-fold in normo-osmotic media, whereas insulin lowered the PEPCK mRNA levels to about 8 %. The effects of cAMP and insulin were also observed during hypo-osmotic and hyperosmotic exposure, respectively, but the anisotonicity effects were not abolished in presence of the hormones. The data suggest that hepatocellular hydration affects hepatic carbohydrate metabolism also over a longer term by modulating PEPCK mRNA levels. This is apparently unrelated to protein kinase C or alterations of cAMP levels. The data strengthen the view that cellular hydration is an important determinant for cell metabolic function by extending its regulatory role in carbohydrate metabolism to the level of mRNA.

INTRODUCTION

Alterations of hepatocellular hydration, which reflect changes of liver cell volume on a short-term time scale, occur within minutes under the influence of hormones, cumulative substrate uptake or oxidative stress. Such alterations of hepatocellular hydration were recently recognized as another ‘second messenger’ of hormone and amino acid action, which influences a variety of metabolic liver functions [for reviews see Häussinger and Lang (1991, 1992) and Lang and Häussinger (1993)], such as protein turnover and carbohydrate metabolism. Regarding the latter, hypo-osmotic cell swelling stimulates glycogen synthesis (Baquet et al., 1990; Meijer et al., 1992) and flux through the pentose phosphate shunt (Saha et al., 1992) and inhibits glycolysis and glycogenolysis (Graf et al., 1988; Lang et al., 1989); opposite effects are found following hyperosmotic cell shrinkage. The effects of aniso-osmolarity on carbohydrate-metabolizing enzymes occur within minutes and are in part due to alterations of the phosphorylation state of key enzymes of glycogen metabolism (Meijer et al., 1992; Baquet et al., 1993; Häussinger et al., 1994a). On the other hand, hepatocellular hydration may affect gene expression. For example, hypo-osmotic liver cell swelling increases the levels of mRNA for tubulin (Häussinger et al., 1994b), β -actin (Schulz et al., 1991; Theodoropoulos et al., 1992), ornithine decarboxylase (Tohyama et al., 1991) and c-jun, but not of c-fos (Finkenzeller et al., 1994). Here we addressed the question whether hepatocellular hydration affects carbohydrate metabolism in a more prolonged way by modulating the mRNA levels of the phosphoenolpyruvate carboxykinase (PEPCK) gene.

PEPCK gene expression has been studied extensively in the past, and among the gluconeogenic enzymes the regulatory elements of the PEPCK gene have been studied most extensively (Warren et al., 1983; Beale et al., 1985; Gurney et al., 1992); for reviews see Pilkis and Granner, 1992; Granner and Pilkis, 1990; McGrane et al., 1992). The present study shows that hepatocellular hydration exerts marked effects on PEPCK mRNA levels in the intact rat liver as well as in H4IIE hepatoma cells.

MATERIALS AND METHODS

Materials

Cyclic AMP (cAMP), 8-(4-chlorophenylthio) cAMP (CPT-cAMP) and restriction enzymes were from Boehringer (Mannheim, Germany). Guanidine thiocyanate and sodium lauroylsarcosinate were from Fluka (Karlsruhe, Germany). Oligonucleotide-labelling kit was from Pharmacia (Freiburg, Germany). Cell-culture medium, fetal-calf serum and glutamine were from Gibco (Eggenstein, Germany). [α - ^{32}P]dCTP (3000 Ci/mmol), the cAMP radioimmunoassay kit and Hybond-N nylon membranes were from Amersham Buchler (Braunschweig, Germany). The salts required for preparation of the Krebs–Henseleit buffer used in liver perfusions were from Merck (Darmstadt, Germany). All other chemicals were from Sigma (Munich, Germany). Gö 6850, which is identical with GF 109203 (Martiny-Baron et al., 1993), was a gift from Dr. C. Schächtele (Gödecke A. G., Freiburg, Germany). The plasmid pBSPCK, containing a 1.6 kb fragment coding for PEPCK, was kindly provided by Dr. R. Hanson (Case Western Reserve

Abbreviations used: PEPCK, phosphoenolpyruvate carboxykinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; cAMP, cyclic AMP; CPT-cAMP, 8-(4-chlorophenylthio) cAMP.

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University, U.S.A.) (Yoo-Warren et al., 1981). The 1.0 kb cDNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used for standardization was from Clontech (Palo Alto, U.S.A.).

Liver perfusion

Livers from male Wistar rats (120–200 g body weight), fed *ad libitum* with standard chow, were perfused as described previously (Sies, 1978) in a non-recirculating manner from the portal to the hepatic vein with bicarbonate-buffered Krebs–Henseleit saline (305 mosmol/l) containing lactate (2.1 mM) and pyruvate (0.3 mM) for 20 min. Thereafter, the perfusion medium was changed to the respective test media. These test media included the above-mentioned normo-osmotic (305 mosmol/l) Krebs–Henseleit medium ('control') supplemented with glutamine (3 mM), cAMP (50 μ M) or insulin (35 nM). Hyperosmotic (385 mosmol/l) media were prepared by adding raffinose (80 mM) or NaCl (40 mM). Hypo-osmotic (225 mosmol/l) test media were obtained by removing 40 mM NaCl from the normo-osmotic control medium. A normo-osmotic low-NaCl medium was prepared by substituting 40 mM NaCl for 80 mM raffinose. All perfusates were equilibrated with O₂/CO₂ (19:1); the temperature was 37 °C. The perfusate flow was approx. 4 ml/min per g of liver tissue and was kept constant throughout the individual perfusion experiment. Perfusion with these test media was carried out for 3 h; then the liver tissue was quickly removed for extraction of total RNA (see below). When cAMP levels were determined, perfusions with the test media were carried out for only 90 min and the liver tissue was immediately frozen in liquid nitrogen with precooled aluminium tongs. The deep-frozen liver tissue was homogenized to a fine powder, which was divided into two parts. After weighing, one part was used to prepare neutralized HClO₄ extracts as described by Häussinger et al. (1975); these extracts were used for determination of cAMP by means of a commercially available radioimmunoassay kit (Amersham Buchler, Braunschweig, Germany). After weighing the other part of the frozen liver powder, protein was precipitated with trichloroacetic acid for determination of the protein content/g of frozen liver powder. This approach allowed us to calculate the cAMP levels in the liver tissue per mg of liver protein and to correct the cAMP levels for the different water contents in the deep-frozen liver samples. Data on cAMP levels are given as means $\pm$ S.E.M. (n = 4 different perfusions).

Cell culture

H4IIE.C3 rat hepatoma cells (A.T.C.C. CRL 1600) were grown to near-confluency in DME/F12 medium, 37 °C, 5% CO₂, pH 7.4, plus glucose (5 mM), supplemented with 10% fetal-calf serum in 100 mm-diam. culture plates. Medium was changed 12–16 h before the experiments. Osmolarity was altered by varying the NaCl concentration in the medium. Cells were maintained under the various test conditions for 6 h, unless indicated otherwise. CPT-cAMP (50 μ M) and insulin (100 nM) were used to demonstrate positive and negative regulation of PEPCK gene expression. In other experiments, cells were pre-treated with the protein kinase C inhibitor Gö 6850 for 15 min and then exposed to test media, also containing the inhibitor, for 6 h.

Northern-blot analysis

Perfused livers were harvested and about 1 g of tissue was immediately homogenized in 10 ml of guanidine thiocyanate solution at 4 °C with an Ultra-Turrax instrument at high speed.

Total RNA from liver or near-confluent plates of H4IIE cells was isolated by using guanidine thiocyanate solution as described by Chomczynski and Sacchi (1987). RNA samples (15 μ g) were electrophoresed in 0.8% agarose/3% formaldehyde and then blotted on to Hybond-N nylon membranes with 20 $\times$ SSC (3 M NaCl, 0.3 M sodium citrate). After brief rinsing with water and u.v. cross-linking (Stratagene UV-Stratalinker 1800), the membranes were observed under u.v. illumination to determine RNA integrity and the location of the 28 S and 18 S rRNA bands. Blots were then subjected to a 3 h prehybridization at 43 °C in 50% deionized formamide, in sodium phosphate buffer (0.25 M, pH 7.2), containing 0.25 M NaCl, 1 mM EDTA, 100 μ g/ml salmon sperm DNA and 7% SDS. Hybridization was carried out in the same solution with [α -³²P]dCTP-labelled random-primed cDNA probes. The *Eco*RI/*Xba*I 1.6 kb fragment from pBSPCK was used to determine PEPCK mRNA levels. All blots were then standardized with GAPDH. Membranes were washed three times in 2 $\times$ SSC/0.1% SDS for 10 min, twice in sodium phosphate buffer (25 mM, pH 7.2)/EDTA (1 mM)/0.1% SDS and twice in sodium phosphate buffer (25 mM, pH 7.2)/EDTA (1 mM)/1.0% SDS. Blots were then exposed to 'preflashed' Kodak AR X-Omat film at –70 °C with intensifying screens. Suitably exposed autoradiograms were then analysed with PDI densitometry scanning (Pharmacia, Freiburg, Germany) to determine the optical densities of the mRNA levels for PEPCK and GAPDH. Relative PEPCK mRNA levels were determined by standardization to the optical density of GAPDH mRNA.

Expression of results

Data are given as means $\pm$ S.E.M. (n = number of perfusion experiments or culture dishes).

RESULTS

Effect of aniso-osmolarity on PEPCK mRNA and cAMP levels in perfused rat liver

Hyperosmotic (385 mosmol/l) exposure of isolated perfused rat liver for 3 h due to addition of NaCl (40 mM) to the Krebs–

Figure 1 PEPCK mRNA levels in isolated perfused rat liver under the influence of anisotonicity, insulin, cAMP, glutamine and leucine

Representative Northern blots are shown from a series of 3–4 perfusion experiments each. For experimental details see legend to Table 1, which also shows the quantification of the relative PEPCK mRNA levels under the respective conditions.

Table 1 Modulation of PEPCK mRNA levels in perfused rat liver by anisotonicity, hormones and amino acids

Livers were perfused as described in the Materials and methods section for 20 min with normo-osmotic medium (305 mosmol/l); thereafter the test conditions were instituted and the single-pass perfusion was continued for another 3 h. Then the liver tissue was harvested and, after RNA isolation, total RNA was subjected to Northern-blot analysis for PEPCK and GAPDH. The PEPCK mRNA levels were normalized to GAPDH mRNA levels; the values found after 200 min in normo-osmotic perfusions ('control') were set to 1. Test conditions consisted of hypo-osmotic (225 mosmol/l) and hyperosmotic (385 mosmol/l) media, a normo-osmotic medium in which 40 mM NaCl was substituted for 80 mM raffinose, a hyperosmotic medium produced by addition of 80 mM raffinose, and normo-osmotic control media supplemented with 35 nM insulin, 50 μ M cAMP, 3 mM glutamine or 3 mM leucine. cAMP tissue levels were determined after a 90 min exposure of perfused livers to the test media. Thereafter, the liver tissue was freeze-clamped and analysed for cAMP and protein content, as described in the Materials and methods section. Data are given as means $\pm$ S.E.M. and are from 3–4 different perfusion experiments for each condition with respect to PEPCK mRNA and from 4 different experiments for cAMP tissue levels. Abbreviation: n.d., not determined.

Condition	Osmolarity (mosmol/l)	Relative PEPCK mRNA level	cAMP tissue levels (pmol/mg of protein)
Normo-osmotic control	305	1.0 $\pm$ 0.3	1.79 $\pm$ 0.06
Hypo-osmotic (–40 mM NaCl)	225	0.4 $\pm$ 0.1	2.00 $\pm$ 0.09
Hyperosmotic (+40 mM NaCl)	385	2.8 $\pm$ 0.5	1.67 $\pm$ 0.11
Hyperosmotic (+80 mM raffinose)	385	5.6 $\pm$ 1.6	n.d.
Normo-osmotic (–40 mM NaCl/+80 mM raffinose)	305	0.9 $\pm$ 0.3	n.d.
Insulin (35 nM)	305	0.6 $\pm$ 0.3	n.d.
cAMP (50 μ M)	305	4.1 $\pm$ 1.1	n.d.
Glutamine (3 mM)	308	3.7 $\pm$ 0.4	1.86 $\pm$ 0.07
Leucine (3 mM)	308	1.0 $\pm$ 0.4	n.d.

Henseleit perfusion medium led to a 2.8-fold increase, whereas hypo-osmotic exposure (225 mosmol/l, by lowering the NaCl concentration by 40 mM) led to a 60% decrease in PEPCK mRNA levels, compared with normo-osmotic (305 mosmol/l) control perfusions (Figure 1, Table 1). GAPDH mRNA levels were chosen in these experiments as standard, because this mRNA was previously shown to be uninfluenced by aniso-osmolarity (Schulz et al., 1991). Substitution of 40 mM NaCl for 80 mM raffinose and accordingly maintenance of normo-osmolarity had no effect on PEPCK mRNA levels (Table 1, Figure 1). Hyperosmolarity due to addition of raffinose also increased PEPCK mRNA levels (Figure 1, Table 1). These findings indicate that the aniso-osmolarity effects on PEPCK mRNA levels in perfused rat liver are not explained by alterations of the extracellular Na⁺ or Cl[–] activity, but are due to osmolarity or cell volume changes. Table 1 also shows the effect of aniso-osmotic rat liver perfusion for 1.5 h on the cAMP tissue levels. Hypo-osmotic exposure increased the cAMP content, when expressed per mg of liver protein, by about 12%, and hyperosmotic exposure decreased it by about 7%. Taking into account that hypo- and hyper-tonic exposure led to roughly parallel changes in the intracellular water space by about 10–15%, as shown by Hallbrucker et al. (1991) and vom Dahl et al. (1991), the data indicate that aniso-osmotic exposure does not significantly affect the hepatocellular cAMP concentration, at least after 1.5 h of aniso-osmotic exposure (Table 1). Also, hypo-osmotic exposure for 30 min had no significant effect on cAMP tissue levels (results not shown). These findings are in line with previous studies demonstrating also that hypo-osmotic exposure for 5 and 30 min did not significantly affect cAMP levels in isolated rat hepatocytes (Baquet et al., 1991; Gaussin et al., 1992).

Effect of aniso-osmolarity on PEPCK mRNA levels in H4IIE rat hepatoma cells: osmolarity dependence and time course

Aniso-osmotic exposure of H4IIE rat hepatoma cells, induced either by changing the NaCl concentration or by addition of raffinose, led to changes in PEPCK mRNA levels similar to those observed with the intact perfused rat liver (Figure 2, Table 2), but

Figure 2 Effect of aniso-osmotic exposure on PEPCK and GAPDH mRNA levels in H4IIE rat hepatoma cells

H4IIE cells were incubated for 6 h in media with different osmolarities. The osmolarity changes were performed by appropriate changes of the NaCl concentration. Thereafter, the cells were harvested for RNA isolation and subjected to Northern-blot analysis for PEPCK and GAPDH (15 μ g of total RNA per lane).

had no detectable effects on GAPDH mRNA levels (Figure 2). Cultured H4IIE cells were then used to characterize further the modulation of PEPCK mRNA levels by aniso-osmolarity. Figure 3 shows that the PEPCK mRNA levels under the influence of a 6 h aniso-osmotic exposure were strongly dependent on the osmolarity of the medium, with most sensitive regulation occurring in the range between 250 and 350 mosmol/l. The time courses of aniso-osmolarity effects on the relative PEPCK mRNA levels are shown in Figure 4. No significant changes in the PEPCK mRNA levels were detectable during the first 1 h of aniso-osmotic exposure, but near-maximal aniso-osmolarity effects were achieved within the next 3 h of aniso-osmotic exposure.

Role of protein kinase C in the aniso-osmolarity-induced changes in PEPCK gene expression

In order to determine whether the effects of aniso-osmolarity on PEPCK mRNA levels involve protein kinase C, the effects of aniso-osmolarity on PEPCK mRNA levels were investigated in the absence or presence of Gö 6850, an inhibitor of Ca²⁺-

Table 2 Effects of aniso-osmotic exposure, CPT-cAMP, insulin and glutamine on PEPCK mRNA levels in H4IIE rat hepatoma cells

For experimental details see the Materials and methods section. Sub-confluent cultures of H4IIE cells were exposed for 6 h to media with different osmoticity or to normo-osmotic medium containing CPT-cAMP (50 μ M), insulin (100 nM) or glutamine (20 mM). Thereafter cells were harvested and analysed for PEPCK and GAPDH mRNA by Northern-blot analysis. The PEPCK mRNA levels were normalized to GAPDH mRNA levels; the value found after 6 h in normo-osmotic (305 mosmol/l) incubations was set to 1.00 (a); in (b) and (c) the value found after 6 h of hyperosmotic (405 mosmol/l) or hypo-osmotic (205 mosmol/l) was set to 1.00, respectively. Osmolarity changes were performed by addition or removal of appropriate amounts of NaCl. Data are given as means $\pm$ S.E.M. ($n = 3-4$).

Condition	Relative PEPCK mRNA level
(a) Normo-osmotic (305 mosmol/l)	1.00
Hypo-osmotic (205 mosmol/l)	0.12 $\pm$ 0.04
Hyperosmotic (405 mosmol/l)	2.79 $\pm$ 0.17
Normo-osmotic (305 mosmol/l) (- 50 mM NaCl/ + 100 mM raffinose)	0.90 $\pm$ 0.20
Hyperosmotic (405 mosmol/l) (+ 100 mM raffinose)	2.17 $\pm$ 0.20
Glutamine (20 mM)	0.38 $\pm$ 0.01
CPT-cAMP (50 μ M)	2.29 $\pm$ 0.11
Insulin (100 nM)	0.08 $\pm$ 0.04
(b) Hyperosmotic (405 mosmol/l) Plus insulin (100 nM)	1.00 0.17 $\pm$ 0.06
(c) Hypo-osmotic (205 mosmol/l) Plus CPT-cAMP (50 μ M)	1.00 5.00 $\pm$ 0.35

Figure 3 Effect of osmolarity of the medium on relative PEPCK mRNA levels in cultured H4IIE rat hepatoma cells

H4IIE cells were incubated for 6 h in media of different osmolarity, with osmolarity changes being performed by corresponding alterations of the NaCl concentration. Thereafter cells were harvested for RNA isolation and subjected to Northern-blot analysis for PEPCK and GAPDH. PEPCK mRNA levels were normalized for GAPDH mRNA levels, and the relative PEPCK mRNA levels found in normo-osmotic incubations (305 mosmol/l) were arbitrarily set to 1. Data are given as means $\pm$ S.E.M. and are from 3 different culture dishes for each condition.

dependent and Ca^{2+} -independent isoforms of protein kinase C (Martiny-Baron et al., 1993). At a concentration of 1 μ M, i.e. sufficiently high to abolish fully the inductive effect of the phorbol ester PMA on the c-jun gene in H4IIE hepatoma cells

Figure 4 Time course of the aniso-osmolarity effects on relative PEPCK mRNA levels

H4IIE cells were incubated for the time periods indicated in normo-osmotic (305 mosmol/l), hypo-osmotic (205 mosmol/l) or hyperosmotic (405 mosmol/l) medium. The osmolarity changes were performed by increasing or lowering the NaCl concentration by 50 mM. At the time points indicated, cells were harvested for RNA isolation and subjected to Northern-blot analysis for PEPCK and GAPDH. PEPCK mRNA levels were normalized for GAPDH mRNA levels, and the relative PEPCK mRNA levels found in normotonic incubations (305 mosmol/l) at each time point were set to 100% and the relative PEPCK mRNA levels found in aniso-osmotic media were expressed as percentages thereof. Data are given as means $\pm$ S.E.M. and are from 3 different culture dishes for each condition.

Figure 5 Effect of the protein kinase C inhibitor Gö 6850 on the aniso-osmolarity effects on PEPCK mRNA levels in H4IIE rat hepatoma cells

H4IIE cells were incubated for 6 h in normo-osmotic (305 mmol/l), hypotonic or hypertonic medium in the absence or presence of Gö 6850 (1 μ M; for details see the Materials and methods section). The osmolarity changes were performed by appropriate changes in the NaCl concentration. Thereafter, the cells were harvested for RNA isolation and subjected to Northern-blot analysis for PEPCK and GAPDH (15 μ g of total RNA per lane).

(Finkenzeller et al., 1994), Gö 6850 had no effect on the aniso-osmolarity effects on PEPCK mRNA levels (Figure 5).

Modulation of cAMP and insulin effects on PEPCK mRNA levels by aniso-osmolarity

In line with previous studies (Lamers et al., 1982; Granner et al., 1983; Sasaki et al., 1984; Pilkis and Granner, 1992; Fillat et al., 1993), cAMP increased and insulin decreased PEPCK mRNA levels in both perfused rat liver (Figure 1 and Table 1) and H4IIE

Figure 6 PEPCK mRNA levels in H4IIE hepatoma cells under the influence of anisotonicity, insulin or cAMP

H4IIE cells were incubated for 6 h in media of different osmolarity, with osmolarity changes being performed by corresponding alterations of the NaCl concentration and insulin (100 nM) or CPT-cAMP (50 μ M). Thereafter, cells were harvested for RNA isolation and subjected to Northern-blot analysis for PEPCK and GAPDH; 15 μ g of total RNA was loaded per lane. For quantification of experiments from a similar series, see Table 2.

rat hepatoma cells (Figure 6 and Table 2). The extent of the hormone effects on PEPCK mRNA was comparable with that of the anisotonicity effects (Tables 1 and 2). Because insulin is known to induce cell swelling (Hallbrucker et al., 1991), we tested to what extent hyperosmotic cell shrinkage counteracted the insulin effect in H4IIE cells. When the PEPCK mRNA level found in hyperosmotic (405 mosmol/l) incubations was set to 100%, addition of insulin (100 nM) to hyperosmotic media lowered the PEPCK mRNA levels to $17 \pm 6\%$ ($n = 3$), indicating that the insulin effect was preserved. However, taking into account that the PEPCK mRNA level in hyperosmotic media was about 2.8-fold the normotonic level (Table 2, Figure 3), the combined exposure to hyperosmolarity plus insulin may result roughly in a 50% decrease of the PEPCK mRNA level compared to normo-osmotic control incubations, whereas insulin in normo-osmotic incubations lowered it by about 90%. The inductive effect of cAMP, which is known to induce cell shrinkage (Hallbrucker et al., 1991) was preserved in hypo-osmotically swollen cells. However, taking into account that hypo-osmotic exposure itself lowered the PEPCK mRNA levels to 10% of the normotonic control values, the 5-fold stimulation by cAMP in presence of hypo-osmotic media may correspond to a PEPCK mRNA level only about half of that found in normo-osmotic control incubations. These data suggest that the repressive/inductive effects of insulin and cAMP are still present in hyperosmotic/hypo-osmotic incubations respectively, but also demonstrate that these hormones do not abolish the anisotonicity effects on PEPCK gene expression.

Effects of glutamine and leucine on PEPCK mRNA levels

Glutamine is known to induce cell swelling due to the cumulative uptake of the amino acid into liver cells (Häussinger and Lang, 1991). As shown in Table 2, glutamine significantly lowered the PEPCK mRNA levels in H4IIE hepatoma cells, compatible with a role of cell volume in regulating PEPCK mRNA levels. However, glutamine had, surprisingly, a strong inductive effect

in the intact perfused rat liver (Table 1), despite cell swelling (Häussinger and Lang, 1991). cAMP tissue levels were not affected after a 90 min exposure to the amino acid at a concentration of 3 mM (Table 1), and only a minor increase of cAMP content in isolated rat hepatocytes exposed to 10 mM glutamine for 30 min was reported (Baquet et al., 1991). Thus cAMP is probably not involved in the inductive effect of glutamine in the intact liver. Possibly, different rates of glutamate and α -oxoglutarate formation from glutamine in perfused liver and hepatoma cells, respectively, and interference with the regulation of PEPCK gene by substrates (for review, see Pilkis and Granner, 1992) may account for the opposing effects of glutamine on PEPCK mRNA in both cell types. This was, however, not investigated further. Leucine, which has no effect on cell volume, did not affect PEPCK mRNA levels in perfused rat liver (Table 1).

DISCUSSION

Control of PEPCK mRNA levels by aniso-osmolarity

The data in this paper show that hepatocellular hydration exerts marked effects on PEPCK mRNA levels, thereby adding a new aspect to the complex regulation of the PEPCK gene. Our study, however, leaves open the question whether modulation of PEPCK mRNA levels by cell volume occurs at the level of gene transcription or mRNA stability. The PEPCK gene is known to be subject to positive and negative regulation by hormones and dietary factors. For example, insulin inhibits PEPCK mRNA transcription, whereas glucagon (via cAMP) and glucocorticoids increase transcription and simultaneously stabilize PEPCK mRNA (for reviews see Granner and Pilkis, 1990; Pilkis and Granner, 1992). It appears unlikely that changes in the cellular cAMP content mediate the effects of aniso-osmolarity on PEPCK mRNA levels. This is suggested by the finding that no effect on the hepatocellular cAMP concentration was found in perfused liver after 90 min exposure to aniso-osmotic media (Table 1), in line with previous studies measuring the cAMP levels after 5 and 30 min (Baquet et al., 1991; Gaussin et al., 1992). Further, hypo-osmotic cell swelling decreased the PEPCK mRNA levels even in presence of high concentrations of CPT-cAMP (Table 2). However, the possibility is not ruled out that aniso-osmotic cell volume modulation interferes in a cAMP-independent way (for example, due to an altered intracellular ionic composition) with the activity of protein kinase A. On the other hand, in view of the data shown in Figure 5 it is unlikely that protein kinase C is involved in the anisotonicity effects on PEPCK mRNA levels. This is similar to the swelling-induced increase of c-jun mRNA in H4IIE cells, which was also found to be insensitive to inhibition by the protein kinase C inhibitor Gö 6850 (Finkenzeller et al., 1994). Recent studies demonstrated binding of Jun homodimers and Fos/Jun heterodimers (activator protein-1; AP-1) to the promoter region of the PEPCK gene, and an increased expression of Jun stimulated, whereas Fos inhibited, PEPCK gene transcription (Gurney et al., 1992). Hypo-osmotic cell swelling was recently shown to increase in H4IIE cells the mRNA levels for c-jun, but not for c-fos (Finkenzeller et al., 1994). Thus an altered expression of these early immediate genes probably does not mediate the cell-volume effects on PEPCK mRNA. However, aniso-osmolarity effects on the physical association of Jun and Fos or on their phosphorylation state, which is crucial for the AP-1 binding activity to DNA (Curran and Franza, 1988; Angel and Karin, 1991), have not been investigated and could be involved in transducing the aniso-osmolarity effects on PEPCK mRNA levels. Although the molecular mechanisms of the cell-volume-dependent regulation of the PEPCK mRNA levels

remain to be elucidated, it is attractive to speculate that the lag phase of about 1 h required to detect anisotonicity effects on PEPCK mRNA levels (Figure 4) may be indicative of the formation of transcription factors. Indeed, recent studies showed an increased histone phosphorylation in response to aniso-osmotic exposure (Santell et al., 1993), and cell-volume changes have been suggested to alter protein phosphorylation due to changes of 'macromolecular crowding' (Minton et al., 1992).

Insulin and cAMP were previously shown to induce cell swelling and cell shrinkage, respectively (Hallbrucker et al., 1991), and the repressive/inductive effects on PEPCK mRNA of these hormones are mimicked by hypo-osmotic cell swelling and hyperosmotic cell shrinkage, respectively. However, our data cannot be taken to suggest that the hormone effects on PEPCK mRNA are primarily explained by hormone-induced cell volume changes, although a contributing role of hormone-induced cell volume changes to PEPCK mRNA regulation is not ruled out. For example, insulin was reported to induce an almost immediate inhibition of PEPCK gene transcription (Granner et al., 1983, Sasaki et al., 1984), whereas the decline in mRNA levels in response to hypo-osmotic cell swelling shows a 1 h lag period (Figure 4).

Possible significance

Hyperosmotic cell shrinkage not only increases PEPCK mRNA levels, but also activates glycogen phosphorylase *a* activity (Häussinger et al., 1994a). Thus, cell shrinkage may augment glucose output from the liver by increasing glycogenolysis (Graf et al., 1988; Lang et al., 1989) and gluconeogenesis and by inhibiting glycogen synthesis (Baquet et al., 1991). Furthermore, both PEPCK induction and stimulation of proteolysis occur in response to cell shrinkage (Häussinger et al., 1991), which should favour gluconeogenesis from proteolysis-derived amino acids. This may be seen as another example of a co-ordination of functionally linked processes in response to changes of cellular hydration. To what extent our findings are of physiological relevance remains to be elucidated, because *in vivo* fluctuations of the osmolarity in portal venous blood are probably small, but cell volume may change markedly under physiological conditions in response to cumulative substrate uptake. However, glutamine, which is known to induce cell swelling, had opposing effects on PEPCK mRNA levels in perfused rat liver and cultured rat hepatoma cells respectively. The reason for this is unclear, but the data indicate that at least the effects of this amino acid or its metabolites on PEPCK mRNA levels cannot be explained exclusively on the basis of glutamine-induced cell swelling. Recently, glutamate was identified as a potent regulator of glycogen synthase phosphatase (Meijer et al., 1992), and different rates of glutamate formation from glutamine in perfused liver and hepatoma cells respectively could influence protein phosphorylation in both cell types differently, and hence PEPCK mRNA levels.

This work was supported by Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 154, the Gottfried Wilhelm Leibniz-Programm, the Schilling Stiftung and the Fonds der Chemischen Industrie.

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