Distinct effects of glucagon and vasopressin on proline metabolism in isolated hepatocytes

The role of oxoglutarate dehydrogenase

James M. STADDON and John D. MCGIVAN

Department of Biochemistry, University of Bristol Medical School, University Walk, Bristol BS8 1TD, U.K.

(Received 11 July 1983/Accepted 26 September 1983)

The hormonal regulation of gluconeogenesis and ureogenesis in isolated rat hepatocytes with 5 mM-proline as precursor was studied, with the following results. (1) The formation of glucose and urea in a 30 min interval were stimulated more by vasopressin than by glucagon, and the effects of the two hormones in combination were additive. (2) The rates of gluconeogenesis during the 30 min were constant under control, glucagon-stimulated and glucagon-plus-vasopressin-stimulated conditions. The stimulated rate in the presence of vasopressin diminished with time; glucagon in combination with vasopressin prevented this diminution, resulting in an additive effect. (3) Coincident with these changes in gluconeogenesis, vasopressin caused a decrease in cell oxoglutarate concentration, which, in contrast with the decrease caused by glucagon, was greater, but not sustained unless glucagon was also present. Changes in cell glutamate concentration similar to those observed for oxoglutarate occurred. (4) The data suggest that activation of oxoglutarate dehydrogenase (EC 1.2.4.2) by glucagon and vasopressin by different mechanisms may explain the relative effects of the hormones alone and in combination on gluconeogenesis from proline.

Hepatic metabolism can be regulated in the short-term by glucagon, which acts via a mechanism involving cyclic AMP, and by hormones (e.g. vasopressin, angiotensin and phenylephrine) acting via a cyclic AMP-independent Ca^{2+} -dependent mechanism (for reviews, see, e.g., Williamson *et al.*, 1981; Exton, 1981). Some of the effects of glucagon and vasopressin on hepatic metabolism are similar, e.g. they stimulate both glycogenolysis (see Hems & Whitton, 1980) and gluconeogenesis (see Hue *et al.*, 1981*b*). In isolated hepatocytes, distinct effects of glucagon and vasopressin have been observed concerning, e.g. ketogenesis (Williamson *et al.*, 1980), protein phosphorylation (Garrison & Wagner, 1982) and fructose 2,6-bisphosphate metabolism (Hue *et al.*, 1981*a*).

Effects of hormones on proline metabolism have been reported. Glucagon stimulated gluconeogenesis from proline in hepatocytes isolated from starved rats (Joseph & McGivan, 1978) and stimulated ureogenesis in the perfused rat liver (Haussinger *et al.*, 1982). Vasopressin stimulated the formation of $^{14}\text{CO}_2$ from $[\text{U-}^{14}\text{C}]$ proline in

hepatocytes isolated from fed rats (Sugden *et al.*, 1980).

In the present paper, comparative aspects of the regulation by glucagon and vasopressin of gluconeogenesis and ureogenesis from proline have been studied. This is of interest, because the metabolism of proline involves potential regulatory sites not involved in gluconeogenesis from substrates such as lactate/pyruvate.

Proline is metabolized to glutamate in the liver via two successive reactions. The first is catalysed by mitochondrial proline oxidase (Johnson & Strecker, 1962) and the product, 1-pyrroline-5-carboxylate, is then oxidized to glutamate by an NAD^+ -linked enzyme (EC 1.5.1.12) (Strecker, 1960) located in both the cytosolic and mitochondrial compartments (Brunner & Neupert, 1969). The glutamate formed can be metabolized to provide ammonia and aspartate for urea synthesis. The oxoglutarate formed in this series of reactions is converted via oxoglutarate dehydrogenase into C_4 intermediates, which serve as precursors of glucose.

In the present paper, distinct quantitative effects of vasopressin and glucagon on the stimulation of gluconeogenesis and ureogenesis from proline are reported. A novel finding was that the effects of the two hormones were additive when metabolism was measured over a 30 min interval. Analysis of pathway intermediates indicates that there may be a causal relationship between the control of oxoglutarate dehydrogenase activity by the hormones and the hormone effects on gluconeogenesis from proline.

Materials and methods

Isolation of hepatocytes

Hepatocytes were isolated from 200–300 g 24-h starved male Wistar rats by the method of Berry & Friend (1969) as modified by Krebs *et al.* (1974).

Incubation conditions

The isolated hepatocytes were suspended in Krebs–Henseleit bicarbonate-buffered saline, pH 7.4 (Krebs & Henseleit, 1932), containing 2% (w/v) dialysed bovine serum albumin (fraction V). In some experiments the cells were incubated in Ca^{2+} -free medium. All incubations were at 37°C under an atmosphere of CO_2/O_2 (1:19), and the cells were kept in suspension by rotatory shaking. Two procedures were used to incubate the cells. In procedure 1, used for time-course experiments, 8 ml of cell suspension was preincubated for 20 min with the required substrate in sealed 50 ml plastic flasks, then 0.9 ml of cell suspension was added to 0.09 ml of 35% (w/v) HClO_4 . Hormones were added as required to the remaining cells, and samples were taken for assay 2, 5, 10, 15, 20 and 30 min later. For all substrates used, control metabolic rates were linear for the 30 min interval after the 20 min preincubation period. In procedure 2, 8 ml of cell suspension was preincubated for 20 min with substrate and a sample was taken for assay as above; then 1.5 ml samples of the remaining cells were added to the required hormones in 25 ml plastic flasks and a sample was taken 30 min later. Product formation was expressed as the amount formed during the 30 min interval between sampling. Cell protein concentration was in the range 7–12 mg of cell protein/ml as determined by a biuret method (Gornall *et al.*, 1949), with bovine serum albumin as standard. Where representative results are presented, similar data were obtained from at least three cell preparations.

Determination of metabolites

Metabolites were determined in protein-free KOH-neutralized extracts of the cell suspension. Glutamate and oxoglutarate were assayed enzymically by procedures outlined in Bergmeyer (1965). Oxoglutarate was always assayed within 1 h after

extraction. Urea was determined as ammonia after hydrolysis with urease. Glucose was determined by using hexokinase, NAD^+ and NAD(P)^+ -specific glucose-6-phosphate dehydrogenase.

Materials

Enzymes and coenzymes used in the enzymic assays were from Sigma Chemical Co., Poole, Dorset, U.K., or Boehringer Corp., Lewes, Sussex, U.K. Collagenase (type II) was from Worthington. Glucagon, vasopressin (type V), phenylephrine and dibutyryl cyclic AMP were from Sigma.

Results and discussion

Effects of glucagon and vasopressin on gluconeogenesis from 5 mM-proline

Vasopressin and glucagon (saturating concentrations) stimulated gluconeogenesis from 5 mM-proline in hepatocytes isolated from starved rats (Table 1). The stimulation by vasopressin was greater than that by glucagon, and the effects of the two hormones were additive when net metabolism was measured in a 30 min interval. The hormonal effects were not due to a stimulation of the rates of glucose production by endogenous metabolism, which were 17.9 ± 0.7 , 16.3 ± 0.8 , 20.8 ± 0.6 and 19.2 ± 1.1 (nmol of glucose formed 30 min per mg) for control, glucagon-stimulated, vasopressin-stimulated and glucagon-plus-vasopressin-stimulated cells respectively (values are means $\pm$ S.D. of triplicate incubations of a representative cell preparation). The control rate of gluconeogenesis from 5 mM-proline was similar to that observed by Hensgens *et al.* (1978).

Detailed time courses (Fig. 1) show that over 30 min the rates of gluconeogenesis from 5 mM-proline were approximately constant under control, glucagon-stimulated and glucagon-plus-vasopressin-stimulated conditions. The rates were 1.0,

Table 1. *Effects of vasopressin and glucagon on gluconeogenesis from 5 mM-proline*

Hepatocytes were incubated in the presence of 5 mM-proline according to procedure 2 as described in the Materials and methods section. Values given are means $\pm$ S.E.M. of results obtained from nine independent cell preparations. Numbers in parentheses are the experimental values expressed as percentages of the control value. By a paired *t* test, all values were significantly different from each other, with $P \leq 0.001$ in each case.

Hormone added	Glucose formed (nmol/mg in 30 min)
Control	36.4 ± 1.8
Glucagon (300 nM)	46.2 ± 2.8 (127)
Vasopressin (15 nM)	61.6 ± 3.6 (169)
Glucagon + vasopressin	84.7 ± 4.7 (233)

Fig. 1. Time courses showing the effects of hormones on the rate of gluconeogenesis from 5 mM-proline in isolated hepatocytes

The cells were incubated according to procedure 1 as described in the Materials and methods section. Glucose formation was measured from the end of the preincubation period. ○, Control; □, 300 nM-glucagon; ●, 15 nM-vasopressin; ■, glucagon plus vasopressin. Results of a representative experiment are shown.

1.3 and 2.3 nmol of glucose/min per mg respectively. The initial rate of glucose production in the presence of vasopressin alone was not sustained. The rates of glucose production in the presence of vasopressin and of glucagon plus vasopressin were very similar during the first 10 min of incubation with the hormones, i.e. 22.6 and 23.5 nmol of glucose formed/mg respectively (Fig. 1). During the last 10 min of the incubation, glucose was 10.1 and 21.5 nmol/mg for vasopressin-stimulated and glucagon-plus-vasopressin-stimulated cells respectively. It appears that glucagon in combination with vasopressin had the effect of maintaining the initially stimulated rate of gluconeogenesis that was observed with vasopressin alone. This resulted in an apparent additive stimulation of gluconeogenesis by the hormones in combination when gluconeogenesis was measured over 30 min, as shown in Table 1.

Hormone effects on the cellular concentrations of oxoglutarate and glutamate

In cells incubated with proline, glucagon caused a small decrease in the amount of oxoglutarate in the cell suspension, and vasopressin caused a larger

Fig. 2. Effects of hormones on the cellular contents of (a) oxoglutarate and (b) glutamate in isolated hepatocytes incubated with 5 mM-proline

For experimental details see Fig. 1. The results shown here were obtained from the same incubation used to obtain the results shown in Fig. 1. The contents of C₅ dicarboxylates are shown under control conditions (○), in the presence of 300 nM-glucagon (□), in the presence of 15 nM-vasopressin (●), and in the presence of glucagon plus vasopressin (■). Results of a representative experiment are shown.

decrease (Fig. 2a). After about 5 min in the presence of vasopressin, the oxoglutarate started to return to control concentrations. Glucagon plus vasopressin caused a decrease in oxoglutarate in the cell suspension that was initially indistinguishable from that caused by vasopressin alone, but, in contrast, the decrease was sustained (Fig. 2a).

The decrease in glutamate concentration caused by the hormones (Fig. 2b) were qualitatively similar to the changes in oxoglutarate concentration (Fig. 2a). Vasopressin caused a larger decrease in glutamate than that caused by glucagon (Fig. 2b). The decrease in glutamate caused by glucagon and vasopressin in combination was similar to that caused by vasopressin alone.

Stimulation of gluconeogenesis from proline necessarily involves an increase in flux through oxoglutarate dehydrogenase. Increased flux through a non-equilibrium enzyme coincident with a decrease in its substrate concentration is indicative of an activation of that enzyme. It follows that the hormonal stimulation of gluconeogenesis from proline may be attributed in part to an activation of oxoglutarate dehydrogenase. In the present paper the measurements of oxoglutarate were in the total cell suspension and not in the mitochondrial matrix, the subcellular location of oxoglutarate dehydrogenase. It was assumed that changes in the oxoglutarate measured were a reflection of changes in oxoglutarate concentration in the mitochondrial matrix. This assumption is supported by the results of Siess *et al.* (1977, 1978).

Consideration of the relative effects of the hormones alone and in combination of gluconeogenesis from proline and the coincident hormone effects on oxoglutarate concentration suggests that the hormone effects on gluconeogenesis may be a consequence of differential control of oxoglutarate dehydrogenase activity. The data indicate that activation of oxoglutarate dehydrogenase by vasopressin was greater than that caused by glucagon and hence could account for the relative magnitudes of the effects of the hormones on gluconeogenesis. The observation that the decrease in oxoglutarate caused by vasopressin was not sustained is indicative of a transient activation of oxoglutarate dehydrogenase. This may explain the attenuation of the stimulated rate of gluconeogenesis in the presence of vasopressin. Glucagon in the presence of vasopressin appears to sustain the activation of oxoglutarate dehydrogenase caused by vasopressin. This may be responsible for the sustained increase in gluconeogenesis caused by the hormones in combination.

Effects of glucagon and vasopressin on ureogenesis from 5 mM-proline

Glucagon and vasopressin stimulated ureogenesis from proline, and the effects of the two hormones were additive (Table 2). If proline is quantitatively metabolized to glucose and urea, a stoichiometric production of the two end products is expected (Krebs *et al.*, 1976). Tables 1 and 2 show that under all conditions the ratio of glucose to urea is close to the unexpected value of 1. These

Table 2. *Effects of vasopressin and glucagon on ureogenesis from 5 mM-proline*

Hepatocytes were incubated with 5 mM-proline according to procedure 2 as described in the Materials and methods section. Values given are means $\pm$ S.D. of triplicate incubations of one cell preparation. A representative experiment is shown. Numbers in parentheses are the experimental values expressed as percentages of the control value. Statistical significance of the results was determined by Student's *t* test; all values were significantly different from each other, with *P* at least ≤ 0.001 in each case.

Hormone added	Urea formed (nmol/mg in 30 min)
Control	38.0 $\pm$ 3.6
Glucagon (300 nM)	49.2 $\pm$ 1.0 (129)
Vasopressin (15 nM)	67.2 $\pm$ 3.7 (177)
Glucagon + vasopressin	83.4 $\pm$ 1.4 (219)

effects may also be explained by hormonal activation of oxoglutarate dehydrogenase. Under certain conditions the mass-action ratios of glutamate dehydrogenase (EC 1.4.1.3) and glutamate:oxaloacetate transaminase (EC 2.6.1.1) are close to their respective equilibrium constants (Williamson *et al.*, 1967; Zuurendonk *et al.*, 1976). It has been shown by perturbing the concentrations of components of the equilibrium system that the enzymes involved have the capacity rapidly to re-establish equilibrium (Brosnan *et al.*, 1970; Brosnan & Williamson, 1974). The changes in glutamate concentration (Fig. 2b) caused by the hormones were qualitatively similar to the changes in oxoglutarate concentration (Fig. 2a). The mechanism by which the hormones increase urea synthesis could be due to a perturbation of the glutamate dehydrogenase/glutamate:oxaloacetate transaminase system as a result of decreases in oxoglutarate concentration caused by activation of oxoglutarate dehydrogenase. This would have the effect of forming more ammonia and aspartate for urea synthesis.

Effects of Ca²⁺ depletion

The rate of gluconeogenesis from 5 mM-proline incubated in Ca²⁺-free medium with the addition of 0.1 mM-EGTA was 95% of the rate observed in the presence of 2.5 mM-Ca²⁺ (Table 3). EGTA added at increasingly higher concentrations than 0.1 mM progressively inhibited the rate of gluconeogenesis (results not shown). In the presence of 0.1 mM-EGTA the stimulation of gluconeogenesis by vasopressin was decreased from 58% to 14% (Table 3), and the stimulation by glucagon was not greatly impaired. In combination glucagon and vasopressin had no additive effect on gluconeogenesis from proline in Ca²⁺-depleted cells

Table 3. Effects of Ca²⁺ on the hormonal stimulation of gluconeogenesis from 5 mM-proline

Isolated cells were incubated according to procedure 2 as described in the Materials and methods section. In the Ca²⁺-depletion experiments the cells were incubated in Ca²⁺-free saline, to which was added EGTA (final concn. 0.1 mM; added as the sodium salt, pH 7.4) 5 min before hormone addition. Results are means ± S.D. of triplicate incubations of one cell preparation. Numbers in parentheses are the experimental values expressed as percentages of the control value. A representative experiment is shown. Student's *t* test was used to calculate the significance of results. All values were significantly different from each other within each column and across the columns: *P* was at least ≤ 0.01 in each case unless indicated otherwise: *N.S. (not significant) with respect to gluconeogenesis in the presence of 2.5 mM-CaCl₂. **N.S. with respect to glucagon-stimulated gluconeogenesis in the absence of CaCl₂.

Hormone added	Glucose formed (nmol/mg in 30 min)	
	2.5 mM-CaCl ₂ present	CaCl ₂ absent
Control	41.4 ± 2.4	39.4 ± 0.5*
Glucagon (300 nM)	54.4 ± 0.6 (131)	51.1 ± 0.4 (130)
Vasopressin (15 nM)	65.3 ± 0.7 (158)	45.1 ± 1.6 (114)
Glucagon + vasopressin	101.9 ± 2.7 (246)	50.6 ± 1.2 (128)**

(Table 3), and the stimulation observed under these conditions was similar to that observed with glucagon alone. The exact role of Ca²⁺ in the hormonal regulation of proline metabolism remains to be established. Interestingly, distinct effects of glucagon and vasopressin alone and in combination on Ca²⁺ metabolism in isolated hepatocytes have been observed (Morgan *et al.*, 1983).

Hormone specificity

The stimulation of gluconeogenesis from proline was also observed with dibutyryl cyclic AMP and with phenylephrine as effectors (Table 4). Combinations of effectors were tested for additive effects on gluconeogenesis when metabolism was measured over 30 min. Table 4 shows that additive stimulation of gluconeogenesis occurred with combinations of an effector involving cyclic AMP and an effector dependent on Ca²⁺. This indicates that the additive stimulation must be a consequence of a general interaction between the cyclic AMP and Ca²⁺ second-messenger systems and not a phenomenon exclusive to glucagon and vasopressin. The concentrations of effectors used were saturating.

Table 4. Comparison of the stimulation of gluconeogenesis from 5 mM-proline by different effectors alone and effectors in combination

The incubation procedure was as follows. Hepatocyte suspension (14 ml) was preincubated in a 125 ml plastic flask for 20 min with 5 mM-proline, then 0.8 ml of cell suspension was removed for assay. Samples (1 ml) of cells were then added to the required hormone(s) in 25 ml plastic flasks, and a sample was taken for assay 30 min later. Glucose formation is expressed as the amount formed in the 30 min interval between sampling. The values are the means of results obtained from duplicate incubations of a one cell preparation. These differed by no more than 7%. Results of representative experiment are shown.

Hormone(s) added	Glucose formed (nmol/mg in 30 min)
Control	45.0
Glucagon (300 nM)	53.6 (119)
Dibutyryl cyclic AMP (50 μM)	56.5 (126)
Phenylephrine (25 μM)	61.4 (136)
Vasopressin (15 nM)	65.1 (145)
Glucagon + dibutyryl cyclic AMP	58.0 (129)
Glucagon + phenylephrine	81.0 (180)
Glucagon + vasopressin	92.5 (206)
Dibutyryl cyclic AMP + phenylephrine	82.5 (183)
Dibutyryl cyclic AMP + vasopressin	91.1 (202)
Phenylephrine + vasopressin	68.2 (152)

Hormone effects on the metabolism of other amino acids

Glucagon, vasopressin and glucagon plus vasopressin in combination stimulated gluconeogenesis from alanine, asparagine and glutamine (Table 5). As observed with proline as substrate, vasopressin was more effective than glucagon in stimulating gluconeogenesis and ureogenesis from glutamine (see also Joseph *et al.*, 1981), but this was not the case when alanine or asparagine was used as substrate (Table 5). Gluconeogenesis from alanine and asparagine does not involve net flux through oxoglutarate dehydrogenase, and glucagon and vasopressin did not additively stimulate the metabolism of these substrates. This supports the contention that it is the hormonal control of oxoglutarate dehydrogenase activity that is responsible for the hormone effects on gluconeogenesis from proline. It is possible, however, that, with the concentrations of alanine and asparagine used, other hormonally regulated enzymes responsible for the control of the metabolism of these amino acids were saturated with substrate. This could prevent the expression of hormone effects if

Table 5. *Effects of vasopressin and glucagon on the metabolism of alanine, asparagine and glutamine*

Isolated cells were incubated with 5mM amino acid according to procedure 2 as described in the Materials and methods section. The results for each amino acid are expressed as the mean $\pm$ S.D. of the values obtained from four incubations of one cell preparation. The concentrations of glucagon and vasopressin used were 300nM and 15nM respectively. The values in parentheses are the experimental values expressed as a percentage of the control values. Results of representative experiments are shown.

Amino acid (5mM)	Hormone	Product formed (nmol/mg in 30 min)	
		Glucose	Urea
Alanine	Control	132.4 $\pm$ 1.9	144.3 $\pm$ 7.2
	Glucagon	162.7 $\pm$ 3.9 (123)	170.4 $\pm$ 12.3 (118)
	Vasopressin	156.5 $\pm$ 3.4 (118)	164.7 $\pm$ 5.5 (114)
	Glucagon + vasopressin	155.3 $\pm$ 3.8 (117)	174.7 $\pm$ 7.8 (121)
Asparagine	Control	82.8 $\pm$ 2.8	172.3 $\pm$ 10.8
	Glucagon	102.0 $\pm$ 3.3 (123)	206.4 $\pm$ 4.0 (120)
	Vasopressin	90.1 $\pm$ 2.5 (109)	174.7 $\pm$ 5.1 (101)
	Glucagon + vasopressin	96.5 $\pm$ 2.8 (117)	188.2 $\pm$ 3.2 (109)
Glutamine	Control	33.0 $\pm$ 0.7	69.5 $\pm$ 1.1
	Glucagon	56.2 $\pm$ 0.7 (170)	128.8 $\pm$ 3.4 (185)
	Vasopressin	83.5 $\pm$ 0.9 (253)	171.0 $\pm$ 3.5 (246)
	Glucagon + vasopressin	107.1 $\pm$ 4.8 (325)	190.4 $\pm$ 8.5 (274)

the hormones caused changes in the K_m of the regulated enzyme for its substrate.

An additive effect of the hormones was observed on gluconeogenesis from glutamine, the metabolism of which involves flux through oxoglutarate dehydrogenase. The hormonally regulated mitochondrial glutaminase (EC 3.5.1.2) complicates the interpretation of these observations with glutamine as gluconeogenic substrate (Joseph & McGivan, 1978).

Work from other laboratories has also provided information on the hormonal regulation of oxoglutarate dehydrogenase. Glucagon caused a decrease in oxoglutarate and glutamate concentrations in the perfused liver (Williamson *et al.*, 1969; Ui *et al.*, 1973). Vasopressin and glucagon caused a decrease in C_5 dicarboxylates in both the mitochondrial and cytosolic compartments of the isolated hepatocyte (Siess *et al.*, 1978). Glucagon caused an increase in $^{14}CO_2$ production from [1- ^{14}C]glutamate in the perfused rat liver, and this has been attributed to an activation of oxoglutarate dehydrogenase (Ui *et al.*, 1973). Vasopressin increased the production $^{14}CO_2$ from [U- ^{14}C]proline in hepatocytes isolated from fed rats (Sugden *et al.*, 1980), and it was suggested that this may involve an activation of oxoglutarate dehydrogenase. More recently, Taylor *et al.* (1983), in perfused-liver studies, observed a transient stimulation by phenylephrine of $^{14}CO_2$ production from 2-oxo[1- ^{14}C]glutarate.

In summary, it has been shown that glucagon and vasopressin have distinct effects on proline metabolism, and this has been attributed to effects on oxoglutarate dehydrogenase. Studies *in vitro*

(McCormack & Denton, 1979; Smith *et al.*, 1974) have shown that the control of oxoglutarate dehydrogenase activity is complex. At the present time it is not possible to describe the mechanisms by which mitochondrial oxoglutarate dehydrogenase is activated by cyclic AMP- and Ca^{2+} -dependent hormones in liver. Studies are required to elucidate these mechanisms.

This work was supported financially by the Medical Research Council, from whom J. M. S. holds a Studentship.

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