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Stimulation by Vasopressin, Angiotensin and Oxytocin of Gluconeogenesis in Hepatocyte Suspensions

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1. In hepatocytes from starved rats, vasopressin, angiotensin (angiotensin II) and oxytocin stimulated gluconeogenesis from lactate by 25–50%; minimal effective concentrations were about 0.02 pM, 1 nM and 0.2 nM respectively. 2. Vasopressin and angiotensin also stimulated gluconeogenesis from alanine, pyruvate, serine and glycerol. EGTA decreased gluconeogenesis from these substrates. 3. Hormonal stimulation of gluconeogenesis from lactate was abolished in the absence of extracellular Ca^{2+}. 4. Insulin did not prevent stimulation of gluconeogenesis by vasopressin or angiotensin. 5. The potency of the stimulatory effects of vasopressin and angiotensin on hepatic gluconeogenesis suggests they are operative *in vivo*. Also, the data suggest that Ca^{2+} plays a role in the stimulation by these hormones.

The ability of a range of hormones to exert rapid catabolic effects on the liver, without acting via cyclic AMP, has recently been demonstrated. The most well-established of these effects is stimulation of hepatic glycogen breakdown. The hormones include α-adrenergic agonists, vasopressin, oxytocin and angiotensin (angiotensin II) (for review see Hems, 1977).

Studies with the perfused liver and isolated cells from fed rats have demonstrated a direct hepatic effect of vasopressin, oxytocin and angiotensin on glycogenolysis and phosphorylase *a* activity (Hems *et al.*, 1976, 1978*b*; Keppens *et al.*, 1977). These effects are totally dependent on the presence of extracellular Ca^{2+} (Stubbs *et al.*, 1976; Keppens *et al.*, 1977; Hems *et al.*, 1978*b*) and do not involve cyclic GMP (Hems *et al.*, 1978*a*).

Gluconeogenesis can also be stimulated by hormones that exert catabolic effects on liver, such as glucagon, adrenaline and vasopressin, as may be shown in the perfused liver of starved rats (Exton *et al.*, 1970; Exton & Park, 1972; Hems & Whitton, 1973). In view of this fact and since hepatic gluconeogenesis from lactate is Ca^{2+}-dependent (Hems *et al.*, 1966; Elliott, 1976), the effects on gluconeogenesis of angiotensin and oxytocin (not previously reported) and of vasopressin have been investigated, and are described in the present paper. The possibility of an interaction between vasopressin or angiotensin and insulin has also been studied, since catabolic effects of hormones on the liver may be counteracted by insulin. Finally the role of Ca^{2+} in gluconeogenesis has been evaluated with respect both to the Ca^{2+}-dependence of gluconeogenesis from various precursors and to the Ca^{2+}-dependence of the hormone effects.

Materials and Methods

Preparation and incubation of hepatocytes

Male Porton–Wistar rats (200 g) were starved for 48 h from about 10.00 h.

Isolated hepatocytes were prepared by perfusion of the liver with collagenase, essentially as described by Krebs *et al.* (1974) with minor modifications (Hems *et al.*, 1978*b*). Viability of hepatocyte preparations was assessed in several ways (Hems *et al.*, 1978*b*). Trypan Blue was excluded by 90–95% of cells. Rates of gluconeogenesis from various precursors were similar to those reported from other laboratories.

Cells were incubated in duplicate, as described elsewhere (Hems *et al.*, 1978*b*), for 40 min in bicarbonate-buffered saline (Krebs & Henseleit, 1932) gassed with O_2/CO_2 (19:1, v/v).

Glycogen was determined in hepatocytes before incubation (Hems & Whitton, 1973; Hems *et al.*, 1978*b*). A value of 4.5 ± 1.3 (10) μmol of glycogen glucose/g of dry cells (mean ± S.E.M. for the number of observations indicated in parentheses) was obtained. Thus any glucose released from glycogen during incubation of hepatocytes did not significantly contribute to the total glucose released.

Analytical methods

Methods for measuring glucose and lactate in the incubation medium have been described (Hems & Whitton, 1973).

Chemicals

Chemicals were of the highest grade available. [Arg^8]vasopressin and oxytocin were the highest grade made by Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Angiotensin (synthetic 5-valine analogue) was kindly provided by the Medical Research Council National Institute of Biological Standards, Hampstead, London N.W.3, U.K. Collagenase (grade II) was from P-L Biochemicals, Milwaukee, WI 53205, U.S.A. Insulin (glucagon-free) was the highest grade commercial ox preparation from Burroughs Wellcome and Co., Dartford, Kent, U.K.

Calculation of results

Basal glucose release in the absence of substrate was subtracted from the total glucose released in the presence of substrate (both determined in duplicate incubations). Increments in glucose release due to hormone (or decrements due to EGTA) were calculated as the difference between the glucose release in the absence of hormone (or EGTA) and that in its presence, for each preparation of cells. The percentage changes were similarly calculated.

Results

Time course of gluconeogenesis in hepatocytes

The time courses of glucose production from 10mM-lactate and of lactate removal were followed in the presence and absence of vasopressin, to establish an optimal period for routine incubations. A small lag in control glucose release was observed during the first 10min of incubation; thereafter the rate was linear with time. Vasopressin (20nM) stimulated glucose production from lactate over the entire time course and perhaps decreased the lag period in gluconeogenesis observed in the absence of hormone (Fig. 1).

Lactate removal from the incubation medium was not linear with time, as might be expected from the non-linearity of glucose production (Fig. 2). Vasopressin (20nM) did not stimulate lactate uptake (Fig. 2).

Effects of hormones on gluconeogenesis in hepatocytes

Vasopressin stimulated gluconeogenesis from lactate over the concentration range 20fM to 20nM (Fig. 3). Angiotensin acted over the range 10pM to 100nM (Fig. 3). The maximum extra glucose released from lactate in response to both these hormones was similar, corresponding to 50% stimulation over the control value. Oxytocin, which

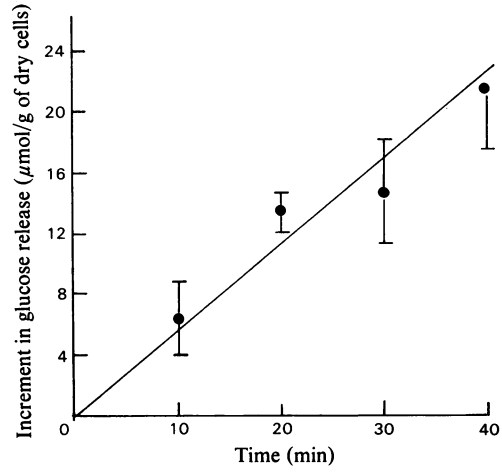

Fig. 1. *Time course of stimulation of gluconeogenesis from lactate by vasopressin*
Hepatocytes were prepared from 48h-starved rats and incubated at 37°C in Krebs–Ringer bicarbonate buffer containing albumin and 10mM-lactate. After various times, the glucose concentration was determined in the medium. When present, vasopressin (20nM) was added at zero time. Data are expressed as the increment in glucose release in response to vasopressin (in μmol of glucose/g dry wt. of cells). Results are means±S.E.M. for four cell preparations. The 20, 30 and 40min points are significantly different ($P<0.05$) from the hormone-free values, as assessed by the paired two-tailed t test.

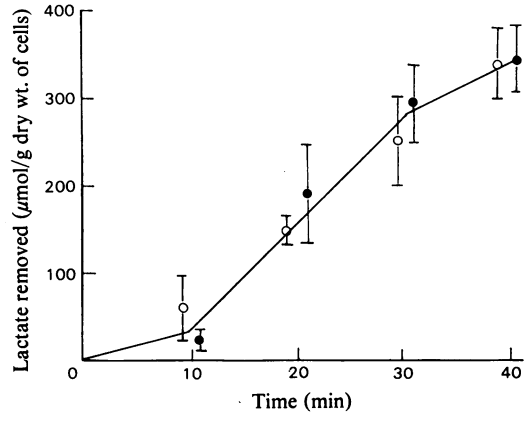

Fig. 2. *Time course of lactate removal by hepatocytes*
In the experiments described in Fig. 1, lactate was assayed in incubation medium in the absence (○) or presence (●) of vasopressin. Results are expressed as μmol of lactate removed/g dry wt. of cells and are the means±S.E.M. for five cell preparations.

stimulated gluconeogenesis by only about 20% w, as effective over the range 0.1–10 nM (Fig. 3).

Endogenous gluconeogenesis was stimulated by 20 nM-vasopressin by about 10%; since the increment due to vasopressin was only 2 ± 1 μmol of glucose/40 min per g dry wt. of cells (mean ± S.E.M. for three observations), this did not significantly contribute to the stimulatory effects of hormones in the presence of substrates.

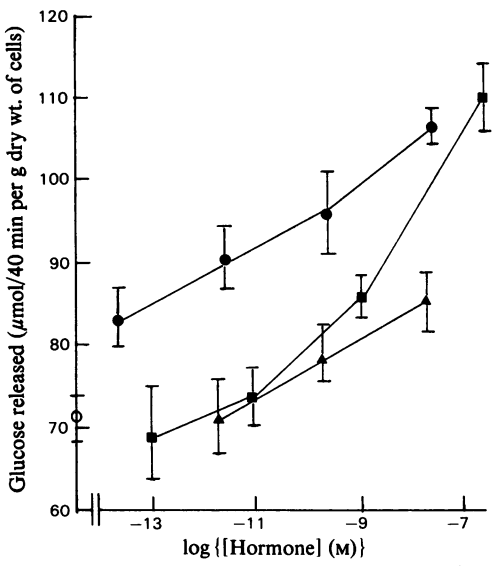

Fig. 3. *Effect of vasopressin, angiotensin and oxytocin on gluconeogenesis from lactate*
Hepatocytes were incubated for 40 min in the absence (○) or presence (●, ■, ▲) of hormone. Vasopressin (●), angiotensin (■) or oxytocin (▲) was added at zero time with 10 mM-lactate. Results, expressed as glucose released (μmol/40 min per g dry wt. of cells) over the substrate-free control value (14.3 ± 2.2), are means ± S.E.M. for four cell preparations. Glucose release in response to maximal concentrations of all hormones was significant as assessed by Student's *t* test: vasopressin, $P < 0.01$; angiotensin, $P < 0.01$; oxytocin, $P < 0.02$.

Substrate-dependence of gluconeogenesis in hepatocytes

To pinpoint sites of action of vasopressin and angiotensin, gluconeogenesis from various precursors was followed in the presence and absence of hormones.

The fastest release of glucose in control experiments was observed with fructose as precursor, whereas serine and alanine gave the slowest rates (Table 1). Pyruvate, glycerol and lactate were poorer glucose precursors than fructose, but better than the two amino acids (Table 1).

In the presence of vasopressin and angiotensin, gluconeogenesis was significantly stimulated from all substrates except fructose (Table 1). The increment in glucose release observed in the presence of either hormone again appeared to be similar, except with fructose and alanine as substrates (Table 1). The increments in gluconeogenesis from alanine caused by both hormones, although smaller than from other substrates, were both significant ($P < 0.05$ for a two-tailed test for paired differences).

Effect of Ca^{2+} depletion on hepatic gluconeogenesis

The effect of Ca^{2+} on gluconeogenesis was assessed by two different procedures. Hepatocytes were either incubated in the absence of extracellular Ca^{2+}, or alternatively EGTA (3 mM) was added to the incubation medium. Both manipulations decreased gluconeogenesis from lactate by about 40% (Tables 2 and 3). The presence of EGTA did not significantly decrease gluconeogenesis from fructose, whereas the

Table 1. *Effect of vasopressin and angiotensin on gluconeogenesis from different precursors*
Hepatocytes were incubated for 40 min. Substrates (10 mM) and hormones (20 nM-vasopressin or 0.1 μM-angiotensin) were added at zero time. The basal (no-substrate) release for each experiment (range 10–25 μmol/40 min per g) has been subtracted from all values. Results are means ± S.E.M. of the numbers of cell preparations shown in parentheses. All increments in glucose release caused by hormone, except in the presence of fructose, were significant ($P < 0.05$) as assessed by the paired two-tailed *t* test.

	Glucose release (μmol/40 min per g of dry cells)				
		Vasopressin		Angiotensin	
Substrate	Control	Increment	Increase (%)	Increment	Increase (%)
Lactate	75.8 ± 4.3 (4)	20.7 ± 3.3	21 ± 1 (4)	24.0 ± 6.3	25 ± 7 (4)
Glycerol	97.5 ± 8.9 (6)	21.6 ± 6.3	23 ± 7 (6)	27.8 ± 8.3	27 ± 7 (5)
Pyruvate	121.9 ± 14.0 (4)	23.9 ± 1.9	17 ± 1 (4)	20.2 ± 2.0	15 ± 2 (4)
Serine	53.6 ± 3.9 (7)	22.8 ± 6.2	30 ± 4 (4)	22.0 ± 5.0	38 ± 9 (7)
Alanine	52.1 ± 6.5 (7)	10.0 ± 3.4	14 ± 6 (7)	16.3 ± 3.1	24 ± 7 (6)
Fructose	375.9 ± 34.9 (4)	5.3 ± 4.6	−2 ± 1 (4)	−11.3 ± 4.4	−3 ± 1 (4)

Table 2. *Effect of EGTA on gluconeogenesis from different precursors*

Hepatocytes were incubated for 40min. Substrates (10mM) and EGTA (3mM) were added at zero time. The basal (no-substrate) release for each experiment (mean 15.9±4.7μmol/40min per g for the four preparations) has been subtracted from all values. Results are means±S.E.M. for four cell preparations. All decrements in glucose release caused by EGTA, except in the presence of fructose, were significant ($P<0.05$) as assessed by the paired two-tailed t test.

	Glucose release (μmol/40min per g of dry cells)		
		Plus EGTA	
Substrate	Control	Decrement	% of control release
Lactate	86.0±6.6	35.7±8.3	61±6
Glycerol	84.0±4.7	18.0±4.3	80±6
Pyruvate	134.4±21.0	43.0±7.1	70±1
Serine	60.5±5.7	25.1±3.9	59±8
Alanine	59.5±8.3	29.7±3.0	58±5
Fructose	476.1±49.9	32.6±15.1	93±4

Table 3. *Role of Ca^{2+} in hormone-stimulated gluconeogenesis*

Hepatocytes were prepared in the usual manner, but washed in Krebs–Ringer bicarbonate buffer either containing Ca^{2+} or in the absence of Ca^{2+}. Lactate (10mM) and hormones (20nM-vasopressin; 0.1μM-angiotensin), were added at zero time and incubation was for 40min. The basal (no-substrate) release for each experiment [in Ca^{2+} media, 15.2±2.0 (5); in Ca^{2+}-free media (17.9±3.6 (4) μmol/40min per g] has been subtracted from each value. Results are means±S.E.M. for the numbers of cell preparations in parentheses.

	Glucose release (μmol/40min per g of dry cells)		
	Control	Increment by vasopressin	Increment by angiotensin
Ca^{2+} (5)	78.0±6.8	38.3±3.6	44.9±3.9
No Ca^{2+} (4)	52.2±11.8	−3.0±6.2	10.8±5.1

rates from the other precursors were decreased by about 30–40% (Table 2). The decrements in glucose release by EGTA were variable depending on the precursor (Table 2). The absolute rate of gluconeogenesis from pyruvate was most affected by the presence of EGTA, whereas that from glycerol was least Ca^{2+}-dependent.

In the absence of extracellular Ca^{2+} the stimulation of gluconeogenesis from lactate observed with vasopressin or angiotensin was abolished (Table 3).

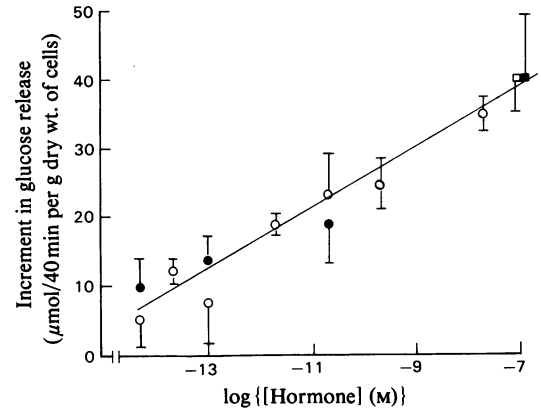

Fig. 4. *Effect of insulin on hormone-stimulated gluconeogenesis*

Hepatocytes were incubated for 40min with 10mM-lactate and vasopressin (○, ●) or angiotensin (□, ■), in the absence (○, □) or presence of 0.4μg of insulin/ml (●, ■). Results, expressed as the increment in glucose released (μmol/40min per g dry wt. of cells) on addition of the hormone(s) over that in the presence of lactate (84.8±11.1), are means±S.E.M. for four cell preparations.

Neither was there any stimulation by oxytocin (results not shown).

Effect of insulin on gluconeogenesis

Insulin did not have any effect on the stimulation of gluconeogenesis from lactate observed with either submaximal concentrations of vasopressin or a maximal concentration of 0.1μM-angiotensin (Fig. 4). Insulin decreased the glucose released by minimally effective concentrations of glucagon (results not shown), showing that the lack of suppression by insulin of the stimulation of gluconeogenesis by vasopressin or angiotensin did not reflect a simple lack of insulin receptors.

Discussion

Sensitivity of hepatic gluconeogenesis to hormonal stimulation

In hepatocyte suspensions from the liver of fed rats phosphorylase activity is significantly more sensitive than is glucose release to activation by vasopressin, angiotensin or oxytocin (Hems *et al.*, 1978*b*). Gluconeogenesis from lactate in hepatocytes from starved rats is even more sensitive to stimulation by vasopressin than the phosphorylase response, the minimal effective concentration being 0.02pM compared with 0.1pM. This may partly reflect a greater sensi-

tivity in liver from starved rats than in fed rats (Hems & Whitton, 1973). The minimal effective concentrations of angiotensin and oxytocin found to stimulate gluconeogenesis and activate phosphorylase in hepatocyte suspensions were similar.

The lactate removed from the incubation medium by hepatocytes (340 μmol/40min per g of dry cells) was sufficient to account for the lactate-dependent production of glucose in the presence or absence of vasopressin, which was 129 or 108 μmol/40min per g of dry cells respectively. Lactate uptake by hepatocytes was not stimulated by 20nM-vasopressin, despite the fact that this was a maximal concentration for stimulation of gluconeogenesis. Therefore vasopressin must redirect pyruvate carbon from alternative fates within the cell (such as oxidation) to glucose. The mechanism of this effect will now be considered.

Substrate-dependence of stimulation of gluconeogenesis by hormones

Hepatic gluconeogenesis from all the precursors studied, except perhaps fructose, was stimulated by vasopressin and angiotensin. This indicates that both hormones may act at a site on the pathway between pyruvate and triose phosphates, yet to be identified. The observed stimulation of gluconeogenesis from glycerol could imply a specific hormonal effect on glycerol metabolism, since glucose synthesis from fructose was apparently unaffected.

In contrast with the findings discussed above, glucagon and adrenaline can stimulate hepatic gluconeogenesis from fructose and dihydroxyacetone in starved rat hepatocytes (Tolbert & Fain, 1974; Pilkis *et al.*, 1976), whereas glucagon does not in general stimulate glycerol-supported gluconeogenesis in the starved state (Garrison & Haynes, 1973; Pilkis *et al.*, 1976). Thus the substrate pattern with vasopressin and angiotensin does not resemble that observed in response to adrenaline and glucagon, which suggests that vasopressin and angiotensin stimulate hepatic gluconeogenesis at a different site(s) from glucagon. This is not surprising, as the mechanisms of action of these hormones are different. Thus vasopressin and angiotensin do not bring about increases in the hepatic content of cyclic AMP or cyclic GMP (Hems *et al.*, 1978*a*), but may act via Ca^{2+} (see the next section).

Role of Ca^{2+} in hormonal stimulation of gluconeogenesis

The present data show that hepatic gluconeogenesis from a number of substrates is Ca^{2+}-dependent. Although gluconeogenesis from lactate is decreased in the absence of extracellular Ca^{2+} (Hems *et al.*, 1966; Elliott, 1976; present work), complete Ca^{2+}-dependence of gluconeogenesis was not observed. Hence it was possible to assess the role of Ca^{2+} in the response to vasopressin and angiotensin. Neither vasopressin nor angiotensin affected gluconeogenesis in Ca^{2+}-free media. Thus their action on the process is as markedly Ca^{2+}-dependent as their action on glycogenolysis and glucose release in liver cells from fed animals (Stubbs *et al.*, 1976; Keppens *et al.*, 1977; Hems *et al.*, 1978*b*).

The Ca^{2+}-dependence of vasopressin- and angiotensin-stimulated glucose release (derived from either gluconeogenesis or from glycogenolysis in liver from fed animals) is in marked contrast with the lack of a role of Ca^{2+} ions in glucagon-stimulated glucose release. Thus the absence of extracellular Ca^{2+} does not alter glucagon-stimulated hepatic glycogenolysis (Pointer *et al.*, 1976; Stubbs *et al.*, 1976; Assimacopoulos-Jeannet *et al.*, 1977; Hems *et al.*, 1978*b*). Although the situation is less clear for gluconeogenesis in cells from starved animals, the effect of glucagon does not appear to be particularly sensitive to Ca^{2+} depletion (Tolbert & Fain, 1974).

The results presented here suggest that gluconeogenesis from substrates that are dependent on Ca^{2+} for maximal rates is particularly stimulated by vasopressin and angiotensin. This is consistent with a role for Ca^{2+} in the effects on liver of both these hormones. The elucidation of the role of Ca^{2+} in gluconeogenesis might not only resolve the mechanism of these hormone responses, but might also clarify an important basic control mechanism in gluconeogenesis.

Role of insulin in gluconeogenesis

Although insulin does not affect basal rates of hepatic gluconeogenesis it can counteract or decrease glucose release by glucagon and adrenaline in both the isolated perfused liver and hepatocyte suspensions (Glinsmann & Mortimore, 1968; Mackrell & Sokal, 1969; Exton *et al.*, 1970; Claus & Pilkis, 1976; Pilkis *et al.*, 1975, 1976). The mechanism of this effect is unclear, but may include prevention of the increase in hepatic cyclic AMP, at least for glucagon (Exton & Park, 1972; Pilkis *et al.*, 1975; Claus & Pilkis, 1976). However, adrenaline stimulates gluconeogenesis partly by an α-adrenergic effect that is cyclic AMP-independent (for reviews see Exton & Harper, 1975; Fain *et al.*, 1975). The mechanism of action of insulin to prevent stimulation in this instance is not understood, although it has been proposed that Ca^{2+} is involved (Clarke *et al.*, 1974).

Insulin did not suppress the stimulation of gluconeogenesis from lactate caused by vasopressin or angiotensin. Since basal gluconeogenesis and the stimulation by these hormones are Ca^{2+}-dependent,

it seems unlikely that Ca^{2+} plays a major or simple role in insulin action on hepatic gluconeogenesis.

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