Regulation of Renal Gluconeogenesis by Calcium Ions, Hormones and Adenosine 3':5'-Cyclic Monophosphate

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1. The effect of Ca^{2+} , glucagon, adrenaline and adenosine 3':5'-cyclic monophosphate on gluconeogenesis by rat kidney-cortex slices was studied. 2. Glucose formation from a range of substrates, with the exception of glycerol, was increased by an increase in extracellular Ca^{2+} concentration. 3. Hormones and adenosine 3':5'-cyclic monophosphate, at low Ca^{2+} concentrations, stimulated glucose production from several substrates, but not from glycerol, fructose, malate or fumarate. 4. Hormonal stimulation was not detected in the absence of Ca^{2+} or at 2.5 mM-Ca^{2+} . 5. Ca^{2+} , hormones and adenosine 3':5'-cyclic monophosphate had no effect on phosphoenolpyruvate carboxylase activity. 6. It is proposed that Ca^{2+} and adenosine 3':5'-cyclic monophosphate-mediated hormone action activate the same rate-limiting step in gluconeogenesis: this step is tentatively identified as the rate of transfer of substrates across the mitochondrial membrane.

Stimulation of renal gluconeogenesis by Ca^{2+} , cyclic AMP (adenosine 3':5'-cyclic monophosphate) and its dibutyl derivative has been reported in several recent papers (Nagata & Rasmussen, 1970*a,b*; Pagliari & Goodman, 1969; Bowman, 1970; Guder & Wieland, 1971). Parathyroid hormone, an activator of renal-cortex adenylate cyclase (Chase & Aurbach, 1968), also increases glucose formation from a range of substrates and the magnitude of stimulation by hormone or 6-*N*-2'-*O*-dibutyladenosine 3':5'-cyclic monophosphate (dibutyl cyclic AMP) shows a marked dependence on extracellular Ca^{2+} concentration (Rasmussen & Nagata, 1970). The changes in metabolite concentrations produced by an increase in extracellular Ca^{2+} or by addition of hormone or dibutyl cyclic AMP are similar, suggesting a common point of action. On the basis of cross-over plots of these results, the point of stimulation has been tentatively identified as Ca^{2+} activation of phosphoenolpyruvate carboxylase (EC 4.1.1.32), possibly coupled with inhibition of pyruvate kinase (EC 2.7.1.40) by Ca^{2+} . Both these enzymes are located in the cytosol of the rat kidney-cortex cell (Flores & Alleyne, 1970; Jiménez de Asúa *et al.*, 1971). It has been suggested that an increase in extracellular Ca^{2+} , parathyroid hormone and cyclic AMP stimulates gluconeogenesis by increasing the Ca^{2+} concentration in the cytosol of the kidney-cortex cell (Rasmussen & Nagata, 1970; Rasmussen, 1970).

In the present paper we report Ca^{2+} -dependent activation of gluconeogenesis in rat kidney-cortex slices by glucagon and adrenaline, and confirm activation by cyclic AMP and dibutyl cyclic AMP. The effects of Ca^{2+} and cyclic AMP on renal-cortex phos-

phoenolpyruvate carboxylase activity were investigated and a new site of activation of renal gluconeogenesis by these agents is proposed.

Experimental

Animals and tissue handling

Adult male Sprague-Dawley rats of a locally bred strain were used. The animals were starved for 18–22 h before the experiment and then killed by cervical fracture. The kidneys were removed and chilled in ice-cold 0.9% NaCl. Slices were cut free-hand with a Stadie-Riggs microtome blade and stored in ice-cold 0.9% NaCl for 15–20 min before the start of the incubation. After a further rinse in ice-cold 0.9% NaCl, each slice (6–10 mg dry wt.) was placed in a 50 ml Erlenmeyer flask containing 5 ml of incubation medium, which was then flushed with $\text{O}_2 + \text{CO}_2$ (95:5), stoppered and then incubated for 1 h, unless otherwise stated, in a shaking water-bath (100 cycles/min) at 37–38°C.

Composition of the incubation medium

A Ca^{2+} -free Krebs bicarbonate buffer was used containing 120 mM-NaCl, 4.8 mM-KCl, 1.2 mM- MgSO_4 , 1.2 mM- KH_2PO_4 and 25 mM- NaHCO_3 . CaCl_2 was added when required to give a final concentration of 0.05–2.5 mM. After gassing with $\text{O}_2 + \text{CO}_2$ (95:5), the pH of these media was 7.35–7.45 at 37°C. Substrates were at a final concentration of 10 mM, with the exception of DL-lactate, which was at 20 mM. Hormones, cyclic AMP and 6-*N*-2'-*O*-dibutyl cyclic AMP were

added in negligible volumes to give the following concentrations: glucagon, 0.1 μM ; adrenaline, 1 μM ; dibutyl cyclic AMP, 0.5 mM; cyclic AMP, 0.1 mM. Stock solutions of substrates and modifiers were adjusted to pH 7.4 with NaOH before use if necessary.

Gluconeogenesis measurements

At the end of the incubation, protein was precipitated with 0.5 ml of 10% (v/v) HClO_4 and then removed by centrifugation. The glucose content of the medium was determined by the glucose oxidase method (Huggett & Nixon, 1957). The slices were placed on weighed planchets and dried at 110°C for 2 h and their dry weight was determined. Glucose formed from added substrate was calculated as the difference between glucose production with and without substrate and was expressed as μmol of glucose/h per g dry wt.

Some variation in the rate of glucose production from the same substrate was found between different groups of rats, e.g. control rates of glucose formation from lactate at 0.25 mM- Ca^{2+} (Tables 1–4). For this reason, the effect of any one modifier on glucose production from a particular substrate was determined by using pooled slices from a single group of animals.

Phosphoenolpyruvate carboxylase assays

Tissue samples were homogenized in 9 vol. of ice-cold 0.25 M-sucrose containing, where indicated, 0.1 M-NaF. In some cases the homogenate was centrifuged at 30000 g for 2 h in a refrigerated MSE 18 centrifuge. The supernatant was retained for phosphoenolpyruvate carboxylase assays. Two assay procedures were used, assay method 1 for supernatant samples and assay method 2 for homogenate and supernatant samples.

Assay method 1. The phosphoenolpyruvate formed after 3 min incubation with oxaloacetate, ITP (6 mM), MgSO_4 (15 mM) and 0.1 ml of supernatant was determined enzymically as described by Flores & Alleyne (1970). Oxaloacetate was at 5 or 0.3 mM and the concentration of NADH for the phosphoenolpyruvate determination was adjusted accordingly.

Assay method 2. A modification of the method

described by Ballard (1970) was used for assays at lower oxaloacetate concentrations. A constant amount of oxaloacetate was generated from L-malate with malate dehydrogenase and NAD^+ . Lactate dehydrogenase was included in the assay mixture to correct for non-enzymic breakdown of oxaloacetate (Scheme 1).

The assay medium contained, in a final volume of 1 ml: 200 μmol of Tris-HCl buffer, pH 8.0; 5 μmol of NAD^+ ; various concentrations of sodium malate; 1.5 μmol of MnCl_2 ; 15 μmol of NaF; 2 μmol of GSH; 4 units of malate dehydrogenase; 2 units of lactate dehydrogenase; 25 μl of supernatant or 10 μl of homogenate. After 2–3 min equilibration, the assay was started by the addition of 5 μmol of ITP. A linear increase in E_{340} was maintained for at least 7 min. Two control cuvettes were included with each assay. A control without malate was used to estimate the concentration of oxaloacetate generated in the malate dehydrogenase reaction from the difference in E_{340} between this cuvette and one without ITP. This correlated well with the value for oxaloacetate concentration calculated by the use of eqn. (1)

$$K'_{\text{eq.}} = [\text{oxaloacetate}][\text{NADH}]/[\text{malate}][\text{NAD}^+] \quad (1)$$

and a value for $K'_{\text{eq.}}$ of 10^{-4} at pH 8.0 (Greville, 1969). The other control without ITP served to correct for any change in E_{340} not associated with phosphoenolpyruvate carboxylase activity. In assays of supernatant samples this was negligible, but with homogenate samples there was a slow decrease in E_{340} in the control cuvette, presumably owing to microsomal NADH oxidase activity.

Fig. 1 shows the relationship between reaction rate and oxaloacetate concentration. The inhibition at high substrate concentrations is probably due to competitive inhibition by the high malate concentrations present. However, the highest concentration of malate used as a routine with assay 2 was 2 mM, which is well below the K_i of 0.25 mM reported for malate inhibition of rat liver cytosol phosphoenolpyruvate carboxylase activity (Ballard, 1970).

Both assays were done in a Unicam SP.8000 recording spectrophotometer at 37°C, by using cuvettes with 10 mm light-path.

Scheme 1. Non-enzymic breakdown of oxaloacetate
For details see the text.

Fig. 1. *Lineweaver-Burk plot of the reciprocal of the velocity against the reciprocal of the oxaloacetate concentration with oxaloacetate-generation assay for phosphoenolpyruvate carboxylase*

Phosphoenolpyruvate carboxylase activity of rat kidney-cortex cytosol was measured at various oxaloacetate concentrations by using assay method 2 as described in the text. Velocity (v) is in nmol of oxaloacetate utilized/min. Oxaloacetate concentration was calculated as described in the text.

Materials

All chemicals were AnalaR grade. Biochemicals and enzymes were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A., with the following exceptions; malate dehydrogenase, Koch-Light Laboratories, Colnbrook, Bucks., U.K.; sodium pyruvate, sodium succinate and sodium fumarate, C.F. Boehringer Corp. (London) Ltd., London W.5, U.K.; glucagon, Eli Lilly and Co., Indianapolis, Ind., U.S.A.

Results

Stimulation of gluconeogenesis

Glucose production from all substrates, with the exception of glycerol, showed a marked dependence on extracellular Ca^{2+} concentration. Fig. 2 shows the Ca^{2+} dependence of glucose formation from glutamine and malate over the range 0 [0.1 mm-ethanedioxybis(ethylamine)tetra-acetic acid] to 2.5 mm-extracellular Ca^{2+} . The effects of glucagon and adrenaline on gluconeogenesis are shown in Tables 1 and 2 respectively. At the three Ca^{2+} concentrations employed, hormonal stimulation was significant at 0.25 mm- Ca^{2+} only. The hormones did not significantly increase glucose formation from glycerol,

Fig. 2. *Relationship between extracellular Ca^{2+} concentration and gluconeogenesis from malate and glutamine by kidney-cortex slices*

Kidney-cortex slices from rats that had been starved for 18–22 h were incubated in Krebs bicarbonate buffer, pH 7.4, containing either 0.1 mm-ethanedioxybis(ethylamine)tetra-acetic acid or CaCl_2 over the concentration range 0.05–2.5 mm, for 1 h at 37°C. Glutamine or sodium malate was present at a concentration of 10 mm. Total glucose formed was determined as described in the text. Each point is the mean $\pm$ S.E.M. of five observations. ●, Malate present; ■, glutamine present.

fructose, malate or fumarate at any of the Ca^{2+} concentrations studied. Stimulation of gluconeogenesis by cyclic AMP and its dibutyl derivative was similar to that observed with the hormones, with two differences which are indicated (*) in Tables 3 and 4. The reason for these apparent anomalies is not understood.

Stimulation of glucose formation from glutamine was apparent after only 30 min incubation and the rates of glucose production with and without glucagon were linear for at least 2 h (Fig. 3).

Fig. 4 shows the relationships between degree of stimulation of glucose formation from glutamine and modifier concentration for each modifier studied. All four modifiers showed a fairly sharp optimum concentration above which there was decreased stimulation or even inhibition of gluconeogenesis. A similar relationship has been reported for the stimulation of gluconeogenesis by cyclic AMP in a rat kidney-tubule preparation (Guder & Wieland, 1971) and has been interpreted as inhibition by 5'-AMP (Weidemann *et al.*, 1969) generated by phosphodiesterase activity. The high concentration of cyclic AMP required to

Table 1. *Effect of Ca²⁺ and glucagon on gluconeogenesis from a range of substrates by kidney-cortex slices*

Slices of kidney cortex from rats that had been starved for 18–22 h were incubated in Krebs bicarbonate buffer, pH 7.4, at the indicated Ca²⁺ concentrations for 1 h at 37°C. Substrates were at a concentration of 10 mM, except for DL-lactate, which was at 20 mM. Glucagon, when present, was at 0.1 μ M. Glucose formed from added substrate was determined as described in the text. Results are means $\pm$ S.E.M., with the numbers of observations in parentheses. n.s., Not significant.

Substrate	[Ca ²⁺] (mM)	Glucose formed from added substrate (μ mol/h per g dry wt.)		P	Stimulation (%)
		Control	+Glucagon		
Glutamine	0	16.0 $\pm$ 2.6 (12)	15.5 $\pm$ 2.3 (12)		0
	0.25	21.6 $\pm$ 2.4 (12)	34.0 $\pm$ 4.2 (12)	<0.02	58.3
	2.5	58.8 $\pm$ 6.0 (8)	57.3 $\pm$ 6.2 (8)		0
Glutamate	0	24.0 $\pm$ 2.9 (4)	24.5 $\pm$ 5.2 (4)		0
	0.25	32.6 $\pm$ 2.6 (8)	50.9 $\pm$ 3.3 (8)	<0.001	56
	2.5	85.1 $\pm$ 4.2 (4)	75.7 $\pm$ 6.2 (4)		0
Succinate	0.25	40 $\pm$ 3.6 (8)	63 $\pm$ 4.1 (8)		57.5
Pyruvate	0	25.4 $\pm$ 4.0 (12)	28.4 $\pm$ 6.6 (4)		0
	0.25	36.9 $\pm$ 6.8 (8)	73.1 $\pm$ 4.1 (8)	<0.001	98
	2.5	128.2 $\pm$ 20.1 (4)	130.3 $\pm$ 11.0 (4)		0
Lactate	0	12.1 $\pm$ 1.7 (5)	12.3 $\pm$ 3.6 (5)		0
	0.25	18.9 $\pm$ 4.2 (6)	35.0 $\pm$ 3.6 (6)	<0.02	85
	2.5	69.4 $\pm$ 6.4 (5)	53.7 $\pm$ 6.0 (5)		0
Glycerol	0	19.1 $\pm$ 2.1 (5)	20.5 $\pm$ 4.5 (5)		0
	0.25	23.8 $\pm$ 4.7 (6)	26.8 $\pm$ 3.1 (6)		0
	2.5	32.7 $\pm$ 6.7 (6)	32.4 $\pm$ 5.0 (5)		0
Fructose	0	252 $\pm$ 14 (5)	229 $\pm$ 13 (5)		0
	0.25	303 $\pm$ 20 (6)	294 $\pm$ 30 (6)		0
	2.5	442 $\pm$ 47 (5)	462 $\pm$ 41 (5)		0
Malate	0	24.9 $\pm$ 8.2 (5)	28.1 $\pm$ 7.3 (5)		0
	0.25	35.8 $\pm$ 2.5 (28)	42.1 $\pm$ 3.8 (27)	n.s.	0
	2.5	179.5 $\pm$ 13.7 (5)	211 $\pm$ 25.4 (5)	n.s.	0
Fumarate	0.25	54.7 $\pm$ 4.5 (13)	55.1 $\pm$ 5.2 (13)		0
	2.5	192.5 $\pm$ 12.8 (6)	193 $\pm$ 14 (6)		0

achieve activation presumably reflects the low permeability of the cortex cell to this agent. Dibutyl cyclic AMP was even less potent than cyclic AMP. It has been suggested that deacylation of this compound is necessary for its action and that this process is slow in renal cortex (Bowman, 1970).

Phosphoenolpyruvate carboxylase activity

The pattern of stimulation by Ca²⁺ and hormones indicated activation of the gluconeogenic sequence between the tricarboxylic acid cycle and the triose phosphates. Exton & Park (1969) from studies on glucagon activation of gluconeogenesis in perfused liver concluded that the point of stimulation by the hormone lay between pyruvate and phosphoenolpyruvate. Since phosphoenolpyruvate carboxylase is considered to be a rate-limiting enzyme in gluconeogenesis,

several different approaches were used in an attempt to detect activation of this enzyme by Ca²⁺, hormones and cyclic AMP.

(i) *Activity in the supernatant fraction.* With assay method 1, in the presence of 5 mM-oxaloacetate and 15 mM-Mg²⁺, 2.5 mM-Ca²⁺ was slightly (20%) inhibitory. With assay method 2 at 7 μ M-oxaloacetate and 1.5 mM-Mn²⁺, 1 mM-Ca²⁺ gave 25% inhibition, whereas addition of cyclic AMP at concentrations of 25 μ M and 0.25 mM had no effect.

(ii) *Activity in the homogenate.* At 30 μ M-oxaloacetate, addition of 50 μ M-cyclic AMP to assay system (2) had no effect on the reaction rate. Preincubation of the homogenate with 50 μ M cyclic AMP for 10 min at 37°C, followed by assay in the presence of 30 μ M-oxaloacetate and 50 μ M cyclic AMP, gave the same activity as the control. It is appreciated that F⁻, necessary in the reaction mixture to prevent break-

Table 2. *Effect of adrenaline on gluconeogenesis*

Glucose formation from added substrate was determined as described in the legend of Table 1, except that adrenaline replaced glucagon and, when present, was at $1\text{ }\mu\text{M}$. Results are means $\pm$ S.E.M., with the numbers of observations in parentheses. n.s., Not significant.

Substrate	[Ca ²⁺] (mm)	Glucose formed from added substrate ($\mu\text{mol/h}$ per g dry wt.)		P	Stimulation (%)
		Control	+Adrenaline		
Glutamine	0	20.1 $\pm$ 4.4 (5)	14.0 $\pm$ 4.0 (5)		0
	0.25	35.6 $\pm$ 2.4 (38)	55.9 $\pm$ 4.4 (38)	<0.001	57
	2.5	74.5 $\pm$ 10.1 (5)	56.0 $\pm$ 6.9 (5)		0
Succinate	0.25	35.1 $\pm$ 5.2 (6)	54.6 $\pm$ 4.4 (6)	<0.02	55
	2.5	135.3 $\pm$ 8.4 (6)	167.8 $\pm$ 11.8 (6)	<0.05	24
Lactate	0	12.9 $\pm$ 2.6 (5)	14.9 $\pm$ 2.1 (5)		0
	0.25	28.0 $\pm$ 2.4 (7)	47.6 $\pm$ 7.3 (7)	<0.025	70
	2.5	82.1 $\pm$ 5.5 (5)	94.6 $\pm$ 8.5 (5)	n.s.	0
Fructose	0	206 $\pm$ 11 (5)	198 $\pm$ 9.8 (5)		0
	0.25	273 $\pm$ 10 (7)	280 $\pm$ 14 (7)		0
	2.5	371 $\pm$ 15 (5)	364 $\pm$ 23 (5)		0
Malate	0	26.0 $\pm$ 2.3 (5)	25.8 $\pm$ 5.2 (5)		0
	0.25	35.7 $\pm$ 2.2 (36)	40.6 $\pm$ 2.6 (34)	n.s.	0
	2.5	223 $\pm$ 7.9 (5)	276 $\pm$ 25.0 (5)	n.s.	0
Fumarate	0.25	54.7 $\pm$ 4.5 (13)	58.6 $\pm$ 5.1 (13)		0
	2.5	192.5 $\pm$ 12.8 (6)	218 $\pm$ 12.3 (6)	n.s.	0

Table 3. *Effect of cyclic AMP on gluconeogenesis*

Glucose formation from added substrate was determined as described in the legend of Table 1, except that cyclic AMP replaced glucagon and, when present, was at 0.1 mM . Results are means $\pm$ S.E.M., with the numbers of observations in parentheses. n.s., Not significant.

Substrate	[Ca ²⁺] (mm)	Glucose formed from added substrate ($\mu\text{mol/h}$ per g dry wt.)		P	Stimulation (%)
		Control	+Cyclic AMP		
Glutamine	0	23.5 $\pm$ 4.0 (10)	24.5 $\pm$ 5.0 (10)	n.s.	0
	0.25	33.3 $\pm$ 3.0 (21)	46.2 $\pm$ 4.7 (21)	<0.05	39
	2.5	60.1 $\pm$ 6.8 (10)	73.6 $\pm$ 6.3 (10)	n.s.	0
Succinate	0.25	40.0 $\pm$ 3.6 (8)	59 $\pm$ 3.0 (8)		47.5
Lactate	0	28.0 $\pm$ 2.1 (11)	29.0 $\pm$ 1.9 (11)		0*
	0.25	42.8 $\pm$ 6.1 (7)	65.4 $\pm$ 6.3 (7)	<0.05	52.3
	2.5	90.6 $\pm$ 7.9 (7)	94.8 $\pm$ 5.9 (7)		0*
Fructose	0	256 $\pm$ 19 (5)	262 $\pm$ 20 (5)		0
	0.25	310 $\pm$ 12 (6)	300 $\pm$ 16 (6)		0
	2.5	381 $\pm$ 2.3 (5)	386 $\pm$ 17 (5)		0
Malate	0	26.1 $\pm$ 6.3 (5)	34.2 $\pm$ 7.6 (5)	n.s.	0
	0.25	35.5 $\pm$ 2.4 (28)	38.2 $\pm$ 2.2 (27)	n.s.	0
	2.5	248 $\pm$ 18.5 (7)	242 $\pm$ 8.2 (7)		0
Fumarate	0.25	59.6 $\pm$ 5.7 (7)	58.4 $\pm$ 3.8 (7)		0
	2.5	192.5 $\pm$ 12.8 (6)	201.9 $\pm$ 11.4 (6)		0

* For details see the text.

Table 4. *Effect of 6-N-2'-O-dibutyryl cyclic AMP on gluconeogenesis*

Glucose formation from added substrate was determined as described in the legend of Table 1, except that 6-N,2'-O-dibutyryl cyclic AMP replaced glucagon and when present was at 0.5 mM. Results are means $\pm$ S.E.M., with the numbers of observations in parentheses. n.s., Not significant.

Substrate	[Ca ²⁺] (mM)	Glucose formed from added substrate (μ mol/h per g dry wt.)			Stimulation (%)
		Control	+Dibutyryl cyclic AMP		
Glutamine	0	15.5 $\pm$ 4.1 (10)	24.6 $\pm$ 4.5 (10)	n.s.	0
	0.25	34.9 $\pm$ 3.7 (10)	57.2 $\pm$ 4.6 (12)	<0.005	64
	2.5	58.5 $\pm$ 5.2 (10)	48.4 $\pm$ 4.0 (10)	n.s.	0
Succinate	0.25	35.1 $\pm$ 5.2 (6)	50.6 $\pm$ 3.8 (6)	<0.05	44.3
	2.5	135 $\pm$ 8.4 (6)	175 $\pm$ 13.6 (6)	<0.05	29.5
Lactate	0	26.2 $\pm$ 3.2 (6)	35.2 $\pm$ 3.6 (6)	<0.05	34.3*
	0.25	38.4 $\pm$ 4.0 (6)	64.0 $\pm$ 5.7 (6)	<0.005	67
	2.5	90.6 $\pm$ 7.9 (7)	112.8 $\pm$ 5.0 (7)	<0.05	24.5*
Malate	0	31.5 $\pm$ 2.7 (5)	47.8 $\pm$ 2.2 (5)	n.s.	0
	0.25	47.9 $\pm$ 5.8 (8)	55.1 $\pm$ 6.9 (8)	n.s.	0
	2.5	178 $\pm$ 17 (5)	172 $\pm$ 10 (5)		0
Fumarate	0.25	59.6 $\pm$ 5.7 (7)	61.0 $\pm$ 7 (7)		0
	2.5	192.5 $\pm$ 12.8 (6)	185.0 $\pm$ 10.0 (6)		0

* For details see the text.

Fig. 3. Time-courses of glucose formation from glutamine by kidney cortex with and without glucagon

Kidney-cortex slices from rats that had been starved for 18–22 h were incubated in Krebs bicarbonate buffer, pH 7.4, containing 0.25 mM-CaCl₂ and 10 mM-glutamine, with (■) or without (●) 0.10 μ M-glucagon, for the indicated times at 37°C. Glucose formed from glutamine was determined as described in the text. Each point is the mean $\pm$ S.E.M. of five observations.

down of phosphoenolpyruvate, will activate adenylate cyclase (Streeto, 1969) and therefore possibly mask any activation. However, the reaction rates for assays of equal volumes of supernatant and homogenate samples in the presence of F⁻ were not significantly different.

(iii) *Activity in preincubated slices.* The results in Table 5 show that after incubation of slices in the presence of 1 μ M-adrenaline, 10 mM-glutamine and 0.25 mM-Ca²⁺ for 1 h, there was no detectable change in the V_{max} or K_m of the supernatant enzyme subsequently isolated. Slices were homogenized in the presence of 0.1 M-NaF, which did not affect the activity of supernatant enzyme, as shown by the activities of fresh tissue samples homogenized with and without F⁻ present. Supernatant enzyme from slices incubated at different Ca²⁺ concentrations showed no significant change in phosphoenolpyruvate carboxylase activity at 5 mM-oxaloacetate with assay method 1. Variations in extracellular Ca²⁺ concentration did not affect the rate of leakage of protein from kidney slices during incubation.

Discussion

The stimulation of renal gluconeogenesis by glucagon and adrenaline reported here is, in many ways,

Fig. 4. Dose-response curves for stimulation of gluconeogenesis from glutamine by glucagon, adrenaline, cyclic AMP and 6-N-2'-O-dibutyryl cyclic AMP

Kidney-cortex slices from rats that had been starved for 18–22h were incubated in Krebs bicarbonate buffer, pH7.4, containing 0.25mM-CaCl₂, 10mM-glutamine and a range of modifier concentrations, for 1h at 37°C. Glucose formed from glutamine was determined as described in the text. The rate of glucose production in the absence of any modifier is taken as 100%. Modifiers were: glucagon (■); adrenaline (▲); cyclic AMP (●); 6-N-2'-O-dibutyryl cyclic AMP (▼). Each point is the mean of six observations.

comparable with the stimulation by parathyroid hormone described by Rasmussen & Nagata (1970). The similarity between activation by these hormones and by cyclic AMP and its dibutyryl derivative suggests that the hormonal activation is mediated by an increase in intracellular cyclic AMP. Both parathyroid hormone (Chase & Aurbach, 1968) and adrenaline (Back *et al.*, 1972) stimulate renal-cortex adenylate cyclase activity; we are not aware of any results about the effect of glucagon on this enzyme. The failure to detect stimulation of renal gluconeogenesis by glucagon, adrenaline and parathyroid hormone reported previously (Nishiitsutsuji-Uwo *et al.*, 1967; Bowman, 1970) can be attributed to the high extracellular Ca²⁺ (2.5mM) used in these experiments.

Extracellular Ca²⁺ concentration is most critical for the action of these hormones. Since Ca²⁺ is not required for hormonal activation of renal-cortex adenylate cyclase (Nagata & Rasmussen, 1970a), and cyclic AMP does not stimulate gluconeogenesis in the absence of Ca²⁺ (Tables 3 and 4), there would appear to be a minimal Ca²⁺ requirement for activation of gluconeogenesis by cyclic AMP. Glucose production from fructose, although Ca²⁺-dependent, is not stimulated by hormones and cyclic AMP (Tables 1–3). This argues against the proposal by Rasmussen (1970) that cyclic AMP activates gluconeogenesis by increasing the Ca²⁺ concentration in the cytosol compartment. Ca²⁺ stimulates glucose formation from fructose by activation of fructose 1-phosphate aldolase (EC 4.1.2.7), a cytosolic enzyme (G. A. O. Alleyne, H. Flores & A. Roobol, unpublished work).

The pattern of stimulation by Ca²⁺ and cyclic AMP from the range of substrates studied suggests

Table 5. Effect of adrenaline on phosphoenolpyruvate carboxylase activity in kidney-cortex slices

Slices of kidney cortex from rats that had been starved for 18–22h were incubated in Krebs bicarbonate buffer, pH7.4, containing 0.25mM-CaCl₂ and 10mM-glutamine, with and without 1 μM-adrenaline, for 1h at 37°C. After incubation the slices were homogenized in 9vol. of ice-cold 0.25M-sucrose, containing 0.1M-NaF. Samples of fresh tissue were homogenized in 9vol. of ice-cold 0.25M-sucrose, with and without 0.1M-NaF. Phosphoenolpyruvate carboxylase activity in the cytosol fraction of the homogenate was determined by assay method 1 with oxaloacetate at a concentration of 5mM or 0.3mM. Results are means ±s.e.m. of four observations.

		Phosphoenolpyruvate carboxylase activity (μmol of phosphoenolpyruvate formed/min per g wet wt.)		
0.1M-NaF in homogenizing medium	Concn. of oxaloacetate in assay (mM)	Control slices	Adrenaline-treated slices	Fresh tissue
—	5			6.50
+	5	3.94 ± 0.15	4.0 ± 0.08	6.61
—	0.3			3.65
+	0.3	2.92 ± 0.18	2.49 ± 0.26	3.86

that activation of gluconeogenesis occurs between the tricarboxylic acid cycle and phosphoenolpyruvate. Activation of pyruvate carboxylase (EC 6.4.1.1) is unlikely to be important, since glucose formation from glutamine, glutamate and succinate is stimulated by Ca^{2+} , hormones and cyclic AMP (Tables 1–4). We have failed to detect either direct activation of phosphoenolpyruvate carboxylase or indirect activation (Table 5) of the type described for the liver phosphorylase system (Sutherland & Cori, 1951; Rall *et al.*, 1956). The failure of hormones and cyclic AMP to stimulate glucose formation from malate and fumarate (Tables 1–4) also argues against phosphoenolpyruvate carboxylase activation. Stimulation by Ca^{2+} with these substrates may be due to inhibition of pyruvate kinase (EC 2.7.1.40) activity (Bygrave, 1966), or Ca^{2+} may activate phosphoenolpyruvate carboxylase in some way that we have failed to detect. Fumarate hydratase (EC 4.2.1.2) and malate dehydrogenase (EC 1.1.1.37) are present in the cytosol and the mitochondria of kidney cortex (Simpson, 1972) so that glucose formation from malate and fumarate can occur entirely in the cytosol. ATP required for this process could be generated from oxidation of endogenous substrates and possibly from a portion of malate entering the mitochondrial membrane. Fumarate cannot penetrate the kidney mitochondrial membrane (Chappell, 1968).

All substrates tested which are sensitive to both

Ca^{2+} and cyclic AMP must enter the mitochondrion during gluconeogenesis. The transfer of substrates in and out of mitochondria may be a major control site for gluconeogenesis (Exton & Park, 1969; Anderson *et al.*, 1971). There is evidence for increased pyruvate uptake by liver mitochondria from glucagon-treated rats (Adams & Haynes, 1969). Energy-dependent anion accumulation by liver mitochondria requires the presence of a permeant cation, e.g. Ca^{2+} or K^+ , in the presence of valinomycin (Robinson & Chappell, 1967; Haslam & Griffiths, 1968). We suggest that at low extracellular Ca^{2+} concentrations, renal gluconeogenesis is limited by the rate of mitochondrial transport, which may be increased by cyclic AMP either directly or by increasing the sensitivity of the system towards Ca^{2+} . An increase in extracellular Ca^{2+} [and hence intracellular Ca^{2+} (Borle, 1968)] activates this rate-limiting process, and sensitivity of gluconeogenesis towards cyclic AMP is consequently decreased.

Scheme 2 summarizes the proposed interactions of Ca^{2+} and cyclic AMP with the gluconeogenic sequence. This scheme indicates that the Ca^{2+} -dependence of gluconeogenesis from malate and fumarate may well differ from that of other substrates giving rise to oxaloacetate. For malate and fumarate this should reflect Ca^{2+} inhibition of pyruvate kinase and/or activation of phosphoenolpyruvate carboxylase. For other substrates, e.g. glutamine, the Ca^{2+} -

Scheme 2. Proposed interactions of Ca^{2+} and cyclic AMP with renal gluconeogenesis

At low extracellular Ca^{2+} concentrations, mitochondrial transport is the slowest step in gluconeogenesis and glucose formation from all substrates except fructose, glycerol, malate and fumarate will be stimulated by cyclic AMP. At high (2.5 mM) extracellular $[\text{Ca}^{2+}]$, mitochondrial transport is stimulated and sensitivity towards cyclic AMP is lost. ---⊕--->, Activation; ---⊖--->, inhibition.

dependence of glucose formation will be associated with activation of mitochondrial transport. The results of Fig. 2 are consistent with this interpretation. Between 0.1 and 0.5 mM- Ca^{2+} there is little increase in glucose formation from glutamine, whereas glucose formation from malate increases steadily. Within this Ca^{2+} concentration range, we propose that the rate of mitochondrial transport becomes limiting for gluconeogenesis from glutamine and hence sensitivity of glucose formation towards cyclic AMP is observed. The Ca^{2+} -dependence of gluconeogenesis from succinate is similar to that of glutamine. This is also consistent with the proposed Scheme 2.

Finally, the physiological significance of these results must be considered. Extra- and intra-cellular concentrations of Ca^{2+} are in the range (Borle, 1968) to be relevant in control of renal gluconeogenesis. The optimum concentrations of hormones required to stimulate glucose production (Fig. 4) are rather higher than concentrations reported for the physiological ranges of glucagon (Ohneda *et al.*, 1969) and adrenaline (Exton *et al.*, 1971). It is possible, however, that in the kidney, which, for example, is known to concentrate a variety of peptide hormones (Rasmussen *et al.*, 1967), the concentrations of these hormones are high enough to play a role in regulating renal gluconeogenesis.

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