Hormonal Control of Gluconeogenesis in Tubule Fragments from Renal Cortex of Fed Rats

EFFECTS OF α -ADRENERGIC STIMULI, GLUCAGON, THEOPHYLLINE AND PAPAVERINE

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1. In incubated tubule fragments from renal cortex of fed rats gluconeogenesis from pyruvate was stimulated by adrenaline ($1\ \mu\text{M}$ optimum) and by the selective α -adrenergic agonists oxymetazoline and amidephrine. The selective β -agonists isoproterenol and salbutamol were ineffective at concentrations up to $10\ \mu\text{M}$. 2. Stimulation of gluconeogenesis by $1\ \mu\text{M}$ -adrenaline was almost completely blocked by $10\ \mu\text{M}$ -phenolamine (α -antagonist), partially blocked by $10\ \mu\text{M}$ -phenoxybenzamine (α -antagonist) and unaffected by $10\ \mu\text{M}$ -propranolol (β -antagonist). 3. Adrenaline stimulation of gluconeogenesis was rapid and was sustained for at least 1 h. 4. Oxymetazoline (α -agonist) was extremely potent in stimulation of gluconeogenesis. This compound stimulated glucose production from pyruvate, lactate and glutamate, but not from succinate or glycerol. 5. In the absence of Ca^{2+} oxymetazoline was ineffective, whereas some stimulatory effect of adrenaline on gluconeogenesis was still observed. 6. Glucagon had no effect on gluconeogenesis from pyruvate in the presence of $1.27\ \text{mM}$ - Ca^{2+} and inhibited the process in the presence of $0.25\ \text{mM}$ - Ca^{2+} . Parathyrin (parathyroid hormone) stimulated gluconeogenesis at $1.27\ \text{mM}$ - Ca^{2+} . 7. In short incubations of tubule fragments glucagon, papaverine and adrenaline significantly increased 3':5'-cyclic AMP. Adrenaline also slightly decreased 3':5'-cyclic GMP. Oxymetazoline had no effect on the amount of either cyclic nucleotide. 8. At all concentrations tested, theophylline and papaverine decreased gluconeogenesis from pyruvate. 9. It is concluded that renal gluconeogenesis may be increased by α - but not β -adrenergic stimuli and that this is probably independent of changes in 3':5'-cyclic AMP or 3':5'-cyclic GMP. An involvement of Ca^{2+} in the action of oxymetazoline appears likely, but this is less certain with adrenaline.

Renal gluconeogenesis represents a significant metabolic activity in non-functioning kidney preparations *in vitro*. The process is localized in the proximal tubule (Guder & Schmidt, 1974) and probably makes immediate localized provision of glucose for other parts of the nephron (McCann, 1962). Contribution to the glucose supply for body tissues in general is also possible.

Renal-cortex preparations are convenient for investigating the control of gluconeogenesis, since this tissue has an extremely low glycogen content (Krebs, 1963). In the presence of appropriate substrates, gluconeogenesis can be observed *in vitro* at rates that related to tissue wet weight, may even exceed those found in liver. Metabolic studies have been carried out mainly in three different systems, incubated cortex slices, the isolated perfused kidney and tubule fragments prepared by collagenase digestion of kidney cortex (Nagata & Rasmussen, 1970a; Guder *et al.*, 1971). In many instances there has been agreement in the results obtained with these different preparations.

Many factors alter renal gluconeogenesis *in vitro*, e.g. the electrolyte composition of media (Krebs *et al.*, 1963; Rutman *et al.*, 1965; Nagata & Rasmussen, 1970a; Kurokawa & Rasmussen, 1973; Alleyne *et al.*, 1973), diet (Krebs *et al.*, 1963), fatty acids (Weidemann & Krebs, 1969; Guder & Wieland, 1972) and several hormones. The observations that parathyrin (parathyroid hormone) or exogenous 3':5'-cyclic AMP stimulate renal gluconeogenesis, that parathyrin can increase renal-cortex 3':5'-cyclic AMP through the action of a parathyrin-sensitive adenylate cyclase and that actions of parathyrin and 3':5'-cyclic AMP on gluconeogenesis are in some cases non-additive (Pagliara & Goodman, 1969; Nagata & Rasmussen, 1970b; Melson *et al.*, 1970; Kaminsky *et al.*, 1970; Guder & Wieland, 1972) have led to the conclusion that 3':5'-cyclic AMP acts as a messenger of this hormone in this process. In addition Roobol & Alleyne (1973) observed stimulation of renal gluconeogenesis by glucagon and implicated 3':5'-cyclic AMP as a mediator of this hormonal effect. However, this renal action of glucagon could

not be demonstrated in the isolated perfused kidney (Nishiitsutsuji-Uwo *et al.*, 1967; Bowman, 1970).

Just before the start of the present study it was reported that catecholamines stimulated gluconeogenesis in renal-cortex slices (Klahr *et al.*, 1973; Kurokawa & Massry, 1973a) and in isolated tubule fragments (Friedrichs & Schoner, 1973). These authors concluded that this effect was mediated by elevation of 3':5'-cyclic AMP concentrations with the involvement of a β -type adrenergic receptor. At the same time Tolbert *et al.* (1973) and Tolbert & Fain (1974) had shown that catecholamine stimulation of gluconeogenesis in isolated liver cells involved a 3':5'-cyclic AMP-independent α -adrenergic action. The main objective was therefore to test for the possibility of α -adrenergic stimulation of glucose production in kidney by using cortex-tubule fragments. This involved testing the effects of both α - and β -adrenergic blocking agents on catecholamine-stimulated gluconeogenesis and also testing the actions of synthetic α - and β -adrenergic agonists. The actions of glucagon were also investigated in the tubule-fragment system, in view of the divergence of opinion about the action of this hormone in other renal preparations. During the course of this work Guder & Rupprecht (1975a) presented evidence that catecholamine stimulation of renal gluconeogenesis is mediated via an α -receptor and is independent of changes in 3':5'-cyclic AMP concentrations. The present study confirms and extends these findings.

Materials and Methods

Chemicals

NADP⁺, ATP, sodium pyruvate, hexokinase and glucose 6-phosphate dehydrogenase were obtained from Boehringer Corp. (London) Ltd. (Lewes, Sussex, U.K.). 3':5'-Cyclic AMP (sodium salt), L-adrenaline, DL-propranolol hydrochloride, theophylline, papaverine (6,7-dimethoxy-1-veratrylisoquinoline), L(+)-lactic acid and DNA (type V, sodium salt, 'highly polymerised, from calf thymus') were from Sigma (London) Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Collagenase (type I from *Clostridium histolyticum*) was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Bovine plasma albumin powder (fraction V) from Armour Pharmaceutical Co. (Eastbourne, Sussex, U.K.) or from Sigma (London) was subjected to a defatting procedure before use (Chen, 1967). Bovine parathyroid and bovine glucagon were from Calbiochem (Hereford, U.K.). 3':5'-Cyclic [8-³H]AMP (ammonium salt) and a 3':5'-cyclic GMP radioimmunoassay kit were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). 3':5'-Cyclic AMP-binding protein prepared from bovine muscle (Tovey *et al.*, 1974) was a gift from

Professor A. L. Greenbaum of University College London. 2,5-Bis-(5-t-butylbenzoxazol-2-yl)thiophene was from CIBA (A.R.L.) Ltd. (Duxford, Cambridge, U.K.). Heparin (Mucous) B.P. was supplied by Weddel Pharmaceuticals (London E.C.1, U.K.). The following were a gift from Dr. D. Osborne, Department of Pharmacology, University College London: L-amidephrine mesylate [*m*-(1-hydroxy-2-methylaminoethyl)methanesulphonanilide methanesulphonate], L-isoproterenol hydrochloride, ($\pm$)-oxymetazoline hydrochloride [2-(4-t-butyl-3-hydroxy-2,6-dimethylbenzyl)-2-imidazoline hydrochloride], phentolamine hydrochloride, salbutamol [1-(4-hydroxy-3-hydroxymethylphenyl)-2-(*t*-butylamino)ethanol]. Phenoxybenzamine hydrochloride was obtained from the dispensary of University College Hospital. All other chemicals were A.R. grade or of the highest purity commercially available.

Animals

These were male Sprague-Dawley rats bred in the animal colony at University College. They were maintained on GR3EK diet (E. Dixon and Sons, Ware, Herts., U.K.) until the time of experimentation, when they weighed 160–180 g.

Isolation of tubule preparations from renal cortex

Rats were injected sequentially at 5 min intervals with 0.05 ml of heparin (38 mg/ml) intraperitoneally, and 20 min after heparin treatment each rat was killed by cervical dislocation. The kidneys were quickly perfused as described by Guder *et al.* (1971) with 20–30 ml of ice-cold bicarbonate buffer (Krebs & Henseleit, 1932) containing 1.27 mM-Ca²⁺, rapidly removed and the medulla and papilla excised. Generally the pooled cortices of three rats were collected, cut into small pieces and shaken at 37°C under O₂/CO₂ (19:1) in a 50 ml silicone-treated Erlenmeyer flask containing 10 ml of Krebs-Ringer bicarbonate buffer (containing 1.27 mM-Ca²⁺), fatty acid-poor albumin (20 mg/ml) and collagenase (2 mg/ml). The tissue suspension was then gently forced through nylon mesh (no. 110; J. Stanier and Co., Manchester, U.K.) to remove undigested pieces and to aid dispersion. This was followed by centrifugation at 100g_{av.} for 1 min and the resulting pellet was then resuspended and washed twice by similar centrifugation with Krebs-Ringer bicarbonate buffer containing fatty acid-poor albumin (10 mg/ml). Finally, the tubule preparation was generally made up in a stock suspension in which the tissue from each original kidney cortex was dispersed in 5 ml of Krebs-Ringer bicarbonate buffer containing albumin (10 mg/ml). Essentially this procedure differed from the preparations used by Nagata & Rasmussen (1970a) and Guder *et al.* (1971) in the absence of

anaesthetics, the presence of albumin and the considerably shorter digestion time. In addition, sieving of undigested tissue (Guder *et al.*, 1971) and Ca^{2+} -free media (Nagata & Rasmussen, 1970a) were not used. Examination by phase-contrast microscopy indicated that the preparations consisted mainly of tubule segments rather than isolated cells. Electron microscopy of preparations fixed in glutaraldehyde and post-fixed in OsO_4 showed intactness of plasma, mitochondrial and nuclear membranes. Mitochondria were not swollen and the cristae appeared regular. Brush-border microvilli were also present and the interdigitating profile of tubule cells was clearly seen. Most of the cells had intracellular structures similar to those found in sections of fresh undigested cortex.

Incubation techniques

Incubations were always commenced immediately after the preparation of tubule fragments. When glucose production was measured duplicate or triplicate 1.0 ml portions of the stock suspension were dispensed into 25 ml silicone-treated Erlenmeyer flasks. When cyclic nucleotides were measured duplicate 2.0 ml portions of the stock were similarly dispensed. These incubation flasks finally contained 4 ml of Krebs-Ringer bicarbonate buffer with albumin (10 mg/ml) and CaCl_2 (1.27 mM) unless otherwise stated. Substrates, hormones and other agents were added as appropriate. Incubation was at 37°C with shaking at approx. 70 oscillations/min. The flask contents were continuously gassed with O_2/CO_2 (19:1).

Analytical methods

Glucose was measured enzymically (Slein, 1965) in extracts prepared by deproteinization of incubation-flask contents with 0.5 ml of ice-cold 45% (w/v) HClO_4 . These extracts were neutralized by addition of 1 M-triethanolamine hydrochloride and concentrated K_2CO_3 (approx. 5 M). In all experiments the small amount of glucose initially present in non-incubated preparations was also determined and subtracted from experimental values.

When cyclic nucleotides were to be measured, flask contents were deproteinized by 2 ml of 20% (w/v) trichloroacetic acid, left on ice for 1 h, centrifuged at 1000 g for 5 min and supernatants of duplicate experiments pooled. These were extracted with 5×20 ml of water-saturated diethyl ether and then freeze-dried. The extracts were dissolved in 0.5 ml of 50 mM-sodium acetate buffer (adjusted to pH 4.2 with HCl). Duplicate 15 μl or 30 μl samples were assayed for 3':5'-cyclic AMP as described by Gilman (1970). Portions of incubation media taken through the extraction procedures were also assayed and the small

blank values so found were subtracted from all experimental values. Extracts in sodium acetate buffer were neutralized (pH 6.5–7.5) by addition of a small amount of 1.67 M-Tris/HCl, pH 8.5, before duplicate 100 μl samples were taken for radioimmunoassay of 3':5'-cyclic GMP as described by The Radiochemical Centre in the assay kit.

DNA was measured by the method of Burton (1956) in triplicate 1.0 ml portions of stock tubule suspensions.

Statistical methods

Analysis of data was performed on a paired basis and statistical significance determined by the Student's *t* test.

Results

General considerations

Ca^{2+} -free buffers, which are often used in preparation of isolated liver cells or renal tubules, were deliberately avoided, since their use did not result in any greater yields. Furthermore, in our hands, tubule fragments prepared in the presence of Ca^{2+} consistently exhibited higher rates of glucose production, e.g. 2–3 μmol of glucose/30 min per mg of DNA was produced by tubules prepared in 1.27 mM- Ca^{2+} and then incubated with 1.27 mM- Ca^{2+} , whereas those prepared in Ca^{2+} -free media and subsequently incubated with 1.27 mM- Ca^{2+} produced only approx. 0.8 μmol /30 min per mg of DNA.

In most experiments 5 mM-pyruvate was used as substrate, since it is rapidly utilized as a gluconeogenic precursor in tubule preparations (Nagata & Rasmussen, 1970a; Guder *et al.*, 1971) and its route of utilization involves most of the putative gluconeogenic control points. Preliminary experiments established the linearity of glucose production with time for at least 1 h.

Glycogen was not detectable in samples of tubule fragments equivalent to those normally incubated, and glucose production from endogenous substrates alone on incubation for 1 h with 1.27 mM- Ca^{2+} was only 3.8% of that when 5 mM-pyruvate was present. It was therefore presumed that glucose formation in the presence of pyruvate or other substrates represented gluconeogenesis from these exogenous substrates.

All experimental values have been standardized by relation to tissue DNA. There was 24.6 μg of DNA/mg tubule dry wt. (mean of 91 values). The use of albumin in incubation media precluded the expression of results per quantity of tissue protein.

Effects of adrenergic agonists and antagonists

The experiments shown in Fig. 1 and Table 1 strongly suggested that adrenergic stimulation of

Fig. 1. Effect of adrenergic agonists on glucose formation from pyruvate

Kidney-tubule fragments were incubated for 30 min in Krebs-Ringer bicarbonate medium containing albumin (10 mg/ml), 1.27 mM- CaCl_2 , 5 mM-sodium pyruvate and the indicated concentrations of agonists ($\bullet$, adrenaline; $\circ$, oxymetazoline; $\blacksquare$, amidephrine; $\blacktriangle$, isoproterenol; $\triangle$, salbutamol). The bars represent S.E.M. The mean tubule DNA/ml of flask contents was: with adrenaline, 56 μg (four experiments); with oxymetazoline, 43 μg (four experiments); with amidephrine, 70 μg (four experiments); with isoproterenol, 75 μg (two experiments); with salbutamol, 79 μg (one experiment). The mean basal rate of glucose production was $2.53 \pm 0.24 \mu\text{mol}/30 \text{ min}$ per mg of DNA.

renal gluconeogenesis in fed rats is through an α -receptor. Using tubule fragments from starved rats, Guder & Rupprecht (1975a) have similarly concluded that noradrenaline stimulation of gluconeogenesis is α -adrenergic. Fig. 1 shows adrenaline stimulation of glucose formation, which is significant at 0.1 μM ($P < 0.02$), 1 μM ($P < 0.001$) and 10 μM ($P < 0.05$). Table 1 shows that the α -blocker phentolamine produced almost complete blockade of adrenaline action, whereas the β -blocker propranolol was completely ineffective and even appeared to permit a small potentiation of the adrenaline action on glucose production. The α -blocker phenoxybenzamine was less effective than phentolamine at the concentrations used. The 10 μM concentrations of α - and β -blockers

appeared to be sufficiently low to cause negligible effects themselves on gluconeogenesis. Time-course experiments (results not shown) established that the action of 1 μM -adrenaline was not a transient phenomenon, but was a sustained effect for at least 1 h.

Amidephrine, which shows selective α -sympathomimetic actions and is inoperative with β -receptors (Dungan *et al.*, 1965), also stimulated glucose production, but with a lower potency than adrenaline. Significant effects of amidephrine were observed at 0.1 μM ($P < 0.01$), 1 μM ($P < 0.01$) and 10 μM ($P < 0.02$). Oxymetazoline (an imidazoline derivative) has also been reported to be devoid of β -sympathomimetic actions but to be particularly potent with some α -receptors (Mujić & van Rossum, 1965): 10 nM-oxymetazoline was as effective as 1 μM -adrenaline in promoting glucose formation. Significant effects of oxymetazoline were observed at 10 nM ($P < 0.02$), 0.1 μM ($P < 0.02$) and 1 μM ($P < 0.05$). On the other hand, salbutamol, a selective β_2 -agonist (Cullum *et al.*, 1969), and isoproterenol, a β_1 - and β_2 -agonist (Lands *et al.*, 1967), were both ineffective in the concentration range 10 nM–10 μM . In fact, isoproterenol stimulation of rat renal gluconeogenesis has been demonstrated only at concentrations of 0.1 mM or higher (Kurokawa & Massry, 1973a; Klahr *et al.*, 1973; Guder & Rupprecht, 1975a).

Since oxymetazoline was obviously a potent selective agonist for this system and may therefore be a potential experimental tool, some further aspects of its action were examined (Table 2); 0.1 μM -oxymetazoline stimulated gluconeogenesis when lactate or glutamate was substrate. Absolute rates of glucose production were lower with these substrates than with pyruvate, but percentage stimulations by oxymetazoline were greater. Oxymetazoline was ineffective in promoting gluconeogenesis from succinate or glycerol.

The influence of Ca^{2+} concentration on stimulation of gluconeogenesis by oxymetazoline or adrenaline was tested (Table 3). Significant effects of adrenaline were apparent even in the absence of Ca^{2+} . In percentage terms the sensitivity to adrenaline at zero Ca^{2+} was similar to that encountered at 1.27 mM- Ca^{2+} (Fig. 1 and Table 1) and the sensitivity to adrenaline in the presence of a total Ca^{2+} concentration of 0.2 mM was greater. In absolute terms, of course, greater stimulation by adrenaline is observed at 1.27 mM- Ca^{2+} . By contrast, oxymetazoline action was absolutely Ca^{2+} -dependent. No stimulation of gluconeogenesis by this drug was ever observed when Ca^{2+} was omitted. Significant effects of oxymetazoline were seen only at relatively high Ca^{2+} concentrations (1 mM and higher).

Effects of peptide hormones

No glucagon stimulation of gluconeogenesis was seen at either 1.27 mM- Ca^{2+} or 0.25 mM- Ca^{2+} (Fig. 2).

Table 1. *Effect of adrenergic blocking agents on stimulation by adrenaline of glucose formation from pyruvate*
Kidney-tubule fragments were incubated for 30 min in Krebs–Ringer bicarbonate buffer containing albumin (10 mg/ml), 1.27 mM-CaCl₂, 5 mM-sodium pyruvate and, where indicated, 1 μ M-adrenaline, 10 μ M-phentolamine, 10 μ M-phenoxybenzamine or 10 μ M-DL-propranolol. The mean tubule DNA/ml of flask contents was 38 μ g, 51 μ g and 63 μ g for Expts. 1, 2 and 3 respectively. The results are means $\pm$ S.E.M. of 8, 16 and 6 determinations for Expts. 1, 2 and 3 respectively and are expressed as μ mol of glucose/30 min per mg of DNA. In testing statistical significance of adrenaline effects, incubations with adrenaline alone were compared with those containing no additions; incubations with adrenaline + blocking agent were compared with those containing blocking agent alone: ** P < 0.02; †† P < 0.001.

Expt. no.	Additions	Glucose formation	Effect of adrenaline
1	None	3.38 $\pm$ 0.04	
	Adrenaline	4.16 $\pm$ 0.07††	+23 $\pm$ 2%
	Phentolamine	3.12 $\pm$ 0.09	
	Adrenaline + phentolamine	3.33 $\pm$ 0.07	+7 $\pm$ 4%
2	None	2.89 $\pm$ 0.10	
	Adrenaline	3.59 $\pm$ 0.12††	+26 $\pm$ 4%
	Phenoxybenzamine	2.85 $\pm$ 0.13	
	Adrenaline + phenoxybenzamine	3.21 $\pm$ 0.08**	+13 $\pm$ 5%
3	None	2.62 $\pm$ 0.13	
	Adrenaline	3.23 $\pm$ 0.13††	+23 $\pm$ 1%
	Propranolol	2.49 $\pm$ 0.10	
	Adrenaline + propranolol	3.24 $\pm$ 0.10††	+30 $\pm$ 2%

Table 2. *Effect of oxymetazoline on glucose formation from various substrates*

Kidney-tubule fragments were incubated for 30 min in Krebs–Ringer bicarbonate buffer containing albumin (10 mg/ml) and 1.27 mM-CaCl₂ together with the sodium salts of substrates (5 mM) in the presence (+) or absence (–) of 0.1 μ M-oxymetazoline. The mean tubule DNA was 62 μ g/ml of flask contents. The results are means $\pm$ S.E.M. of six determinations and are expressed as μ mol of glucose/30 min per mg of DNA. †† P < 0.001 for effects of oxymetazoline versus the appropriate controls.

Substrate	Oxymetazoline	Glucose formation	Effect of oxymetazoline
Pyruvate	–	2.39 $\pm$ 0.07	
	+	2.96 $\pm$ 0.07††	+24 $\pm$ 2%
L-Lactate	–	1.32 $\pm$ 0.05	
	+	1.76 $\pm$ 0.04††	+34 $\pm$ 1%
L-Glutamate	–	0.99 $\pm$ 0.05	
	+	1.35 $\pm$ 0.04††	+36 $\pm$ 4%
Succinate	–	1.07 $\pm$ 0.02	
	+	1.14 $\pm$ 0.06	+3 $\pm$ 3%
Glycerol	–	1.82 $\pm$ 0.04	
	+	1.92 $\pm$ 0.04	+6 $\pm$ 2%

Indeed, at 0.25 mM-Ca²⁺ glucagon caused significant decreases in glucose production (P < 0.02 and < 0.05 with 0.1 and 0.5 μ g/ml respectively). Fig. 2 also shows that the preparations retained sensitivity to another polypeptide, parathyrin. In the presence of 1.27 mM-Ca²⁺, 0.25 μ g of parathyrin/ml stimulated gluconeogenesis by 16 $\pm$ 3% (P < 0.05). Expressed in percentage terms, other workers have found similar effects of parathyrin on pyruvate- or lactate-dependent gluconeogenesis with 1–2 mM-Ca²⁺ (Rasmussen & Nagata, 1970; Kurokawa *et al.*, 1973; Guder & Rupprecht, 1975a).

In a single experiment (results not shown) [arginine]vasopressin was tested for ability to stimulate gluconeogenesis from pyruvate in 1.27 mM-Ca²⁺-containing media. This agent was totally ineffective in this respect over a concentration range of 0.15–3 ng/ml.

Effects of nucleoside 3':5'-cyclic monophosphate phosphodiesterase inhibitors

The methylxanthine derivatives theophylline and aminophylline cause strong inhibition of renal

Table 3. Effect of Ca^{2+} concentration on stimulation of glucose formation by oxymetazoline and adrenaline

Renal-tubule fragments prepared with 1.27 mM- CaCl_2 were washed three times with Ca^{2+} -free Krebs-Ringer bicarbonate and then incubated for 30 min in Krebs-Ringer bicarbonate buffer containing albumin (10 mg/ml), 100 μM -EGTA, 5 mM-sodium pyruvate, the indicated CaCl_2 additions and oxymetazoline or adrenaline where shown. The mean tubule DNA was 65 and 52 $\mu\text{g}/\text{ml}$ of flask contents for Expts. 1 and 2 respectively. In both experiments the results are means $\pm$ S.E.M. of four determinations and are expressed as μmol of glucose/30 min per mg of DNA. The values in parentheses indicate percentage changes caused by the agonists. * $P < 0.05$, $P^\dagger < 0.01$, $P^{\dagger\dagger} < 0.001$ for effects of agonists versus the appropriate controls.

Expt. no.	Agonist	CaCl_2 addition (mM) ...	Glucose formation					
			0	0.2	0.5	1.0	1.5	2.0
1	None	0.1 μM -Oxymetazoline	0.36 ± 0.03	—	0.96 ± 0.03	1.38 ± 0.08	1.76 ± 0.07	2.02 ± 0.07
			0.33 ± 0.04	—	1.10 ± 0.13	$1.71 \pm 0.11^\dagger$	$2.16 \pm 0.10^\dagger$	$2.44 \pm 0.11^\dagger$
2	None	1 μM -Adrenaline	$(-8.7 \pm 4.5\%)$	—	$(+14.1 \pm 9.9\%)$	$(+23.4 \pm 2.8\%)$	$(+22.9 \pm 2.6\%)$	$(20.7 \pm 2.6\%)$
			0.23 ± 0.04	0.52 ± 0.06	—	—	—	—
			$0.28 \pm 0.04^*$	$0.78 \pm 0.10^{\dagger\dagger}$	—	—	—	—
			$(+21.9 \pm 5.5\%)$	$(+49.7 \pm 1.1\%)$	—	—	—	—

Fig. 2. Effects of glucagon and parathyrin on glucose formation from pyruvate

Kidney-tubule fragments were prepared as described in the Materials and Methods section, except that, where appropriate, washing of the preparations was performed with Krebs-Ringer bicarbonate medium containing 0.25 mM- CaCl_2 . In all other cases medium containing 1.27 mM- CaCl_2 was used. Incubations were for 30 min in Krebs-Ringer bicarbonate medium containing albumin (10 mg/ml), 5 mM-sodium pyruvate and the indicated Ca^{2+} and hormone concentrations. The bars represent S.E.M. ■, 1.27 mM- Ca^{2+} with parathyrin (four experiments). ●, 1.27 mM- Ca^{2+} with glucagon (three experiments). ○, 0.25 mM- Ca^{2+} with glucagon (five experiments). The mean tubule DNA/ml of flask contents was 63 μg (■), 64 μg (●) and 33 μg (○). Basal rates of glucose formation ($\mu\text{mol}/30\text{ min}$ per mg of DNA) were 2.39 ± 0.35 (■), 2.76 ± 0.17 (●) and 1.19 ± 0.20 (○).

gluconeogenesis (Pagliara & Goodman, 1970; Guder & Wieland, 1971; Kurokawa & Massry, 1973a,b). Quite high concentrations of these compounds have to be used to inhibit cyclic nucleotide phosphodiesterases, and undesirable non-specific effects are therefore likely (Kurokawa & Massry, 1973b). The smooth-muscle relaxant papaverine, which is structurally different from the methylxanthines, is a far more potent and possibly more specific inhibitor of cyclic nucleotide phosphodiesterases (O'Dea *et al.*, 1970; Triner *et al.*, 1970; Pösch & Kukovetz, 1972). Fig. 3 shows that theophylline inhibited glucose formation, confirming previous reports (see above), and that papaverine showed similar, though more potent, effects. No stimulation of glucose formation was seen at any tested concentration of papaverine (0.3 μM –0.1 mM); 50% inhibition of gluconeogenesis was found with 55 μM -papaverine. In addition, 50 μM -papaverine did not potentiate the effect of 0.25 μg of

Fig. 3. Effects of theophylline and papaverine on glucose formation from pyruvate

Kidney-tubule fragments were incubated for 30 min in Krebs-Ringer bicarbonate medium containing albumin (10 mg/ml), 1.27 mM-CaCl₂, 5 mM-sodium pyruvate and the indicated concentrations of theophylline (●) or papaverine (○). The results are means of two experiments in each case. For experiments with theophylline, mean basal glucose production was 2.39 μmol/30 min per mg of DNA, and the tubule DNA was 58.1 μg/ml of flask contents. In experiments with papaverine, basal glucose production was 2.47 μmol/30 min per mg of DNA and the mean tubule DNA was 71.0 μg/ml of flask contents.

parathyrin/ml on glucose production (results not shown).

Changes in cyclic nucleotide contents

Measurements were made of 3':5'-cyclic AMP and 3':5'-cyclic GMP in incubations of tubule fragments (Table 4). These were short-time incubations under similar conditions to the studies presented above, except that the tissue concentration in the incubations was doubled. Cyclic nucleotide phosphodiesterase inhibitors, which have previously been included in many studies of cyclic nucleotides in tissues, were deliberately omitted. Eight experiments were performed, involving four agonists at three times together with the appropriate controls. The means of all eight of these control incubations are shown, indicating that basal contents of both cyclic nucleotides were essentially unchanged over the period of incubation. The values represent the cyclic nucleotide content of the whole incubation (tubules + medium).

Papaverine (10 μM) doubled 3':5'-cyclic AMP after 4 min. This was the shortest incubation time that could be satisfactorily resolved with the techniques used. Thereafter 3':5'-cyclic AMP decreased. Glucagon (1 μg/ml) caused a significant increase in 3':5'-cyclic AMP at 4 min. This then decreased rapidly and returned to the basal value by 15 min.

Table 4. Effects of papaverine, glucagon, adrenaline and oxymetazoline on 3':5'-cyclic AMP and 3':5'-cyclic GMP contents
Kidney-tubule fragments were incubated for the indicated times in Krebs-Ringer bicarbonate medium containing albumin (10 mg/ml), 1.27 mM-CaCl₂ and 5 mM-sodium pyruvate. The results are means ± s.e.m. of the numbers of experiments indicated in parentheses. The mean tubule DNA (μg/ml of flask contents) was: with papaverine, 138; with glucagon, 134; with adrenaline or oxymetazoline, 189. *P < 0.05, **P < 0.02, †P < 0.01, ††P < 0.001 for comparison of drug- or agonist-treated tubules with controls.

Incubation time (min)	Basal contents of cyclic nucleotides (pmol/mg of DNA)		Percentage changes in 3':5'-cyclic AMP contents				Percentage changes in 3':5'-cyclic GMP contents			
	3':5'-Cyclic AMP (8)	3':5'-Cyclic GMP (8)	With 10 μM-papaverine (4)	With glucagon (1 μg/ml) (4)	With 1 μM-adrenaline (4)	With 0.1 μM-oxymetazoline (4)	With glucagon (1 μg/ml) (4)	With 1 μM-adrenaline (4)	With 0.1 μM-oxymetazoline (4)	
0	127 ± 10	6.0 ± 0.8	—	—	—	—	—	—	—	—
4	117 ± 7	6.2 ± 0.7	+97 ± 17**	+74 ± 14**	+23 ± 7*	+9 ± 6	+28 ± 15	-10 ± 3*	0 ± 5	
7.5	126 ± 6	7.3 ± 0.9	+69 ± 4††	+41 ± 21	+16 ± 5*	+5 ± 11	+1 ± 7	-17 ± 5*	-2 ± 5	
15	108 ± 4	6.7 ± 0.8	+48 ± 12*	-3 ± 6	+14 ± 2†	+3 ± 1	-12 ± 5	-4 ± 4	+1 ± 5	

Similar transient changes in 3':5'-cyclic AMP are seen in several mammalian tissues after hormonal stimulation. With glucagon, 3':5'-cyclic GMP also showed a tendency to increase at early times and then to decrease. However, none of these changes led to values significantly different from basal. Possibly techniques permitting resolution of shorter incubation times might reveal greater changes in 3':5'-cyclic GMP with glucagon. Adrenaline (1 μ M) caused small but extremely consistent increases in 3':5'-cyclic AMP. Conversely 3':5'-cyclic GMP was decreased by small but significant amounts at both 4 and 7.5 min in the presence of adrenaline. Oxymetazoline (0.1 μ M) had no effect on the content of either cyclic nucleotide at any of the sampling times.

Glucagon and adrenaline both increased the ratio 3':5'-cyclic AMP/3':5'-cyclic GMP. Glucagon increased the ratio by $50 \pm 20\%$ at 4 min, $38 \pm 12\%$ at 7.5 min ($P < 0.05$) and $15 \pm 4\%$ at 15 min ($P < 0.05$). With adrenaline, increases were $37 \pm 6\%$ at 4 min ($P < 0.01$), $40 \pm 12\%$ at 7.5 min ($P < 0.05$) and $20 \pm 3\%$ at 15 min ($P < 0.05$). Oxymetazoline caused no significant changes in the 3':5'-cyclic AMP/3':5'-cyclic GMP ratio.

Discussion

Assuming that α -adrenergic receptors are located on the plasma membrane of cells and that α -agonists are unlikely to work through a direct interaction with enzymes of gluconeogenesis, a 'second messenger' must be postulated to transmit the agonist stimulus. Both Ca^{2+} and 3':5'-cyclic GMP have been implicated in mediation of α -adrenergic actions in various systems (Kaminsky *et al.*, 1970; Goldberg *et al.*, 1973; Selinger *et al.*, 1973; Butcher, 1975). Kneer *et al.* (1974) have shown that exogenous 3':5'-cyclic GMP can stimulate gluconeogenesis in rat hepatocytes. However, although 3':5'-cyclic GMP in hepatocytes is increased after α -adrenergic stimulation, these changes do not correlate with changes in gluconeogenesis (Bosch *et al.*, 1976). The present observations with oxymetazoline (Table 4) show that the effects of this α -agonist are not related to changes in 3':5'-cyclic GMP concentration, nor indeed to 3':5'-cyclic AMP either, unless some subtle inter- or intracellular compartmentation of cyclic nucleotides is invoked. The unlikelihood of 3':5'-cyclic GMP being involved in α -adrenergic stimulation of renal gluconeogenesis is further strengthened by the observation that adrenaline slightly decreased 3':5'-cyclic GMP (Table 4). Adrenaline differed from oxymetazoline in this respect, but it is not known where in the tubule this decrease in 3':5'-cyclic GMP occurs. Possibly it may be related to the small adrenaline-induced increase in 3':5'-cyclic AMP (Table 4), which Guder & Rupprecht (1975a) have suggested occurs through β -adrenergic stimulation of distal structures contaminating the preparation.

Since extracellular Ca^{2+} itself has such a pronounced effect on renal gluconeogenesis, it is not easy to separate basal actions of Ca^{2+} from those necessary for or mediating agonist actions. De Wulf & Keppens (1976) have reported that extracellular Ca^{2+} is essential for α -adrenergic stimulation of hepatic glycogenolysis. Similarly, Tolbert & Fain (1974) have found that α -adrenergic stimulation of hepatic gluconeogenesis is abolished by omission of Ca^{2+} , although gluconeogenesis itself is almost completely abolished at the same time. The results with oxymetazoline (Table 3) show that the action of this α -agonist is clearly Ca^{2+} -dependent. Present data, however, cannot discriminate between the possibilities that Ca^{2+} may be required for oxymetazoline binding to a receptor or alternatively that oxymetazoline may act to influence some movement of Ca between the cell and the extracellular space. Although the action of adrenaline on gluconeogenesis was clearly an α -effect (Table 1), its action was not abolished by omission of Ca^{2+} (Table 3), thus showing a difference between the catecholamine and imidazoline agonists. Either the α -adrenergic effect of adrenaline does not operate through Ca^{2+} or it is able to influence a Ca^{2+} pool that is insensitive to oxymetazoline. These results suggest diversity in α -adrenergic effects and indicate that similar studies with several α -agonists might be rewarding. It is noteworthy that Mujić & van Rossum (1965) have reported that oxymetazoline acts only on certain kinds of α -receptors.

Several investigators have sought to clarify the role of glucagon in renal gluconeogenesis. In perfused kidneys glucagon either slightly decreased (Nishiitsutsuji-Uwo *et al.*, 1967) or was without effect on (Bowman, 1970) gluconeogenesis from pyruvate. In cortex slices from starved rats Roobol & Alleyne (1973) showed that glucagon considerably stimulated glucose formation from several substrates. These effects were seen only at low Ca^{2+} concentrations (0.25 mM). Using tubule fragments, also from starved rats, Guder & Rupprecht (1976) found small increases in gluconeogenesis from pyruvate after addition of glucagon. In percentage terms this effect was also more apparent at low Ca^{2+} concentrations. Guder & Rupprecht (1976) also demonstrated that glucagon may increase tubule 3':5'-cyclic AMP, but questioned the physiological relevance of this observation, since large hormone doses were needed. We confirm (Table 4) the effect of glucagon on tubule 3':5'-cyclic AMP. At present the location in the nephron from which this change in cyclic nucleotide concentration originates is unknown. With regard to glucagon and renal gluconeogenesis, we conclude that this hormone does not stimulate the process in tissue from fed rats. Possibly the discrepancy between the present study and that of Roobol & Alleyne (1973) reflects differences in nutritional status. Alternatively it is possible in the slice system used by Roobol & Alleyne (1973)

that 3':5'-cyclic AMP released from some other structure in the cortex after glucagon stimulation may act to increase proximal-tubule gluconeogenesis in the same manner as added exogenous 3':5'-cyclic AMP.

The effects of cyclic nucleotide phosphodiesterase inhibitors remain perplexing. One of the criteria developed by Sutherland and his colleagues (Sutherland *et al.*, 1965; Sutherland & Robison, 1966) for 3':5'-cyclic AMP to be established as second messenger mediating a hormone action was that cyclic nucleotide phosphodiesterase inhibitors should mimic or potentiate the hormone action (methylxanthines were cited in Sutherland's original criteria). Although theophylline (Kurokawa & Massry, 1973a) and papaverine (Table 4) increase renal-cortical 3':5'-cyclic AMP, stimulation of renal gluconeogenesis is obviously not seen (Fig. 3). Side effects of theophylline and papaverine on membrane transport of substrates or Ca^{2+} (Carpenedo *et al.*, 1971; Kadlec *et al.*, 1973; Plagemann & Sheppard, 1974) cannot be discounted, but it is particularly noteworthy that the relative potencies of these drugs in inhibiting gluconeogenesis (Fig. 3) agree with their relative potencies as cyclic nucleotide phosphodiesterase inhibitors in other systems (Triner *et al.*, 1970; Pösch & Kukovetz, 1972). In isolation these results suggest that intracellular 3':5'-cyclic AMP is an unlikely second messenger for stimulation of gluconeogenesis. This is at variance, however, with the current hypothesis that the action of parathyrin on gluconeogenesis is mediated by 3':5'-cyclic AMP and is also difficult to reconcile with the well-documented observation that exogenous 3':5'-cyclic AMP stimulates renal gluconeogenesis. However, the involvement of 3':5'-cyclic AMP in parathyrin stimulation of gluconeogenesis should be regarded with caution, since optimal concentrations of parathyrin and exogenous 3':5'-cyclic AMP show partially additive effects under certain conditions (Fig. 2 of Guder & Wieland, 1972). In addition, noradrenaline antagonizes the effects of parathyrin on tubule 3':5'-cyclic AMP (Guder & Rupperecht, 1975b), whereas noradrenaline and parathyrin have completely additive effects on gluconeogenesis (Guder & Rupperecht, 1975a).

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