THE STIMULATORY EFFECT OF GLUCAGON AND DIBUTYRYL CYCLIC AMP ON UREOGENESIS AND GLUCONEOGENESIS IN RELATION TO THE MITOCHONDRIAL ATP CONTNET

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1. Introduction

The stimulation by glucagon of both ureogenesis and gluconeogenesis has been known for some years [1,2]. Recently Yamazaki reported that pretreatment of the rats with glucagon yields mitochondria having
(i) An increased phosphorylation rate [3]
(ii) An increased rate of citruline synthesis [4]
(iii) An enhanced ATPase activity in presence of uncoupler and an increased rate of K^+ uptake [5].
Garrison and Haynes [6] in their description of the hormone-induced stimulation of pyruvate carboxylation concluded that changes of mitochondrial content did not appear to account for the effect, which they attributed to an increased uptake and metabolism of pyruvate.

The results presented here show that the stimulatory effect of both glucagon and cyclic AMP (cAMP) on ureogenesis and gluconeogenesis in isolated rat liver cells was accompanied by an increase of the mitochondrial ATP/ADP ratio which consequently furnishes more energy for the mitochondrial steps of these two processes. Higher rates of citrulline production in liver mitochondria isolated from rats pretreated with glucagon or cAMP were associated with a raised mitochondrial ATP content and an enhanced adenine nucleotide translocase.

2. Methods and materials

2.1. *Isolation and incubation of rat-liver cells*

Liver parenchymal cells were isolated from 36 h fasted Wistar rats of about 250 g weight by a procedure based on that of Berry and Friend [7] with some later modifications [8,9]. The cells at 10–20 mg dry wt/ml were incubated at 37°C in Krebs-Ringer bicarbonate buffer containing 10 mM glutamine and 2 mM ornithine. Incubations were carried out in 25 ml Erlenmeyer flasks under 95% O_2 + 5% CO_2 in a Dubnoff shaker. To measure glucose and urea formation the reactions were terminated after 30 min by adding $HClO_4$ (3% final concentration). Samples were cooled, centrifuged and after neutralising with KOH, glucose and urea were assayed in the supernatants. Ammonia release was not observed.

The distribution of adenine nucleotides between cytosol and mitochondria was estimated according to Zuurendonk and Tager [10] with the following modification. After 30 min incubation a 1.5 ml sample of the cell suspension was rapidly mixed with 5 ml of an ice-cold medium containing 0.25 M sucrose, 20 mM MOPS buffer, pH 7.0, 3 mM EDTA, 0.5 mg/ml digitonin (Calbiochem) and 0.12 mM atractyloside (Sigma). After 20 s the suspension was

centrifuged for 20 s at 3000 × *g* at approx. 0–4°C. The supernatant was acidified with $HClO_4$ (6% final concentration). The particulate matter was washed with 1 ml of an ice-cold medium containing 0.25 M sucrose, 3 mM EDTA and 20 mM MOPS buffer, pH 7.0, centrifuged as before and extracted with 6% perchloric acid. The total time taken for mixing the cell suspension with digitonin medium, centrifuging, washing the pellet and acidifying both supernatant and particulate fractions was approx. 90 s. The concentrations of ATP and ADP were estimated after centrifugation and neutralisation of two fractions.

2.2. *Isolation and incubation of rat-liver mitochondria*

Liver mitochondria were isolated from fed Wistar rats as previously. Glucagon (Lilly Research Ltd.) was injected as an alkaline saline–0.1% serum albumin solution at 10 μg/100 g body wt while dibutyryl cAMP (Sigma) was used at 100 nmol/100 g body wt. Injections were made into the portal vein of the nembutal anaesthetised rat 6 min before excision of the liver. The control animals were injected with a saline–0.1% serum albumin solution. The blood sample was withdrawn just before removing the liver and used for the glucose measurement after deproteinisation [12].

The mitochondria (2–3 mg protein/ml) were incubated at 30°C under 95% O_2 + 5% CO_2 for 10 min in a medium containing 15 mM KCl, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM NH_4Cl, 40 mM Tris–HCl, pH 7.4, 10 mM ornithine, 30 mM $KHCO_3$, 20 mM potasium phosphate buffer, pH 7.4, 9 mM succinate, 1 μg/ml rotenone and 3% dextran as before [11]. For measurement of citrulline production 1 ml samples were withdrawn at 3 min intervals and acidified with $HClO_4$ (3% final concentration). The intramitochondrial ATP content was measured in samples taken at 6 min and 10 min incubation periods following the centrifugation through silicone oil (General Electric F.50) into 1.5 M perchloric acid to obtain mitochondrial extracts [13].

Translocation of [^{14}C]ADP into the mitochondria (0.25 mg protein/ml) was measured at 1°C in the incubation medium without succinate, dextran, ammonia and ornithine using an atractylate stopping method [14]. [^{14}C]ADP (Amersham) was used at 37 μM concentration.

Respiratory rates and P/O ratios were measured polarographically at 30°C using the Clark-electrode in a medium containing 125 mM KCl, 20 mM Tris–HCl, pH 7.2 and various substrates at 3 mM concentrations; succinate was supplemented with rotenone (1 μg/ml). The respiratory rates in this medium were higher than in that used for citrulline production.

2.3. *Assays*

Glucose was assayed according to Huggett and Land [12]. Urea was measured as ammonia [15] following the pretreatment with urease (Sigma). Citrulline was determined colorimetrically as described previously [11]. ATP and ADP were estimated fluorometrically by the enzymic methods [16]. Mitochondrial protein was determined by a biuret reaction as described by Cleland and Slater [17].

3. Results and discussion

The content of ATP and ADP and their ratios which were measured in the two fractions obtained by digitonin treatment of the isolated hepatocytes are shown in table 1. Incubation with either glucagon or dibutyryl cAMP stimulated production of both urea and glucose from glutamine and increased the ATP/ADP ratio in the pellet containing the mitochondria. This is consistent with mitochondrial ATP production being stimulated. The cytosolic ATP certainly does not fall despite the increased demand for it in the processes of gluconeogenesis and ureogenesis so it is likely that and enhanced efflux of ATP is also occurring.

Using the mitochondria prepared from control and pre-treated rats the rates of citrulline production were measured (table 2). The results depended either on the season or the source of the rats; over the period September–October, glucagon increased the citrulline production by about 1.6 times; from October–February the rates were less but the same factor applied both to glucagon and dibutyryl cAMP stimulation. In March–April the two agents induced inreases of rate by a factor of 2 which also applies to the internal ATP content. Blood glucose determined just before removing the liver from animal was also increased.

The effects on ADP-stimulated respiration of

Table 1
Effects of glucagon and dibutyryl cAMP on urea and glucose synthesis from glutamine by rat liver cells and the related changes of adenine nucleotide distribution within the cells

Additions	Rates of synthesis		Nucleotides in pellet			Nucleotides in supernatant		
	Urea (μmol/g dry wt cells)	Glucose	ATP (μmol/g dry wt cells)	ADP	ATP/ADP	ATP (μmol/g dry wt cells)	ADP	ATP/ADP
None	161.7 ± 15.5	51.2 ± 5.4	1.52 ± 0.08	0.86 ± 0.05	1.8	8.17 ± 0.73	1.20 ± 0.19	7.1
Glucagon	215.5 ± 24.5	75.0 ± 9.3	1.80 ± 0.08	0.62 ± 0.02	2.9	8.90 ± 0.64	1.11 ± 0.05	8.0
Dibutyryl cyclic AMP	217.3 ± 21.2	78.2 ± 6.3	1.80 ± 0.11	0.68 ± 0.01	2.9	9.02 ± 0.32	1.20 ± 0.24	8.0

The isolated hepatocytes were incubated for 30 min in Krebs-Ringer bicarbonate buffer containing 10 mM glutamine and 2 mM ornithine. Glucagon and dibutyryl cAMP were added at 2 μM and 0.1 mM concentrations, respectively. The distribution of nucleotides was estimated as described in Materials and methods. Values are means ± SEM of 4 experiments

Table 2
Effect of prior injection of glucagon or dibutyryl cAMP into the rat on the rate of production of citrulline by the isolated liver mitochondria

Period	Treatment	Citrulline production ($nmol.min^{-1}.mg^{-1}$)	p	Intramitochondrial ATP (nmol/mg)	p	Blood glucose (mg%)
Sept.–Oct.	Control	34.1 ± 2.9 (7)				
	Glucagon	54.5 ± 13.2 (6)	<0.1%			
Nov.–Dec.	Control	18.3 ± 3.5 (5)				
	Glucagon	31.4 ± 5.3 (5)	<1.0%			
Feb.	Control	21.9 ± 1.9 (4)				
	cAMP	30.0 ± 0.8 (5)	<2.0%			
Mar.–Apr.	Control	22.1 ± 1.3 (5)	<0.1%	2.3 ± 0.1		140.6 ± 7.8
	Glucagon	45.6 ± 4.0 (5)	<0.1%	4.5 ± 0.1	<0.1%	197.0 ± 12.8
	cAMP	47.2 ± 2.2 (5)	<0.1%	5.1 ± 0.2	<0.1%	209.8 ± 13.6

Values are expressed as mean ± SD with the number of experimental animals shown in parenthesis. Probability values were determined using the Student's t-test

different substrates in this period are summarised in table 3. Except with pyruvate + malate the rates are doubled. The lesser increment of pyruvate oxidation is doubtless consequent upon the counter effect of the raised ATP, which will tend to inactivate pyruvate dehydrogenase [18].

The correlation between the ATP content of the mitochondria and the rate of citrulline synthesis seen in the latter experiments noted in table 2, was supplemented by results obtained using a glucose–hexokinase trap to lower the internal ATP concentration. These together with results for ATP content and citrulline production rates are plotted in fig.1. The citrulline rate (in $nmol.mg^{-1}.min^{-1}$) is 5–9 times the ADP content (in nmol.mg $protein^{-1}$). This relation approximates to that found by Bryła et al. [19] using external ATP applied to mitochondria with uncoupler in order to stimulate ATP entry in the presence of oligomycin to inhibit ATPase.

Although the fatty acids freed by the lipolytic action of glucagon [20,21] might have impeded nucleotide translocation [22] measurement of ADP uptake rates show that they are also increased by glucagon or cAMP treatment of the rat (table 4). This result seems to express still another feature of the hormone response which has been noted to accelerate pyruvate transfer [6,23]. Whether there may be an actual increase of physical turnover of adsorbing sites between exposure to interior and exterior is a matter for speculation.

Table 3
Effects of prior treatment of the rats with glucagon or cAMP on ADP-stimulated respiration rates

Substrate	Control		Glucagon-treated		Dibutyryl cAMP-treated	
	Rate	P/O	Rate	P/O	Rate	P/O
Pyruvate + malate	48	2.7	64	2.8	67	2.75
Glutamate + malate	46	2.7	117	2.8	110	2.8
3-Hydroxybutyrate	39	2.7	64	2.75	69	2.7
Succinate	113	1.65	188	1.7	185	1.75

Respiratory rates are expressed in ngatom $O.min^{-1}.mg$ $protein^{-1}$

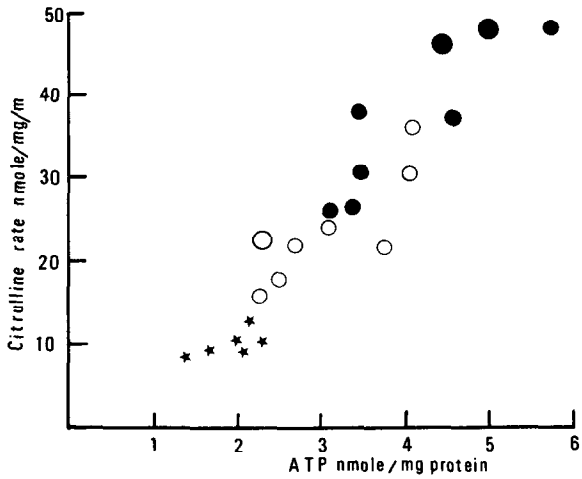

Fig.1. The relation between the rate of citrulline production and the internal ATP content of the mitochondria. The points (●) and (○) are the means from table 1, the others (○), (•) are from individual experiments, the filled-in points being from experiments with mitochondria from treated rats. Points marked as (*) are from state 3 incubations.

In view of manifold effects of glucagon [2–6,20,21] the formulation of hypotheses concerning the site of the hormonal action is very difficult. To some authors [2,24] the amino acid transport into the hepatocyte could be the first step susceptible to hormone activation. Recently, several investigators have reported glucagon [3,5,6,23] as well as cAMP stimulation [25–27] of transport processes across the mitochondrial membrane. On the basis of these reports, as well as the data presented in this paper, it seems likely that besides hormonal effect on the cellular membrane [2,24] glucagon, acting via cAMP, might also alter permeability of the mitochondrial membrane resulting in an enhanced transport of pyruvate [6,23], ions [3,5,24–26] and ADP (table 4). The latter phenomenon could cause an increase of the mitochondrial ATP/ADP ratio (tables 1 and 2) which consequently furnishes more energy for the mitochondrial steps of both gluconeogenesis and ureogenesis. An enhancement of both pyruvate carboxylation [6,28] and citrulline formation ([4] and table 2) following the administration of glucagon supports this suggestion.

Table 4
Effect of prior treatment of the rats with glucagon or dibutyryl cAMP on ADP uptake by the isolated mitochondria

Treatment	Rate (nmol.min^{-1}.mg protein^{-1})	p
Control	3.10 ± 0.03	
Glucagon	3.90 ± 0.10	<0.1%
Dibutyryl cAMP	3.95 ± 0.08	<0.1%

Values are means ± SD of experiments from 5 rats in each group. Uptakes were measured in quadruplicate at each of 5 times spaced from 5–30 s. Regression lines were calculated for each member of the group and slopes were then averaged. Probability values were determined using Student's t-test

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