GLUCAGON STIMULATION OF MITOCHONDRIAL ATPase AND POTASSIUM ION TRANSPORT

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Received 10 February 1977

1. Introduction

The hormone glucagon stimulates hepatic gluconeogenesis. Studies on the mechanism of this stimulation have centered upon those changes manifested in the hepatic mitochondria after the hormonal treatment [1–5]. Recently, acute glucagon treatment of fed, intact rats has been shown to produce a stimulation of respiration with a number of substrates in isolated, hepatic mitochondria [5]. Rates of respiration-dependent ADP phosphorylation, Ca^{2+}-uptake, and uncoupled oxygen-uptake are stimulated after the hormonal treatment. Rates of pyruvate metabolism [1–3] and citrulline formation [4] are also stimulated under the same conditions. In an attempt to determine which components of the mitochondria have been altered by the hormonal treatment, a survey of a number of mitochondrial properties is being carried out. In this report, the effect of acute glucagon treatment upon rates of uncoupler-induced ATPase activity and K^+-accumulation in intact mitochondria has been determined. Glucagon treatment has been found to cause a stimulation of both processes.

2. Experimental

Acute glucagon treatment of fed, male Wistar rats and the subsequent isolation of hepatic mitochondria were done as previously described [5]. In brief, the hormonal treatment consisted of injection of 20 μg crystalline glucagon into the tail vein of intact rats previously anesthetized with sodium pentobarbital. After 6 min the liver was quickly excised, chilled, and then homogenized in a buffered sucrose solution. Oxidative phosphorylation measurements were carried out at 30°C as described [5]. ATPase activity was monitored by measurement of proton-ejection at 21°C with a Beckman 39505 combination electrode connected to a pH-meter with attached strip-chart recorder. The medium used was that of Alexandre et al [6] and contained 5 mM Tris–Cl (pH 7.4) 50 mM KCl and 50 mM NaCl. Each experiment was initiated by adding mitochondria (approximately 1–2 mg protein) to the assay medium to yield a 2 ml total vol. After a 1 min preincubation period to allow temperature equilibration and oxidation of endogenous substrates, ATP at a final concentration of 1 mM was added. FCCP at a final concentration of 0.5 μM was added 1 min later, and rates of proton-ejection into the medium were measured. Absolute changes in hydrogen ion concentration were calibrated by internal standardization with HCl and NaOH additions. In the experiments utilizing additions of organic acids and respiratory-chain inhibitors, these compounds were added to the medium before addition of mitochondria. In some experiments, ATPase activity was assayed in parallel by following phosphate-liberation. The micromethod of Itaya and Ui [7] was utilized in these experiments. The validity of the pH-method for assaying ATPase activity has been discussed by Gear [8].

Rates of K^+-uptake were measured at 20°C with a Beckman 39660 combination cation-electrode. The medium used was patterned after Höfer and Pressman [9] and contained 20 mM Tris–Cl, 250 mM sucrose, 2.5 mM phosphate (added as H_3PO_4), 3 mM L-

Abbreviation: FCCP Carbonyl cyanide *p*-trifluoromethoxy-phenyl-hydrazone

Table 1
Effect of glucagon treatment on initial rates of ATPase activity in intact mitochondria

Treatment	ATPase activity (ng-ion H^+/min/mg)	Succinate oxidation (ng-atom 0/min/mg)
Control (17)	436 ± 21	144 ± 5
	$p < 0.001$	$p < 0.001$
Glucagon (17)	527 ± 24	234 ± 6

ATPase activity was monitored using a pH-method as described in Experimental section. In two of these experiments, phosphate-liberation was also measured during the first minute of the reaction. The ratio of H^+-ejected to phosphate-released was 0.99 ± 0.086 for control mitochondria and 1.04 ± 0.06 for glucagon mitochondria. Data are expressed as mean ± SE with the number of experimental animals shown in parentheses. Probability values were determined using the paired Student *t*-test.

glutamate and 3 mM L-malate. The assay medium was titrated to pH 7.2 with HCl. For the assay, the medium was placed in a thermostatted beaker and standard KCl solution was added to achieve a final K^+-concentration of 2 mM. Mitochondria (approximately 5 mg) were then added to yield a 2 ml final vol. After a 3 min preincubation period, potassium uptake was initiated by the addition of valinomycin (3 μg/mg protein). The cation-electrode was connected to a Keithley Model 610C electrometer to which was attached a strip-chart recorder. Changes in potassium ion concentration were calibrated by internal standardization using additions of KCl solutions of known concentration. In the measurements of ATP-dependent K^+-accumulation, either rotenone (8.8 μM) or antimycin (1.6 μg/mg protein) was added to block substrate respiration and ATP at a final concentration of 1 mM was used to drive K^+-accumulation.

3. Results

The data of table 1 show that glucagon treatment of animals induces a significant increase in the ATPase activity of isolated mitochondria. The results obtained using the pH-method were also verified by measurements of inorganic phosphate released. Comparison of the data collected by the two methods showed that the stoichiometry of protons released to phosphate-liberated was not altered by the hormonal treatment. Data showing the stimulation of succinate oxidation in these mitochondria are also included to indicate the presence of a defined hormonal effect.

In table 2 are shown the effects of organic acids and respiration inhibitors upon the ATPase activity

Table 2
Effect of substrates and respiratory inhibitors on ATPase activity of isolated mitochondria

Additions	Control	Glucagon
None	100	100
β-Hydroxybutyrate[a]	23 ± 2	24 ± 7
Acetoacetate	97 ± 5	106 ± 11
Pyruvate	101 ± 9	113 ± 10
Succinate	63 ± 8	63 ± 5
Succinate + Rotenone	62 ± 7	70 ± 7
Rotenone	90 ± 7	117 ± 6
Antimycin (1.3 μg/mg)	28 ± 12	44 ± 11
Antimycin (3.3 μg/mg)	4 ± 4	19 ± 8
Oligomycin[a]	11 ± 4	13 ± 4

[a] Indicates ATPase measurements carried out utilizing phosphate-release assay.

ATPase activity was measured utilizing the pH-method except where noted. Substrates and inhibitors were added at the beginning of the preincubation period of the assay at the following concentrations: β-hydroxybutyrate 2.5 mM, pyruvate 2.5 mM, acetoacetate 2.5 mM, succinate 2.5 mM, antimycin 1.3 μg/mg mitochondrial protein and 3.3 μg/mg mitochondrial protein, oligomycin 1.0 μg/mg mitochondrial protein and rotenone 1.8 μM. Data are presented as percentage of rate in the absence of any additions. The actual rates of ATPase activity were 404 ± 28 nmol/min/mg and 530 ± 64 nmol/min/mg for control and glucagon mitochondria respectively ($n = 4$).

of control and glucagon mitochondria. The addition of β-hydroxybutyrate was found to severely inhibit ATPase activity in both control and glucagon mitochondria. The presence of β-hydroxybutyrate also altered the stoichiometry of H^+-released to phosphate-liberated, a ratio of 0.45 being found for both control and glucagon mitochondria.

Inhibition of ATPase activity by β-hydroxybutyrate might be hypothesized to be exerted by reduction of NAD or proximal components of the respiratory-chain [6]. However, this inhibitory effect would appear to be specific to β-hydroxybutyrate since another NAD-linked substrate, pyruvate, did not exhibit any effect. Further, the addition of acetoacetate in order to oxidize the NAD pool of the mitochondria showed no effect.

Addition of succinate was found to cause a 37% inhibition of ATPase activity. Inclusion of rotenone with the succinate did not alter the inhibition. However, when rotenone was added independently, it was found to cause a slight inhibition of control mitochondria whereas glucagon mitochondria were somewhat stimulated.

Antimycin addition at two different concentrations was found to cause a profound inhibition of ATPase activity. Mitochondria isolated from control animals appeared to be more sensitive to this inhibitory action. The inhibitory effect of antimycin may be related to an interaction with ATPase more direct than binding to cytochrome *b* since the concentrations of antimycin required for inhibition of ATPase were found to be approximately 10-times those required to give complete inhibition of succinate-dependent respiration in control and glucagon mitochondria under similar assay conditions (data not shown; see also ref. [10]). The fact that oligomycin inhibited ATPase activity in both control and glucagon mitochondria indicated that the activity measured in both cases was indeed due to the mitochondria.

The data of table 3 indicate that glucagon treatment causes a stimulation of the rate of K^+-accumulation in isolated hepatic mitochondria. This hormonal effect is seen whether the data are analyzed using initial rates or by fitting to a first-order process. In these experiments, a mixture of malate and glutamate was used as substrate to provide respiratory-energy for the ion-accumulation. The finding of an hormonal stimulation of this respiration-dependent process in the mitochondria is consistent with the previous finding of the stimulation of the process of oxidative phosphorylation after glucagon treatment [5]. In terms of the total amount of K^+ taken up by the mitochondria as measured after approximately 1 min when net K^+- transport had ceased, a slight increase in accumulation was noted after the hormonal treatment, but the difference from the control-value was not found to be statistically significant.

Glucagon treatment also stimulates ATP-dependent K^+-accumulation as shown in table 4. Comparison of the means derived for the first-order rate constants show a significant difference. The means for the initial rates of K^+-uptake were higher after glucagon treatment, but the difference was not statistically significant due to the larger variance of the data. Derivation of the first-order rate constant, by using a large number of experimental points, reduces the

Table 3
Effect of glucagon treatment on rates of K^+-uptake and total K^+-accumulation by isolated, hepatic mitochondria

Parameter	Control	*p*-Value	Glucagon
Initial rate (nmol/s/mg)	15.4 ± 1.9 (7)	0.0003	21.4 ± 2.2 (10)
k (10^3/s/mg)	1.88 ± 0.16 (7)	0.007	2.99 ± 0.16 (10)
Total uptake (nmol/mg)	259 ± 17 (7)	0.2	296 ± 19 (10)

Initial rates of K^+-accumulation were estimated in two different ways. Initial rates (nmol/s/mg) were read directly from the charts. The data were also analyzed assuming a first-order process and rate constants were derived from the data collected during the first 30 s. The rate constant k has units/s/mg. The oxidation of malate–glutamate provided the energy for ion-accumulation in these experiments. Probability values were derived utilizing the unpaired Student *t*-distribution.

Table 4
Effect of glucagon treatment on rates of ATP-dependent K^+-uptake

Parameter	Control	*p*-Value	Glucagon
Rotenone as inhibitor			
Initial rate (nmol/s/mg)	10.0 ± 1.1 (6)	0.06	14.7 ± 1.8 (8)
k (10^3/s/mg)	1.06 ± 0.19 (6)	0.03	1.58 ± 0.11 (8)
Total uptake (nmol/mg)	202 ± 9 (6)	0.2	338 ± 23 (8)
Antimycin as inhibitor			
Initial rate (nmol/s/mg)	6.71 ± 0.78 (5)	0.13	9.32 ± 1.26 (6)
k (10^4/s/mg)	5.18 ± 0.55 (5)	0.03	7.56 ± 0.66 (6)
Total uptake (nmol/mg)	93 ± 11 (5)	0.4	109 ± 15 (6)

Rates of K^+-accumulation in isolated mitochondria were estimated directly from charts (initial rate) and by fitting the data to a first-order equation (rate constant, *k*). Either rotenone (8.8 μM) or antimycin (1.6 μg protein) was added to the standard assay medium to prevent respiration and ATP at a final concentration of 1 mM was added as an energy source.

variation in reproducibility within each experiment.

Comparison of the rates of ATP-dependent K^+-accumulation with rotenone and antimycin as respiratory inhibitors indicate that rotenone supports higher rates of ion-accumulation. These data are in agreement with the data of table 1 on FCCP-induced ATPase activity showing an inhibition of ATPase activity with antimycin.

Since an increase in the rate of ATP-dependent K^+-accumulation should exhibit a concomitant increase in the rate of ATP-utilization, experiments on the effect of glucagon treatment upon valinomycin-induced ATPase activity were carried out. In data submitted for publication (Yamazaki, R. K. and Sax, R. D., (1977) an hormonal stimulation of the valinomycin-induced ATPase activity was observed. In spite of somewhat different assay conditions, glucagon treatment produced a 47% stimulation increase in the ATPase activity. This stimulation corresponds very well to the 47% stimulation of K^+-uptake seen here.

4. Discussion

An earlier investigation [5] as to the site of mitochondrial stimulation by glucagon had inferred that the immediate steps of ATP-formation probably were not involved. This conclusion was based on indirect data showing that ATP-dependent Ca^{2+}-transport was not affected by the hormonal treatment. The observation that uncoupled respiration is stimulated by hormone was also taken as indirect evidence for a lack of effect on the phosphorylating system since these steps would presumably be bypassed in an uncoupled system.

The finding of an hormonal stimulation of FCCP-induced ATPase activity adds a degree of complexity to the formulation of hypotheses concerning the site of the hormonal effect. It would appear that an hormonal stimulation of the adenine nucleotide translocase might be ruled out since Mitchell and Moyle [11] have presented evidence that translocase activity is not limiting in ATPase measurements of this type. One difference in the experimental conditions used by Mitchell and Moyle may be noted. Valinomycin was present in their experiments to collapse the membrane potential. In data not shown in this paper (Yamazaki and Sax, submitted for publication) valinomycin has been used to stimulate ATPase activity. Rates of proton-expulsion using valinomycin were found to be very similar to those measured when ATPase activity was induced by FCCP.

The results reported in this paper concerning a stimulation of ATPase activity and ATP-dependent K^+-accumulation appear to be at variance with the earlier report of a lack of stimulation of ATP-dependent Ca^{2+}-uptake [5]. In the study of ATP-dependent Ca^{2+}-transport, rotenone and antimycin were added to the mitochondria to prevent respiration. Since antimycin strongly inhibits ATPase activity, the presence of an hormonal effect on Ca^{2+}-transport may have been obscured in the earlier report.

The finding that substrates and respiratory-chain inhibitors affect ATPase activity and ATP-dependent K^+-accumulation implies a functional interaction between the ATPase and the respiratory-chain. Thus a primary effect of glucagon upon a component of the respiratory-chain might well exert an effect upon ATPase activity. The difference in the sensitivity of control and glucagon mitochondria to inhibition by antimycin suggests a possible hormonal effect in the region of Complex III of the mitochondria.

The data in this communication have been expressed as rates/mg protein in the mitochondrial fraction. If glucagon treatment caused less non-mitochondrial contaminating protein to be isolated in the mitochondrial fraction after differential centrifugation, a systematic error resulting in an apparent hormonal stimulation of mitochondrial function would be produced. Previously published data [5] indicate that such is not the case. Rates of ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine oxidation (States 3, 4, and uncoupled) are not affected by the hormonal treatment. Likewise, the specific activity of NAD-malate dehydrogenase in mitochondria after lysis is not affected by glucagon treatment. Finally, the fact that the respiratory control index is increased by glucagon treatment also gives assurance that the changes lie in the mitochondria themselves since the respiratory control index is independent of the amount of mitochondria and protein in the assay.

Titheradge and Coore [12] have recently demonstrated that the pH-gradient across the mitochondrial membrane is increased by acute glucagon treatment. These authors also confirmed the previously reported findings [5] that glucagon treatment causes a stimulation of State 3 respiration with pyruvate–malate but not with ascorbate-*N,N,N',N'*-tetramethyl-*p*-phenylenediamine as substrate. The finding of an increase in the transmembrane pH-gradient, taken together with the data reported previously [5] and in this paper, indicate that acute glucagon treatment affects the fundamental reactions of mitochondrial energy metabolism. Glucagon treatment, therefore, may provide a powerful tool in investigations of the primary reactions of oxidative phosphorylation.

Acknowledgements

This work was supported by grants from the United States Public Health Service, AM 17849 and 5T32, GM 07055-02. The competent and enthusiastic technical assistance of Peggy Aggson and Donna Mickey is gratefully acknowledged. We thank Eleanor Cassada for her capable assistance in the preparation of this manuscript.

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