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RAPID ACTION OF GLUCAGON ON HEPATIC MITOCHONDRIAL CALCIUM METABOLISM AND RESPIRATORY RATES

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Summary

The time course for the effects of acute, in vivo glucagon treatment on energy-linked functions of isolated hepatic mitochondria has been studied. After 1 min of glucagon treatment, two changes are observed in mitochondrial function. State 4 (nonphosphorylating) respiratory rates with L-glutamate as substrate are decreased. No significant change is observed in the State 4 respiratory rates with succinate as substrate at 1 min of treatment. Concurrent with the change in nonphosphorylating respiratory rates is a decrease in the half time of spontaneous calcium release from mitochondria preloaded with calcium in a phosphate-containing medium.

After 2—4 min of treatment, the previously reported stimulations in rates of State 3 (phosphorylating) respiration and calcium influx into mitochondria are observed. After approximately 6 min of treatment, these changes have reached their maxima. The combined effects of increased calcium uptake rate and decreased calcium efflux rate leads to a decrease in the calcium cycling rate of mitochondria. This decrease in the cycling rate should lead to the increased efficiency of mitochondrial energy transduction and may be responsible, in part, for the increased functional capability of mitochondria isolated from glucagon-treated animals. A correlate of the reduced cycling rate is a decrease in the steady-state concentration at which the mitochondria can buffer the calcium concentration of the incubation medium. The changes observed in calcium efflux rates and respiratory rates exhibit a time course consistent with

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Abbreviations used: Tes, *N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid; Mops, morpholinoethanesulfonic acid.

possible intermediates in the glucagon-induced stimulation of hepatic gluconeogenesis.

Introduction

The pancreatic hormone glucagon stimulates hepatic gluconeogenesis. Studies on hepatic mitochondria isolated from animals treated acutely with glucagon have revealed a number of stable changes induced by the hormonal treatment. The primary effect reported to data is a stimulation of the flux rate in the electron transport chain [1—4]. The increased activity of the respiratory chain leads to an increase in the steady-state transmembrane pH gradient [2,5] and membrane potential [5]. The increase in the transmembrane pH gradient is probably responsible for the observed stimulations of pyruvate transport [6] and phosphate transport [7] since both of these species are transported by exchange with hydroxyl ion and thus would be expected to be sensitive to changes in transmembrane pH. Similarly, the increase in the membrane potential is probably responsible for the increased content of Mg^{2+} [5,9] and K^{+} [5] in mitochondria from glucagon-treated animals. The possibility that increased electron transport chain flux is secondary to increased rates of substrate entry has been ruled out by the observation that stimulated rates of respiration are obtained in submitochondrial particles which no longer have a substrate entry requirement due to their inverted configuration [10].
increase in the steady-state ATP/ADP ratio [4,11] leads to increased rates of pyruvate carboxylation [12] and citrulline formation [13] by isolated mitochondria. Since these increased rates of the initial steps of gluconeogenesis and ureogenesis are manifested over 6 min of glucagon treatment, the activation of the mitochondria is a candidate for one of the primary events in the stimulation by glucagon of these two processes. Glucagon stimulates glucose production from lacate in hepatocytes within a period of minutes, and the rate of mitochondrial pyruvate carboxylation has been shown to be well correlated timewise with the increase in rates of glucose production [14]. The mitochondrial stimulation by glucagon appears to be mediated by the effects of increased intracellular cyclic AMP levels as indicated by the observation that treatment of isolated hepatocytes with cyclic AMP elicits a mitochondrial stimulation just as does treatment with glucagon [14].

Hughes and Barritt [15,16] and Prpic et al. [17] have recently reported that glucagon treatment of animals causes an enhancement of the retention time for accumulated calcium in isolated hepatic mitochondria. This effect has been characterized by both groups as a process completely blocked by inhibitors of protein synthesis and relatively slow in onset (15—60 min). Since the glucagon effects on mitochondria previously reported by Haynes [9], Garrison and Haynes [14], and our laboratory [1] were seen within a period of minutes of treatment (a time course probably incompatible with a requirement for de novo protein synthesis), this study of the time course for acute glucagon effects on hepatic mitochondrial function including early effects on calcium accumulation and release half time was undertaken. As detailed in this communication, alterations of hepatic mitochondrial function including half time for calcium release are observable within 1 min of treatment with glucagon.

Materials and Methods

Acute glucagon treatment of fed, male Wistar rats and the subsequent isolation of hepatic mitochondria were carried out as previously described [1] except that EDTA was omitted from the isolation medium. 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. Control animals were anesthetized and treated with vehicle alone for the corresponding period of time. Highly excited animals do not respond well to these acute glucagon treatments. For this reason, it is important to keep the animals calm during the initial handling and administration of anesthetic. After induction of anesthesia, animals were allowed to sleep for a period of at least 15 min prior to treatment to lessen the influence of the handling. After various durations of hormonal or vehicle treatment as shown in the figures, the livers were excised, chilled and then homogenized in a solution containing 0.3 M sucrose and 5 mM Tes (adjusted to pH 7.4 with KOH). Oxidative phosphorylation measurements were carried out at 25°C using the protocol previously described [1] except that $MgCl_2$ and EGTA were omitted from the assay medium. Thus the medium used was identical to that used for the calcium flux studies as described below.

Cytochrome levels were determined in mitochondrial samples by the method of Williams [18]. Spectra of the deoxycholate dispersed mitochondria were taken with either a Hitachi-Perkin Elmer 256 or Aminco DW-2a dual wavelength spectrophotometer.

Measurements of calcium fluxes and total mitochondrial calcium content were carried out as previously described [19]. The method makes use of a Radiometer Selectrode F2112Ca calcium ion specific electrode and a laboratory pH meter. Techniques for the calibration and the electrode are discussed in the cited reference. The medium in these experiments consisted of 80 mM KCl, 5 mM K_2HPO_4 and 50 mM Mops (all adjusted to pH 7.0 with KOH). In most of the experiments, succinate was included as an energy source for the mitochondria and was added as the disodium or dipotassium salt at a final concentration of 12.5 mM. The temperature for these incubations was maintained at 25°C with the use of a jacketed vessel attached to a circulating water bath. Stirring was accomplished with a small magnetic stirring bar.

Proton permeability measurements were carried out at 25°C with a Beckman 4500 pH meter equipped with a Beckman combination electrode following the method of Mitchell and Moyle [20]. The data were collected digitally using a Beckman AutoPro Intercoupler (analog-to-digital converter) with a sampling rate of 1 Hz. The data were then fitted to straight line segments using a Hewlett-Packard 2000 Access time-sharing computer.

All animals treated with glucagon were paired with controls similar in weight and age but treated for the same time period with vehicle alone. These pairings were used in the paired *t* analysis. The data, unless otherwise noted, are expressed as means ± S.E.M. with the number of experiments shown in parentheses.

ADP, EGTA, rotenone, glucagon, and ruthenium red were obtained from Sigma Chemical Company, St. Louis, MO. Disodium succinate, Mops and Tes

were obtained from Calbiochem-Behring, La Jolla, CA. All other chemicals were reagent grade from Fisher Scientific Company, Pittsburgh, PA.

Results

Lack of effect of glucagon treatment on mitochondrial cytochrome levels

Previous work demonstrated that acute glucagon treatment of intact animals causes a stimulation of hepatic mitochondrial function but does not cause a change in the specific activities of two mitochondrial matrix enzymes, pyruvate carboxylase [21] and NAD-malate dehydrogenase [1]. To establish further that the hormonal changes were not arising from changes in mitochondrial specific activity, cytochrome levels in mitochondria isolated from control and glucagon-treated animals were determined. As shown in Table I, glucagon treatment produces no change in the levels of cytochromes *a*, *b*, c_1, and *c* of the isolated mitochondria.

Measurements of calcium fluxes

In Fig. 1 is shown the effect of various durations of glucagon treatment on the time course of calcium metabolism by hepatic mitochondria. Because the calcium electrode used for these measurements yields a signal proportional to the logarithm of the free calcium concentration, the ordinate for the figure corresponds to log calcium concentration. The numbers immediately below the traces are the calculated free calcium concentrations in the assay medium. The times for glucagon treatment refer to the period between injection of the intact animal with hormone and the removal and chilling of the liver prior to homogenization. The top curve shows that, after the addition of mitochondria isolated from control animals, the calcium concentration in the medium drops until a low steady state is achieved. This steady state is determined by the balance between ongoing influx and efflux as previously discussed [19]. Subsequently, all of the calcium taken up plus the internal store is released to the phosphate-containing medium. The steady state nature of the trough is more easily observable with a medium lacking permeant anions since the osmotic swelling associated with the uptake of permeant anions and release of calcium is avoided (Khazan, U. and Yamazaki, R.K., (1979), unpublished results).

With increasing duration of hormonal treatment as depicted in the lower

TABLE I

CYTOCHROME LEVELS IN MITOCHONDRIA ISOLATED FROM CONTROL AND GLUCAGON-TREATED RATS

Treated animals were injected with 20 μg glucagon 6 min before sacrifice and removal of the liver. Mitochondria were isolated by differential centrifugation. Cytochrome levels were described in Methods. The data are expressed as the mean ± S.E.M. with the number of determination shown in parentheses.

Treatment	Cytochrome content (pmol/mg protein)			
	a	*b*	c_1	*c*
Control (7)	317 ± 11	223 ± 25	127 ± 25	234 ± 8
Glucagon (7)	313 ± 11	245 ± 25	138 ± 13	241 ± 10

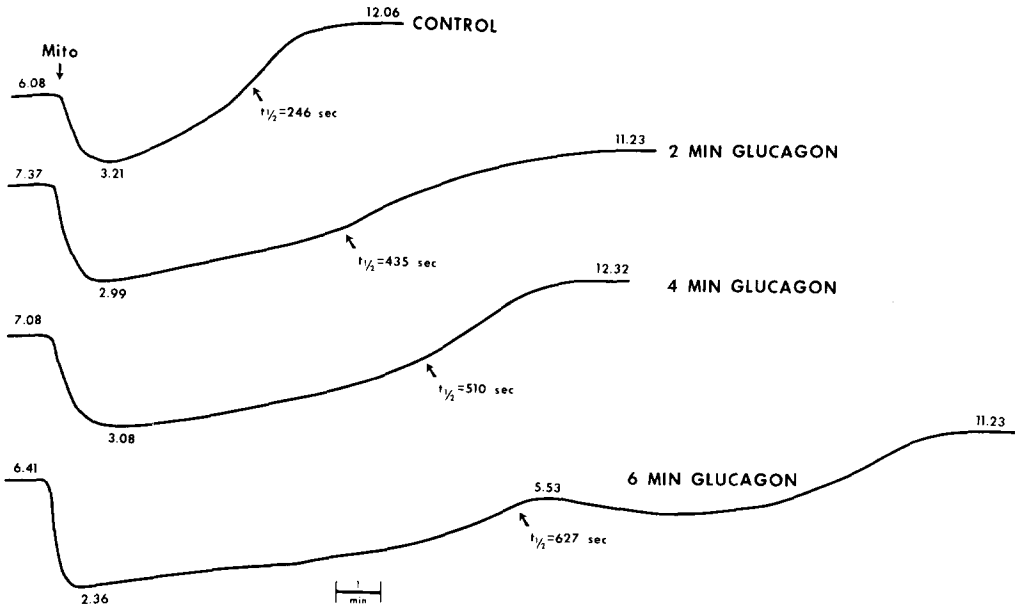

Fig. 1. Effect of various durations of glucagon treatment on hepatic mitochondrial calcium metabolism. Animals were treated in vivo as described in Methods. The times indicated for glucagon treatment refer to the time from injection of glucagon until the animal was sacrificed and the liver was chilled. The explanation for the arrows indicating the $t_{1/2}$ is given in the text. Free (ionized) calcium concentrations were determined using a calcium electrode as explained in Methods.

traces of Fig. 1, the first order rate constant for calcium uptake is increased. This uptake rate constant is determined from the initial straight line portion of the uptake as described previously [19]. More prominent changes are to be found in the release process. Glucagon treatment causes a decrease in the rate of calcium efflux. Because of the complexity of the release process, the data for release have been expressed as a half time for release. This value is calculated by determining the time required for half maximal release as shown by the arrows in Fig. 1. With the 6 min treatment sample shown, a biphasic release was observed. The first plateau was completed with the release of about 8—10 nmol Ca^{2+} per mg mitochondrial protein. This point is elaborated further under Discussion.

In these experiments, the total amount of calcium released represents that due to uptake from the assay medium plus the internal store of calcium isolated within the mitochondria. Thus, the difference between the total calcium in the medium after complete release and the total calcium before the addition of mitochondria represents the intramitochondrial store of calcium (see Ref. 19 for details). For example, in the 6 min glucagon sample of Fig. 1, this difference would be (11.23 μM—6.41 μM) or 4.82 μM. In the assay volume of 4.0 ml, the change in concentration corresponds to 19.28 nmol free calcium. After multiplying by the factor 1.50 to correct for binding to 5.0 mM phosphate and 12.5 mM succinate and dividing by the amount of mitochondrial protein in the assay, the intramitochondrial store of calcium for this sample is

found to be 13.1 nmol Ca^{2+} per mg mitochondrial protein. As discussed previously [19], the values for the intramitochondrial stores of calcium determined using the calcium electrode agree closely with those values determined by other methods such as atomic absorption.

Measurements of calcium release

With any given preparation of mitochondria, the time required for half maximal release ($t_{1/2}$) is a function of the calcium load within the mitochondrial. The load is calculated in a fashion similar to that of the intramitochondrial store of calcium except that the difference between the final total medium concentration and the minimum or trough concentration is used. Again the total amount of calcium calculated is multiplied by 1.50 to correct for binding to anions. The rationale for using the calcium load is as follows. Release is a process associated with swelling caused by the combination of phosphate and calcium in the mitochondrial matrix. The initial event is calcium entry followed by phosphate entry as evidenced by the observation that calcium uptake is completed before the maximal rate of osmotic swelling as measured by light scattering changes is achieved (Khazan, U. and Yamazaki, R.K., (1979) unpublished results). Since this release process is triggered by the presence of calcium within the mitochondria, the total amount of calcium due to uptake plus the endogenous level is used. The $t_{1/2}$ for release is a linear function of the calcium load. In order to compare various samples of mitochondria, the value of $t_{1/2}$ extrapolated to zero calcium load is used, since different preparations of mito-

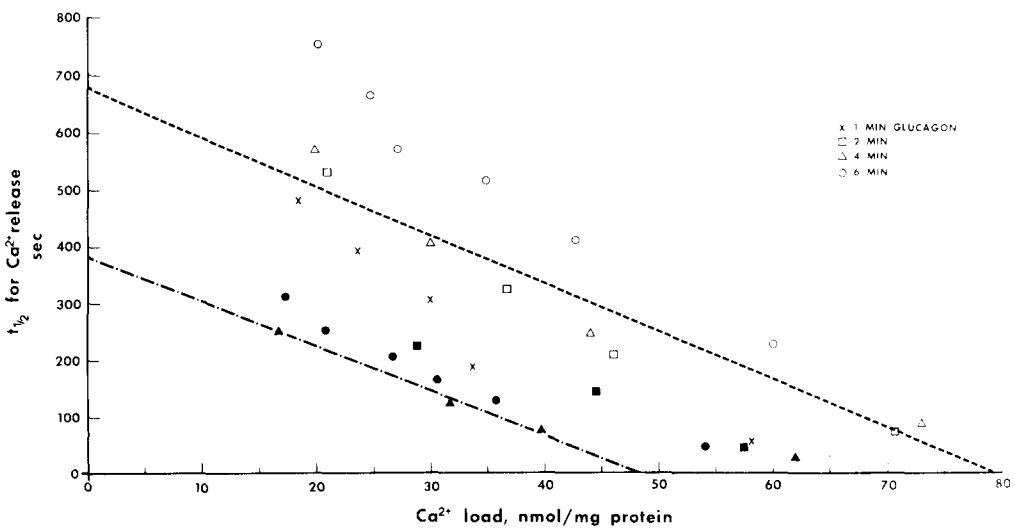

Fig. 2. Half time for calcium release from mitochondria as a function of the calcium load within the mitochondria. Treatments were carried out as described in Methods. The methods for calculation of the calcium load and $t_{1/2}$ are described in the text. The open symbols show data collected from various treatment times as indicated in the symbol legend in the figure. The solid symbols show the data for the corresponding control animals treated with vehicle only for the same duration of time. The symbol X shows data for a 1-min treatment period with glucagon. The data shown are representative samples of the total data, the remainder of which was omitted for clarity. For the control samples, there were a total of 8 experiments. For each of the glucagon treatment times, three to five experiments were carried out.

chondria may have different amounts of internal stores present after isolation.

An example of some data treated in this manner is shown in Fig. 2. In this figure, it may be observed that glucagon treatment causes an increase in the $t_{1/2}$ for release at any given calcium load. The dotted lines show the least squares fit to the data for the 3-min control and glucagon treatment samples. The slopes of the fitted lines do not show any significant differences for the pooled data.

Time course for glucagon action

An analysis of the time course for the various parameters of mitochondrial function is shown in Fig. 3. In these experiments, glucagon has been injected into the tail vein of anesthetized animals, and the livers have been excised, chilled and homogenized after the various durations of treatment as indicated on the abscissa. Control animals were anesthetized and treated with vehicle only for the same period of time prior to sacrifice. No differences with respect to time were seen in the control animals. After 1 min of glucagon treatment,

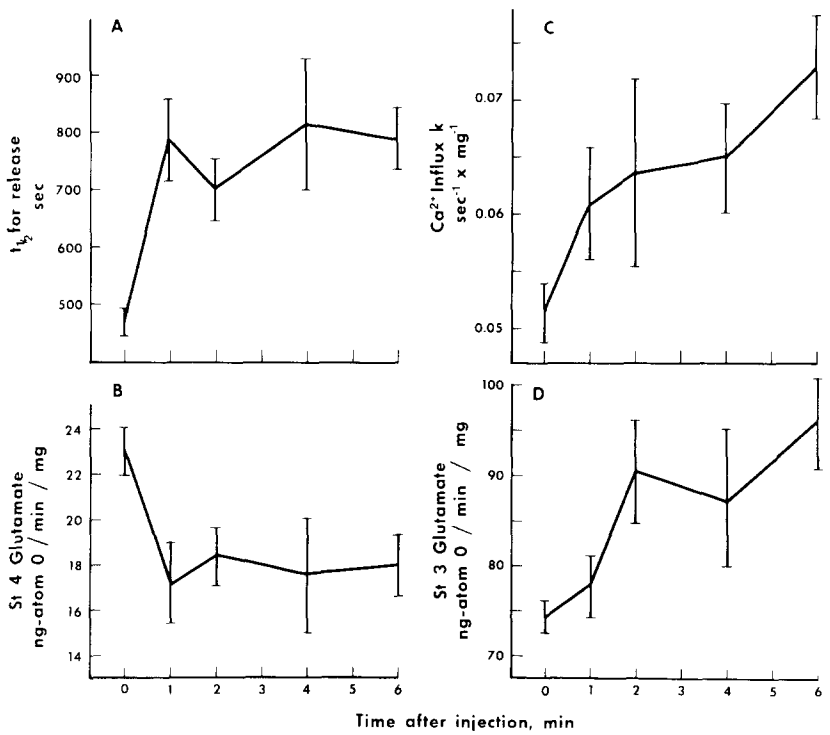

Fig. 3 Effect of duration of glucagon treatment on mitochondrial function. Treatments were carried out as in Figs. 1 and 2. Panel A displays the effect of duration of glucagon treatment on the half time for calcium release from mitochondria. This half time is extrapolated to zero calcium load as shown in Fig. 2 and explained in the text. Panel B shows the State 4 (nonphosphorylating) respiratory rate with glutamate as substrate. Panel C shows the first order rate constant for calcium influx; and Panel D displays the State 3 (phosphorylating) respiratory rate with L-glutamate as substrate. Each time point is shown as a mean ± S.E.M. for the following number of observations: 0 time or control, $n = 8$; 1 min, $n = 4$; 2 min, $n = 4$; 4 min, $n = 3$; 6 min, $n = 5$.

the values of the $t_{1/2}$ for release (extrapolated to zero calcium load) and State 4 (nonphosphorylating) respiration have reached their maximum and minimum values respectively. The apparent rate constant for calcium uptake shows a biphasic behavior. The increase in State 3 (phosphorylating) respiration takes place after a lag with respect to the other changes and does not reach its maximum until 4—6 min of treatment. The observed changes in the mitochondrial function are not due to the effects of molecular glucagon isolated with the mitochondria as evidenced by the fact that no changes in mitochondrial function are observed when glucagon is injected directly into the portal circulation immediately before removal of the liver.

Panel B of Fig. 3 indicates that glucagon treatment causes a decrease in the State 4 or nonphosphorylating respiratory rate. Since State 4 respiratory rate should be determined by the sum of intrinsic ATPase activity and the rate of leakage of protons from the medium into the matrix, it was of interest to determine if glucagon treatment causes a change in the proton permeability of the mitochondrial membrane. The data of Table II indicate that no difference in the proton permeability of the mitochondrial membranes is seen after glucagon treatment. These data must be viewed with caution, however, since the conditions of the mitochondrial isolation were different. Because of the need to quantify small pH changes in these measurements of membrane proton permeability, no buffer was included in the mitochondrial isolation medium. This lack of buffering capacity during the isolation may induce differences in the functional properties of the mitochondria as evidenced by the observation that no hormonally-induced changes are seen in the State 4 respiratory rates after mitochondrial isolation without buffer (Table II) whereas a significant decrease in State 4 respiratory rates was noted after mitochondrial isolation with buffer present (Fig. 3). These differences are presently being investigated.

The respiratory rates shown in Fig. 3 are those data obtained using the NAD-linked substrate L-glutamate. The data obtained using succinate as substrate were similar in character except that the decrease in State 4 respiratory rates

TABLE II

PROTON PERMEABILITY OF MITOCHONDRIAL MEMBRANES FROM CONTROL AND GLUCAGON-TREATED ANIMALS

Treatments of animals were performed as described in Methods. Mitochondria were isolated in 0.3 M sucrose adjusted to pH 7.0 but without buffer present. Proton permeability measurements were carried out by the method of Mitchell and Moyle [20]. In these experiments, a small quantity of acid is added to a mitochondrial suspension and the decay curve for the resultant transmembrane pH difference is recorded. In our experiments, two phases of proton relaxation were observed. These are designated as the early and late phases below. The half time ($t_{1/2}$) for decay is used as an estimate of the proton permeability. Data for respiratory rates with glutamate as the substrate are included to show that State 3 (phosphorylating) respiratory rates were affected by glucagon treatment. The data are expressed as mean ±S.E.M. with the number of observations shown in parentheses.

Treatment	$t_{1/2}$ for decay(s)		Respiration rate (ngatom O/min/mg protein)	
	early	late	State 3	State 4
Control (3)	21.5 ± 1.4	103 ± 2	97.5 ± 9.6	19.9 ± 1.7
Glucagon (3)	21.3 ± 0.7	103 ± 3	124 ± 11	18.1 ± 2.1

was not statistically significant. The data for the State 3 respiratory rates (expressed as ngatom O per min per mg protein) are as follows: 134 ± 4 for the pooled controls; 136 ± 14 for the 1-min glucagon treatment; 159 ± 8, 2-min glucagon treatment; 168 ± 19, 4-min glucagon; and 180 ± 9 for the 6-min glucagon treatment. For the State 4 respiratory rates (expressed as ngatom O per min per mg protein), the values are as follows: 40.3 ± 1.5 for the pooled controls; 39.2 ± 1.4 for the 1-min glucagon treatment; 37.4 ± 1.5 for 2 min; 39.0 ± 3.4, 4 min; and 38.4 ± 1.2 for 6-min glucagon treatment.

The reason for production of a significant decrease in State 4 respiratory rates with L-glutamate without a corresponding decrease in the rate with succinate is not clear. It is a general observation, however, that the respiratory control ratio (State 3 rate/State 4 rate) is higher with NAD-linked substrates than with succinate. Although the State 3 rates obtained with succinate are higher, the State 4 rates show a proportionally larger increase so that the ratio is decreased with succinate. If the effect of glucagon treatment is to alter proton permeability either directly or through a reduction in the rate of calcium cycling, State 4 respiratory rates with NAD-linked substrates may be affected to a proportionally greater extent since the oxidation of these substrates is more tightly and coupled and therefore probably more sensitive to slight changes in the membrane permeability.

Calcium content of mitochondria

In the experiments examining the time course of glucagon action on mitochondrial calcium flux rates, the intramitochondrial calcium content was also estimated using the calcium electrode technique as discussed above. Control animals were found to have an intramitochondrial calcium content of 12.7 ± 0.9 nmol per mg mitochondrial protein (n = 8). After 1 min of treatment, this value fell slightly to 12.2 ± 0.4 nmol per mg (n = 3) and then rose until it reached 14.1 ± 0.5 nmol per mg (n = 5) at 6 min of treatment.

Discussion

As outlined in the Introduction, a number of mitochondrial functions have been demonstrated to be altered by glucagon treatment of intact animals before removal of the liver and subcellular fractionation. These changes lie in the mitochondria themselves rather than the levels or specific activity of the mitochondria isolated as evidenced by the lack of effect of glucagon treatment upon matrix enzyme specific activity [1] or the cytochrome levels as reported in this communication. As discussed previously [8], perhaps most convincing in this respect is the increase in respiratory control ratio produced by glucagon treatment since the respiratory control ratio is independent of the amount of protein present in the assay.

In this communication it is demonstrated that acute glucagon treatment causes changes in mitochondrial function which are evident after 1 min of hormonal exposure. The first observable changes are a decrease in the non-phosphorylating rate of respiration (State 4) and an increase in the half time for calcium release after accumulation in the mitochondria. This change in the t_{1_2} for calcium release reaches its maximum value in the first minute of

glucagon treatment. Thus, the time course observed here is considerably more rapid than that reported by Hughes and Barritt [15] who indicated the maximal prolongation of the calcium retention time took 1 h of glucagon treatment. The same authors also reported that the actions of glucagon on calcium retention time under their experimental conditions were completely abolished by cycloheximide, an inhibitor of protein synthesis. Although such experiments were not carried out in our study, the relatively rapid onset of the observed effect (maximal within 1 min) makes it unlikely that de novo protein synthesis is required.

In order for an event such as changes in the mitochondrial calcium release rates to be considered as contributing to the physiological actions of glucagon, a time course for the event consistent with the time course of the glucagon actions must be obtained. It is known that exposure of hepatocytes to glucagon causes a rapid (within 5 min) stimulation of gluconeogenesis from lactate [14] and an equally rapid stimulation of rates of mitochondrial pyruvate carboxylation [14]. The changes in half time for calcium release and rates of respiration reported here are rapid enough to be considered as primary events in the stimulation of hepatic gluconeogenesis by glucagon. The longer time course and inhibition by protein synthesis inhibitors reported by Hughes and Barritt [15] and Prpic et al. [17] are more suggestive of a trophic action of glucagon on the liver.

Although this report and that of Hughes and Barritt [15] both find glucagon treatment to increase the time that accumulated calcium can be retained by mitochondria, the data are not directly comparable in a quantitative sense because of differences in the experimental conditions and the definitions of the half times for calcium release from mitochondria. Hughes and Barritt [15] used a sucrose-based incubation medium which contained 1 mM phosphate at pH 7.4. In an attempt to simulate cellular conditions, we have used a high salt (80 mM KCl) medium with 5 mM phosphate at pH 7.0. Also, the values reported in this paper for $t_{1/2}$ of calcium release were calculated by extrapolation to zero intramitochondrial calcium levels in order to correct for differences in the endogenous calcium levels of the various mitochondrial preparations. Hughes and Barritt [15] have preloaded mitochondria with 100 nmol calcium per mg protein and measured the half time of release.

The increase in State 3 (phosphorylating) respiration previously reported [1] is seen in Fig. 3 to be evident within 2 min of treatment time. The question as to whether or not the differences in time course for the $t_{1/2}$ values and State 4 respiration (maximal changes evident at 1 min treatment) and State 3 respiration (maximal change observed at 2 min treatment) are significantly different is not totally resolved. The data for these measurements were derived from the same samples, however, suggesting that the changes in calcium release rates and State 4 respiration do precede in time the changes in State 3 respiratory rates.

As seen in the 6-min glucagon sample of Fig. 3, two stages of calcium release from mitochondria are sometimes observed after glucagon treatment. The first plateau of release, when it is distinct, occurs with the release of 8—10 nmol of calcium per mg mitochondrial protein. The second phase involves the remainder of the intramitochondrial calcium, regardless of the total accumulated calcium level. It is possible that the first plateau of release may be

related to either ATP or pyridine nucleotide levels since the intramitochondrial levels of both these factors are in the range of 5—10 nmol per mg protein. Fiskum and Lehninger [24] have indicated that the intramitochondrial redox potential may control calcium efflux. Unpublished data from our laboratory (Khazan, U., Story, M. and Yamazaki, R.K. (1979)) indicate that intramitochondrial ATP levels drop concurrently with the onset of calcium efflux and mitochondrial swelling. Current studies are underway in our laboratory to determine the relationships between these parameters.

Nicholls [22] has indicated that rat liver mitochondria can effectively buffer calcium ion concentrations around pCa^{2+} equal to 6.1 (0.8 μM)at 30°C and pH 7.0 in the absence of permeant anions. Under our incubation conditions which include the presence of 5 mM phosphate which is a permeant anion, we find mitochondria from control animals to take the calcium ion concentration in the medium to pCa^{2+} values around 5.5 (3 μM), depending on the calcium load within the mitochondria (see, for example, Fig. 1). After glucagon treatment of animals, the mitochondria will reduce the medium calcium ion concentrations to somewhat lower levels (2.36 μM or a pCa^{2+} value of 5.63 in 1). Since glucagon treatment also affects the internal stores of calcium, the concentration ratio of calcium ion across the mitochondrial membrane which can be maintained is more meaningful. This ratio is calculated by assuming that the calcium load (endogenous stores plus that amount internalized when uptake ceases) is free within the mitochondrial matrix and is distributed in a volume of 1 μl per mg mitochondrial protein. When the ratio is calculated in this manner, control mitochondria maintain a concentration ratio ($[Ca^{2+}]_{in}/[Ca^{2+}]_{out}$) of $1.46 \cdot 10^4$ ($n = 14$). After 6-min glucagon treatment, this ratio is increased to $2.52 \cdot 10^4$ ($n = 5$). Thus, faced with the same calcium loading conditions, mitochondria isolated from glucagon-treated animals are able to reduce the calcium ion concentration in the incubation medium to a lower level by a factor of 1.7.

The changes observed in the State 4 respiratory rates with glutamate and the half time for calcium release may actually be reflections of a single alteration in the mitochondria induced by glucagon treatment. Mitochondrial State 4 respiratory rates are determined by the sum of intrinsic ATPase activity (included in this activity are those metabolic reactions such as pyruvate carboxylation and carbamyl phosphate synthesis which consume ATP) and those processes which dissipate the protonmotive force. Included in these dissipative processes are the slow backflow of protons inherent to the inner mitochondrial membrane, proton backflow mediated by lipophilic weak acids such as endogenous fatty acids, and inward movements of substrate anions coupled directly or indirectly to the proton gradient.

Mitochondrial calcium movements appear to involve two distinct transport systems [23]. Current evidence indicates that inward movements of calcium involve an electrophoretic uniport mechanism whereby calcium ion moves into the mitochondria in response to the negative-inside membrane potential [23]. Matrix calcium can then be transported out in exchange for protons [24]. If calcium ion is allowed to cycle in and out of the mitochondria, the net reaction will cause a decrease in the transmembrane potential and pH gradient. Thus, calcium cycling produces a partial uncoupling of mitochon-

drial respiration which manifests itself as an increase in State 4 (nonphosphorylating) respiratory rates. No calcium chelator such as EGTA was used in medium for the respiratory studies reported in this paper. Since glucagon treatment of animals causes a decreased mitochondrial calcium cycling rate by decreasing calcium efflux from mitochondria, a decrement in the State 4 respiratory rate proportional to the fraction of energy expended in the cycling of calcium would be expected.

Two primary hypotheses may be invoked to explain the decrease in calcium efflux rates observed after glucagon treatment. The first hypothesis is that the effects on calcium movements are secondary to the glucagon-induced stimulation of phosphate transport [7]. Phosphate transport effects could be exerted directly at the level of calcium influx of efflux or indirectly at the level of internal calcium binding. Use of phosphate transport inhibitors such as *N*-ethylmaleimide [25] or a phosphate-free medium may distinguish whether phosphate transport effects are involved. The second hypothesis involves direct effects of the hormonal treatment on the influx or efflux mechanisms. A direct effect on the calcium efflux mechanism may be studied using submitochondrial paticles since these preparations have an inverted (with respect to intact mitochondria) configuration. Studies to distinguish between these possibilities are underway in our laboratory.

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