Glucagon Effects on the Membrane Potential and Calcium Uptake Rate of Rat Liver Mitochondria*

(Received for publication, April 20, 1984)

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It has been widely reported that the in vivo administration of glucagon to rats results in the stimulation of calcium influx in subsequently isolated liver mitochondria. The mechanism of this effect is investigated through simultaneous measurements of calcium uptake rate and mitochondrial membrane potential. This allows the measurement of the calcium uniporter conductance independent of hormonal effects on electron transport or respiration. Two experimental approaches are used. The first involves measuring the uptake of 40-50 nmol of Ca²⁺/mg of mitochondrial protein with the calcium dye antipyrylazo III; the second uses ⁴⁰Ca²⁺ to follow uptake in the presence of 0.5 to 1.5 μM free calcium, buffered with HEDTA. In both cases a tetrathenyl phosphonium electrode is used to follow membrane potential, and membrane potential is varied using either malonate or butylmalonate in the presence of rotenone. The relative merits of these two approaches are discussed. The conductance of the calcium uniporter is found not to be stimulated by glucagon pretreatment. Also, the relative glucagon stimulation of both calcium influx and membrane potential is found to increase with increasing malonate concentration. These results imply that there is no direct stimulation of calcium uptake into liver mitochondria following glucagon treatment. The results are consistent with a glucagon stimulation of substrate transport, substrate oxidation, or a stimulation of electron transport resulting in an increased membrane potential and secondary stimulation of calcium uptake.

Hepatic mitochondria isolated from rats previously treated with glucagon have been shown to be stimulated in a number of functions compared with untreated mitochondria. Those parameters stimulated include state 3 respiration rate (1-3), uncoupler-dependent ATP hydrolysis (4-6), pyruvate carboxylation (9-11), an increase in the influx of Ca²⁺ (20). Effects on calcium transport are of particular interest because of recent evidence that intracellular Ca²⁺ redistribution is involved in the mediation of the liver's response to glucagon and α-adrenergic agents (21-23). Although most reports agree that glucagon treatment results in an increase of the proton electrochemical gradient, there is disagreement as to the nature of this stimulation. Some have reported a stimulation solely in the pH gradient (17, 24) while others observe stimulation of both pH gradient and membrane potential (ΔΨ) (19).

It is generally accepted that Ca²⁺ uptake occurs through an electrogenic uniport (25, 26). The rate of uptake of Ca²⁺ can, therefore, be affected by either a change in the membrane potential or a change in the kinetics of the Ca²⁺ uniporter itself. The fact that glucagon treatment leads to a stimulation of Ca²⁺ influx in isolated mitochondria (12, 16-19) but poorly characterized. Andia-Waltenbeugh et al. (14) reported an increase in the effective Vₘₚ of the Ca²⁺ uniporter, but their study ignored the question of the role of the membrane potential. Shears (27) has proposed that many of the effects of glucagon on isolated mitochondria can be explained by a hormone-induced stimulation of the proton electrochemical gradient and, under conditions where this manifests itself as an increase in membrane potential, an increase in Ca²⁺ influx can result.

To understand the effects of glucagon on Ca²⁺ uptake, any effect on the actual Ca²⁺ conductance of the uniporter must be separated from the hormone's effect on membrane potential. To accomplish this we have measured the rate of Ca²⁺ uptake and membrane potential simultaneously over a range of membrane potentials. A change in the uniporter would then manifest itself as an increase in the Ca²⁺ influx at a specific membrane potential (i.e. an increase in the Ca²⁺ conductance). Crompton et al. (28) have shown that the Ca²⁺ uniporter of isolated heart mitochondria is activated by pretreatment with an α-adrenergic agent. The results of this study indicate that such activation does not occur in isolated liver mitochondria following glucagon pretreatment.

 MATERIALS AND METHODS

Male Sprague-Dawley rats (200-300 g) (ed ad libitum were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg). After anesthesia took effect, the rats were injected with either 100 μg of glucagon in 1 ml of isotonic saline (two-thirds subcutaneous, one-third intraperitoneal) or 1 ml of saline alone. After 20 min, the livers were removed and mitochondria prepared in 0.3 M sucrose by differential centrifugation as described in Jensen et al. (29). Mitochondrial protein was assayed using the biuret method and resuspended at 40-50 mg/ml.

The rate of ATP hydrolysis under coupled conditions was measured as described by Hamman and Haynes (7). Respiration rates were measured at room temperature using a
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Clark oxygen electrode in a 300 mm anilin-succrose medium containing 1 mM succinate, 2 mM K phosphate, 1 mM MgCl\(_2\), and 0.75 mg/ml of mitochondria. ADP was added at 250 nmol/ml to initiate state 3 respiration.

Intramitochondrial water volume was measured with a \(^{3}H_{2}O\)-[\(^{14}C\)] sucrose exclusion technique, similar to that of Rottenberg (31). A TPP electrode and reference electrode were constructed as described in Kamo et al. (32) and the TPP electrode filled with a 2 mM solution of TPP CI in double distilled water. The TPP electrode was calibrated against data obtained with \(^{14}C\)TPP which in turn was calibrated using 86Rb-valinomycin in low potassium media as in Shen et al. (33).

Inorganic phosphate was measured by the technique of Berenblum et al. (34) on mitochondrial samples following precipitation in 6% perchloric acid. Endogenous calcium was measured by atomic absorption using an air-acetylene flame on samples digested with 30% nitric acid and heated for an hour at 80-90 °C. Calibration was achieved by the method of standard additions.

**Antipyrylazo III Dye Technique—**An Amino DW-2a spectrophotometer was used at the wavelength pair 720-790 nm (35) with a homemade cuvette holder and an adaptor built so that the TPP and reference electrode could be used simultaneously on the same cuvette. The cuvette was filled with 1.8 ml of a succrose medium containing 3 mM succinate, 0.5 mM MgCl\(_2\), 20 mM K Heps, pH 7.40, 60 mM KCl, 5 mM TPP, antipyrylazo III at 100 μM, and from 0-100 μM potassium phosphatase in 2 μg of protein per ml. The output of the TPP and reference electrodes were connected to a Keithley 616 digital electrometer and both this and absorbance data recorded on a Hewlett-Packard 7100 BM strip chart recorder. Uptake was initiated by the addition of 40-50 nmol of calcium per mg of mitochondrial protein. The absorbance change versus time data stored in a Midan microprocessor and later transferred to a Prime 500 computer for calculations. The absorbance of the dye was calibrated by adding known amounts of calcium to a cuvette containing both active mitochondrial and ruthenium red absorbance to prevent uptake and then correcting for the ruthenium red absorbance. The mitochondrial membrane potential was varied by adding butylinomalate (up to 1 mM), an inhibitor of succinate transport.

**Ruthenium Red Quench Technique with TPP Electrode—**The media for this technique contained 2.0 mM HEDTA, 20 mM K Heps, 3 mM Na succinate, 5 mM TPP, 50 mM KCl, 0.2 mM potassium phosphate, and 160 mM sucrose at pH 7.4. Mitochondria were then added to yield 8.0 ml at 4 mg of mitochondrial protein per ml and 6.0 μM rotenone added (final concentration). \(4Ca^{2+}/H^{+}\) was added when a stable membrane potential was achieved as measured with the TPP electrode. The amounts of added calcium needed to achieve the free calcium concentrations of 0.5, 1.0, and 1.5 μM in the presence of 2.0 mM HEDTA were calculated using the stability constants of Martel and Smith (28) and were 0.57, 0.57, and 0.77 μM, respectively. Every 20 s after the addition of the \(4Ca^{2+}/1.0-ml\) sample was taken, added to a tube containing 20 μl of 10 mM ruthenium red, vortexed, and placed on ice. After 5 samples were taken, EGTA was added to each sample (25 μl of 0.5 M) to prevent external binding of Ca\(^{2+}\). The samples were then centrifuged at 12,000 × g for 2 min. Pellet and supernatant were counted to determine the mitochondrial content of calcium which was corrected for calcium in the external water space of the pellet using the \(\text{H}_{2}\text{O}\) counter label. Parallel measurements of Ca\(^{2+}\) efflux were made on mitochondria loaded with calcium in the absence of HEDTA. Efflux was induced by the addition of ruthenium red and samples centrifuged as above every 1/4 min. The above procedure was carried out at various concentrations of malonate in order to vary the membrane potential.

**RESULTS**

**Observation of Glucagon Effect—**It was important to verify that the techniques used in these studies showed glucagon effects similar to those reported in the literature. Therefore, in addition to observing Ca\(^{2+}\) uptake rates and membrane potential, the following were also measured: rate of uncoupler-induced ATP hydrolysis, rate of pyruvate carboxylation, and respiratory control ratios. During the course of this work, there were periods when the control values of the above parameters increased to very near that of the glucagon-treated values, decreasing the apparent hormone effect. In agreement with Yamazaki (1), this problem seemed to correspond with times when the rats appeared agitated. Following the suggestion of R. C. Haynes\(^2\), we moved our rats to a quiet room where they were isolated from female rats and the temperature kept at 73-74 °F. These changes resulted in larger more consistent hormone effects. Glucagon stimulation of respiration and pyruvate carboxylase activity appeared to correlate well with the stimulation of rates of uncoupled ATPase activity. Consequently, the ATPase assay was used as an indication of the hormone effect for every experiment. Ca\(^{2+}\) influx and membrane potential data were collected only from those preparations where the stimulation of ATPase was 50% or greater. Data from these preparations is shown in Table I.

**Measurement of Ca\(^{2+}\) Uptake Rate with Antipyrylazo III and Membrane Potential with TPP—**This approach involved using the TPP electrode to measure membrane potential and the calcium-sensing dye antipyrylazo III to follow calcium uptake and is similar to the method used by Crompton et al. (28). The mitochondria were initially respiring in the presence of 3 mM succinate under state 4 conditions. When Ca\(^{2+}\) was added (40-80 nmol/mg of mitochondrial protein), uptake proceeded rapidly along with a substantial drop in membrane potential. As the uptake of Ca\(^{2+}\) became complete the membrane potential slowly recovered, although usually not to its initial value. Data using the faster responding cyanine dyes have indicated that during uptake of Ca\(^{2+}\) the membrane potential drops rapidly to a plateau value, remains there during most of the period of Ca\(^{2+}\) uptake, and then slowly

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1 The abbreviations used are: TPP, tetraphenylphosphonium; Heps, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HEDTA, N-(2-hydroxyethyl)ethylenedinitriilo-N',N',N'-trietic acid; EGTA, ethylene glycol b(β-aminohydroxy ether)-N,N',N',N'-tetrasacetic acid; Δψ, mitochondrial membrane potential (internally negative).

2 R. C. Haynes, personal communication.
recoveries. For this experiment the relatively constant early influx of Ca\(^{2+}\) was correlated with this plateau value of membrane potential. Although the Ca\(^{2+}\) uptake rate changed with time during uptake, the initial one-third of uptake was approximately linear, and a least squares linear regression was used to calculate Ca\(^{2+}\) influx. Data obtained using this method are shown in Fig. 1. Despite the scatter, an analysis of covariance indicates that these lines are different at the p ≤ 0.05 level of confidence. The difference, however, is small and is probably due to a systematic error of the membrane potential measurement due to the slow time response of the TPP electrode (see "Discussion"). The time response of the electrode is not a problem with the ruthenium red-EGTA quench technique.

**Ruthenium Red-EGTA Quench Technique**—Reed and Bygrave (37, 38) have shown that calcium buffers can be used to achieve relatively slow steady uptake of Ca\(^{2+}\) over a period of 30 s to 2 min. Under proper experimental conditions this can also lead to a stable membrane potential during uptake. It was necessary to adjust both the media phosphate concentration and free Ca\(^{2+}\) concentration to obtain this steady state. Phosphate concentrations below 100 µM caused the membrane potential to continue to drop during Ca\(^{2+}\) uptake when the free concentration of Ca\(^{2+}\) was 1 µM or higher. A phosphate concentration of 200 µM was chosen with a Ca\(^{2+}\) concentration buffered from 0.5 to 1.5 µM.

As can be seen in Fig. 2B, the TPP recording remained constant over the 1½ to 2 min that Ca\(^{2+}\) uptake data are taken. This allows sufficient time for the electrode to respond to the change in ΔΨ suggesting that the TPP has had time to equilibrate across the mitochondrial membrane. The Ca\(^{2+}\) uptake rate was also nearly constant over the same period, beginning to show downward curvature only in the last 1 or 2 time points (Fig. 2A). Using this technique, simultaneous measurements of Ca\(^{2+}\) uptake and ΔΨ can be made with confidence.

Malonate, an inhibitor of both succinate dehydrogenase and succinate transport was used to vary ΔΨ (and consequently Ca\(^{2+}\) influx). These data were obtained for both glucagon-treated and control mitochondria and are shown in Fig. 3 at three different buffered concentrations of Ca\(^{2+}\). As can be seen, at a given external Ca\(^{2+}\) concentration the relationship between Ca\(^{2+}\) influx and ΔΨ is a straight line. This linear relationship is in agreement with the observation of Akerman (39) on untreated mitochondria. As with Akerman’s data this linear relationship appears to extrapolate to a membrane potential of 60–80 mV at zero calcium influx. More importantly, at any given concentration of free calcium, both glucagon-treated and control points scatter around the same line. This leads to the conclusion that the calcium conductance of the calcium uniporter is not affected by glucagon pretreatment. No significant difference was found in the calcium efflux rate between the glucagon-treated and control mitochondria (Table I). The efflux rate was low (less than 1 nmol of Ca\(^{2+}\) per mg of protein) at the highest calcium loads (50 nmol of Ca\(^{2+}\) per mg of protein) and decreased at very low calcium loads. Consequently, the data shown in Fig. 3 are not corrected for efflux of calcium. Another interesting fact emerges from these data when membrane potential (or Ca\(^{2+}\) influx) is plotted as a function of malonate concentration. It can be seen (Fig. 4) that the relative difference between glucagon-treated and control mitochondria increases significantly as malonate concentration is increased. For example, at a free calcium concentration of 0.5 µM, the difference in membrane potential at 0 µM malonate is 4 mV (2.3% difference) while at 1.8 µM malonate the difference is 28 mV (26% increase).

**DISCUSSION**

These data (Fig. 3) demonstrate conclusively that there is no direct glucagon stimulation of the Ca\(^{2+}\) uptake mechanism.
were obtained in this study using two different procedures. The TPP electrode potential was averaged over the 100 s that uptake data were taken and this average electrode potential used to calculate the membrane potential. Malonate was added at concentrations between 0 and 4.0 mM in order to vary the membrane potential. Data of three different experiments are shown and are completely consistent with all seven experiments performed.

in isolated rat liver mitochondria. That is, there is no hormonal stimulation of the Ca\(^{2+}\) conductance of the uptake mechanism. The stimulation of Ca\(^{2+}\) uptake rate that is observed in liver mitochondria is due solely to the ability of glucagon-treated mitochondria to respire at a higher rate and consequently maintain a higher membrane potential than control mitochondria under identical experimental conditions. This conclusion is quite different from that presented by Crompton et al. (28) concerning \(\alpha\)-adrenergic activation of the calcium uniporter in cardiac mitochondria. These workers report a substantial increase in the calcium conductivity of the uniporter following treatment of rats with methoxamine. The fact that the present study sees no difference in calcium conductance of liver mitochondria following glucagon treatment suggests that either there is a fundamental difference in the regulation of calcium influx between mitochondria of heart and liver or a fundamental difference in the cellular response to the two different agents.

It is important to note that qualitatively the same results were obtained in this study using two different procedures. The antipyrylazo/TPP technique used here is similar to the calcium dye/safranine dye technique used by Akerman (39) as well as the arsenazo dye/TPP technique used by Crompton (28). This technique, however, has a number of difficulties associated with it that are largely eliminated by the ruthenium red-EGTA steady state procedure. One problem with the dye technique was that the membrane potential measured using the TPP electrode almost never came to the well defined plateau region seen with the cyanine dyes. Consequently, an average of the lower membrane potential readings was taken, even though the electrode potential was changing slowly through this time. The major difficulty was the relatively slow response time of the TPP electrode. The half-time of response was approximately 5 to 15 s, varied with the “age” of the TPP-selective membrane, and increased with decreasing TPP concentrations. In most cases, the membrane potential drop and recovery occurred faster than the electrode could accurately follow it. This results in underestimating the drop in membrane potential during Ca\(^{2+}\) influx. The underestimation is worse in mitochondria from animals treated with glucagon since the decrease and recovery of \(\Delta \Psi\) occur even faster. Several things were done in an attempt to overcome these problems. 1) The external inorganic phosphate concentration was increased to slow Ca\(^{2+}\) influx. This resulted in slowing the influx of Ca\(^{2+}\) (but also caused a larger drop in \(\Delta \Psi\)). 2) The calcium load was decreased to cause a smaller drop in \(\Delta \Psi\) (however, uptake then occurred over a shorter period of time). 3) The magnesium concentration was increased to slow Ca\(^{2+}\) influx. This was partially successful. An alternative approach (28) is to look at an arbitrary concentration point of the Ca\(^{2+}\) uptake curve, calculate the slope at this point, and find \(\Delta \Psi\) from the simultaneous point on the TPP electrode trace. This approach is subject to many of the same problems of electrode response time as discussed above. Also, the TPP is in flux across the

![Figure 3: Relationship between calcium uptake and the magnitude of the membrane potential as measured with the ruthenium red quench/TPP electrode technique in liver mitochondria from control (○) and glucagon (●)-treated rats. The numbers in the figure represent the free calcium concentration for each set of points. The calcium uptake rate was obtained from the slope calculated by linear regression of the intramitochondrial calcium data. The TPP electrode potential was averaged over the 100 s that uptake data were taken and this average electrode potential used to calculate the membrane potential. Malonate was added at concentrations between 0 and 4.0 mM in order to vary the membrane potential. Data of three different experiments are shown and are completely consistent with all seven experiments performed.](http://www.jbc.org/)

![Figure 4: Relationship between calcium uptake rate and the magnitude of the membrane potential during calcium uptake as a function of malonate concentration. Two different free calcium concentrations are shown: 0.5 \(\mu\)M calcium for glucagon-treated (●) and control (○) and 1.5 \(\mu\)M calcium for glucagon-treated (▲) and control (△) mitochondria. Calculations of calcium uptake rate and membrane potential are described in the previous figure.](http://www.jbc.org/)
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mitochondrial membrane at this point and not fully equilibrated as is assumed in the calculation of $\Delta \Psi$. The time response of the TPP electrode was not a problem using the ruthenium red-EGTA quench technique. The mitochondria were in a steady state of calcium influx and respiration and thus maintain a steady membrane potential. This allows more than sufficient time for the TPP electrode to stabilize and yield an accurate membrane potential measurement.

It has been reported that the presence of Mg\textsuperscript{2+} in the media causes an increase in the relative difference in Ca\textsuperscript{2+} influx rate between glucagon and control (14). We have been unable to observe this relative stimulation using the antipyrilazo dye technique of Ca\textsuperscript{2+} flux measurements. Furthermore, it should be clear from the data of Fig. 4 that a substantial hormonal stimulation of Ca\textsuperscript{2+} influx exists even in the absence of Mg\textsuperscript{2+}. It remains unclear what role, if any, Mg\textsuperscript{2+} plays in the effect of glucagon on Ca\textsuperscript{2+} uptake.

The fact that the relative glucagon stimulation of both $\Delta \Psi$ and Ca\textsuperscript{2+} influx increases with increasing molal concentration is relevant to the mechanism of action of this hormone on isolated mitochondria. Under conditions of molalate inhibition, the rate of succinate transport and/or oxidation should be rate limiting. Titheradge and Haynes and Titherage et al. (11, 40) have reported evidence that the rate of succinate transport is not affected by glucagon treatment. The present data are consistent with at least one site of glucagon action being that of succinate dehydrogenase. It is impossible from the present data to determine if this is the only site of action of glucagon on subsequently isolated mitochondria. A simultaneous stimulation of the electron transport chain, as has been reported (11), would also not be inconsistent with these observations.

It has been suggested that many of the effects of glucagon on mitochondria may be artifacts of mitochondrial isolation (11). In support of this is the observation that changes in the proton electrochemical gradient (42, 43) and in respiration (44) present in isolated mitochondria are not found in glucagon treated hepatocytes. Jensen et al. (29) have concluded that glucagon effects on mitochondria are not artifacts of the isolation procedure based on observations of persistent glucagon effects on respiration, pyruvate carboxylation, and citrulline synthesis in crude liver homogenates prepared under a variety of conditions. Until it can be conclusively demonstrated that mitochondria in isolated hepatocytes are undamaged, caution should be exercised in using negative data obtained with these cells to extrapolate to in vivo systems. Even a relatively small plasma membrane leak to Ca\textsuperscript{2+} in isolated hepatocytes could damage the mitochondria and make them appear to be unresponsive. Regardless of this controversy, the present report demonstrates that there is no direct effect of glucagon on the mechanism of calcium influx in rat liver mitochondria.

Acknowledgments — We wish to thank Dr. Robert C. Haynes, Jr. for giving D. E. W. the opportunity to visit his laboratory and for the advice and help that he and the members of his laboratory rendered. We also thank Dr. Bruce Jensen for his invaluable help in the construction and use of the tetraphenyl phosphonium electrodes.

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J. Biol. Chem. 1984, 259:9390-9394.

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