Effects of Glucagon and N^6O^2' -Dibutyryladenosine 3':5'-Cyclic Monophosphate on Calcium Transport in Isolated Rat Liver Mitochondria

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1. The administration of glucagon to fed rats by intraperitoneal injection, or the perfusion of livers from fed rats with glucagon by the method of Mortimore [Mortimore (1963) *Am. J. Physiol.* **204**, 699–704] was associated with increases of 15- and 5-fold respectively, in the time for which a given load of exogenous Ca^{2+} is retained by mitochondria subsequently isolated from the liver. This effect of glucagon was (a) also induced by N^6O^2' -dibutyryl cyclic AMP, (b) completely blocked by cycloheximide, (c) relatively slow in onset (15–60 min) and (d) associated with a stimulation of about 20% in the rates of ADP-stimulated oxygen utilization and Ca^{2+} transport measured in the presence of succinate. 2. Perfusion of livers with glucagon resulted in the isolation of mitochondria which showed a 50% increase, no significant change and a 40% increase in the concentrations of endogenous Ca, Mg and P_i respectively, when compared with mitochondria isolated from control perfused livers. 3. The administration of insulin or adrenaline to fed rats induced increases of 10- and 8-fold respectively, in the time for which Ca^{2+} is retained by isolated liver mitochondria. Perfusion of livers with insulin had no effect on mitochondrial Ca^{2+} retention time. 4. The perfusion of livers from starved rats with glucagon, or the administration of either glucagon or insulin to starved rats, increased by about 2.5- and 15-fold respectively, the time for which isolated mitochondria retain Ca^{2+} . 5. Mechanisms which may be responsible for the observed alterations in Ca^{2+} -retention time are discussed.

Recent evidence indicates that changes in the intracellular concentration of Ca^{2+} may mediate the actions of a number of hormones on hepatic metabolism (Pilkis *et al.*, 1975; Friedmann, 1976; Assimakopoulos-Jeannet *et al.*, 1977; Keppens *et al.*, 1977; van de Werve *et al.*, 1977). In conjunction with the plasma membrane and endoplasmic reticulum, mitochondria appear to play an important role in the regulation of intracellular Ca^{2+} concentrations through their ability to actively transport and accumulate this cation (for review see Bygrave, 1977). Our previous studies have shown that the administration of insulin to starved rats is associated with a stimulation of the initial rates of Ca^{2+} and phosphate transport, and an increase in the time for which Ca^{2+} is retained in mitochondria subsequently isolated from the liver (Dorman *et al.*, 1975; Barritt *et al.*, 1975, 1978). Increases in the concentration of Ca^{2+} in isolated mitochondria were shown to have marked effects on the activity of the gluconeogenic enzyme, pyruvate carboxylase, and on the transport of pyruvate and ATP across the mitochondrial membrane (Foldes & Barritt, 1977). In order to elucidate further the possible role of mitochondria in the hormonal regulation of intracellular Ca^{2+} in the liver, the nature of the hormones which can induce changes in mito-

chondrial Ca^{2+} transport and the mechanisms by which these changes are achieved have been investigated. The results indicate that glucagon can act directly on the liver to increase the time for which isolated mitochondria retain Ca^{2+} as well as the initial rate of Ca^{2+} transport.

Experimental

Administration of hormones to rats

Male hooded Wistar rats (Institute of Medical and Veterinary Science, Adelaide) weighing 200–300 g and fed *ad libitum* except where indicated otherwise were used for all experiments. Rats were starved for 15 h by the removal of food at 16:00 h on the day before the administration of hormones. Hormones and other agents were administered to rats at 09:00–10:00 h by intraperitoneal injection as described previously (Dorman *et al.*, 1975). After a period of 1 h, unless otherwise stated, the rats were killed by decapitation and liver mitochondria isolated.

Perfusion of livers

Liver perfusions were conducted by the method of

Mortimore (Mortimore *et al.*, 1958; Mortimore, 1963) with two modifications. The inferior vena cava was cut after cannulation of the portal vein, and the medium was continually oxygenated with O_2/CO_2 (19:1) (3 litres/min) by means of an artificial lung (Berry *et al.*, 1973). The initial volume of the perfusion medium was 150 ml. Approx. 40–50 ml of this medium was discarded after perfusion once through the liver before recirculation was begun. The temperature of the medium and the liver was maintained at 35–38°C by a thermostatically controlled cabinet.

Two perfusion media were used. In the presence of erythrocytes, perfusions were performed with a medium consisting of Krebs–Henseleit salts (Krebs & Henseleit, 1932), gassed for 15 min with O_2/CO_2 (19:1), 3% (w/v) bovine serum albumin and 20% (v/v) human erythrocytes (twice-washed and aged for 4–8 weeks) (Hems *et al.*, 1966), adjusted to pH 7.4 with NaOH. Once recirculation of the medium had begun, the flow rate was adjusted to 8–12 ml/min. After a pre-equilibration period of 15 or 30 min, the hormone or agent under test was added directly to the reservoir in about 1 ml of 0.9% (w/v) NaCl, containing 50 mg of bovine serum albumin/l, adjusted to pH 7.4 with NaOH (NaCl/albumin). Control perfusions received an addition of NaCl/albumin only. A mixture of amino acids (John & Miller, 1969) was added in one batch (dissolved in 5 ml of Krebs–Henseleit salts, pH 7.4) at this time.

In the absence of erythrocytes, the perfusion medium consisted of Krebs–Henseleit salts [gassed for 15 min with O_2/CO_2 (19:1)], 3.0% (w/v) bovine serum albumin, 10 mM-Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulphonic acid) and amino acids (John & Miller, 1969) adjusted to pH 7.4 with 10% (w/v) NaOH. The flow rate was maintained at 24–29 ml/min (Friedmann & Rasmussen, 1970). Agents under test were added to the reservoir in about 1 ml of NaCl/albumin after a pre-equilibration period of 15 or 45 min, as indicated in the Tables and Figures.

Perfusions conducted in the absence of erythrocytes were judged to be successful by these criteria. (i) The rates of respiration and respiratory control ratios of mitochondria isolated from the perfused livers were only slightly lower than the values obtained for livers removed immediately after rats were killed (Table 7) (see Ross, 1972). (ii) In the presence of glucagon, the rate of glucose release from the livers of fed rats was stimulated about 2.5-fold (see Exton *et al.*, 1971). (iii) Similar values for livers from fed rats perfused in the presence or absence of erythrocytes were obtained for both control and glucagon-stimulated rates of glucose release into the perfusion medium and for the rates of respiration and respiratory control ratios of mitochondria isolated from the livers (results not shown).

Isolation of mitochondria

Liver mitochondria were isolated by homogenization in 250 mM-sucrose/2 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH/0.5 mM-EGTA (pH 7.4 at 0°C) followed by differential centrifugation (Johnson & Lardy, 1967). The mitochondria were washed and finally suspended at a concentration of 30–50 mg of protein/ml in 250 mM-sucrose/2 mM-Hepes/KOH (pH 7.4). Mitochondria concentration (as mitochondrial protein), respiratory activity and integrity were measured as described previously (Dorman *et al.*, 1975). In the presence of 12.5 mM-succinate, values of 4–7 were obtained for the respiratory control ratio (the ratio of the rate of ADP-stimulated oxygen consumption to the rate obtained after depletion of the ADP) for mitochondria isolated from the livers of rats immediately after the animals had been killed, and values of 3.5–6.5 for mitochondria isolated from perfused livers.

Ca^{2+} transport and retention

Initial rates of Ca^{2+} transport and the time for which a given load of Ca^{2+} is retained by isolated mitochondria were measured as described by Dorman *et al.* (1975) in a medium containing, in a final volume of 2.0 ml, 250 mM-sucrose, 2.5 mM-Hepes, 2 mM-succinate, 1 mM-potassium phosphate, 150 μ M- $^{45}CaCl_2$ (0.13 μ Ci) and mitochondria, 1.5 mg of protein/ml. Except where indicated otherwise, the pH was 7.4. Initial rates of Ca^{2+} transport were measured at 0°C, and Ca^{2+} retention at 25°C. The time for which mitochondria retained a load of exogenous Ca^{2+} decreased markedly in the first 100 min after completion of the isolation procedure (Table 1). Since little further change in retention time occurred after this period, and minimum variation in retention time between one preparation of mitochondria and another was obtained for mitochondria

Table 1. *Effect of age of liver mitochondria on the time for which a given load of exogenous Ca^{2+} is retained*

Isolation of the mitochondria from the livers of fed rats and measurement of Ca^{2+} -retention time (100 nmol of exogenous Ca^{2+} /mg of protein) were performed as described in the Experimental section. After completion of the isolation and wash procedures, the mitochondria were maintained at 0°C.

Time after completion of isolation of the mitochondria (min)	Ca^{2+} -retention time (min)
45	4.4 ± 2.0 (3)
75	2.3 ± 1.1 (5)
105	0.7 ± 0.2 (6)
135	0.8 ± 0.3 (4)

of this age, values for Ca^{2+} retention time were routinely measured 2 h after completion of isolation of the mitochondria. The time for which mitochondria retained Ca^{2+} was defined as the time at which 50% of the accumulated Ca^{2+} had been released. Initial rates of Ca^{2+} transport were determined from the slope of plots of the amount of Ca^{2+} transported as a function of time, by linear-regression analysis.

Endogenous mitochondrial Ca, Mg and P_i

For the analysis of Ca and Mg, four samples (0.3 ml) of each mitochondrial suspension were placed in glass tubes and stored at -20°C . Each sample was dissolved in 3.0 ml of 0.9% (w/v) sodium deoxycholate, 0.1% (w/v) NaCl and 1 mM-EDTA, adjusted to pH 9 with KOH (Hutson *et al.*, 1976). The concentrations of Ca in this extract, and of Mg in a 9-fold dilution of the extract in deoxycholate/NaCl/EDTA, were determined by atomic absorption spectroscopy using an air/acetylene flame on a model AA6 atomic absorption spectrometer (Varian Techtron, North Springvale, Vic., Australia). Calibration standards of Ca and Mg were prepared in the deoxycholate/NaCl/EDTA solutions.

To assess whether mitochondria take up Ca^{2+} during the isolation procedure, livers from control fed rats were homogenized in the presence of $^{45}\text{CaCl}_2$, in the presence and absence of EGTA (see Claret-Berthon *et al.*, 1977). The inclusion of EGTA in the isolation medium decreased the amount of Ca^{2+} taken up by the mitochondria by about 10-fold (Table 2). Similar results were obtained with livers removed from rats treated with glucagon (Table 2). Since the amount of Ca^{2+} taken up by the mitochondria in the presence of EGTA ranged from 2.5 to 4.5% of the total Ca present, it is concluded that under the conditions used (0.5 mM-EGTA present) the amount of Ca^{2+} taken up by the mitochondria does

not contribute significantly to the total amount of Ca^{2+} present. Possible loss of Ca^{2+} from the mitochondria during the washing steps was assessed by allowing the mitochondria (45 mg of protein per ml of 250 mM-sucrose/2 mM-Hepes/KOH, pH 7.4), isolated as described above from a control fed rat to take up exogenous CaCl_2 (150 μM) in the presence of 2.5 mM-succinate for 10 min at 25°C . The mitochondrial suspension (1.0 ml) was then diluted with 250 mM-sucrose/2 mM-Hepes/KOH, pH 7.4. (35 ml), centrifuged at 16000g for 7 min and the mitochondrial pellet washed twice by the same procedure as that used for the isolation of mitochondria. The amount of $^{45}\text{Ca}^{2+}$ associated with the washed mitochondria was 3.3 ± 0.2 (8) nmol per mg of protein compared with 3.2 ± 0.1 (8) nmol per mg of protein present before the wash steps (eight independent estimations). These results indicate that there is no significant loss of Ca^{2+} from the mitochondria during the wash stages of the isolation procedure.

Endogenous P_i was measured in trichloroacetic acid extracts of mitochondria by the method of Baginski *et al.* (1967).

Glucose and plasma glucagon

The amount of glucose released from the liver during perfusion was determined by removing samples (0.2 ml) of the perfusate at 10 min intervals into tubes containing 0.1 ml of 24% (w/v) HClO_4 at 0°C . At the end of the perfusion, the samples were centrifuged at 7000g for 2 min. The amount of glucose present in each supernatant was determined by the glucose oxidase/peroxidase-coupled enzyme assay (Werner *et al.*, 1970).

The concentration of plasma glucagon-like immunoreactivity was determined with a pig glucagon standard (Novo Industri, Copenhagen, Denmark) and Unger's anti-serum 30K (Wise *et al.*, 1973).

Table 2. Effect of the inclusion of EGTA in the isolation medium on the uptake of Ca^{2+} by mitochondria during their isolation. Liver mitochondria were isolated from control fed rats or fed rats treated with glucagon (75 μg per 100 g body wt. administered by intraperitoneal injection 60 min before removal of the liver) as described in the Experimental section, with these modifications. Liver tissue (2 g wet wt.) was homogenized in 20 ml of 250 mM-sucrose/2.5 mM-Hepes/KOH (pH 7.4) which contained tracer amounts of $^{45}\text{CaCl}_2$ (9 μCi) in either the presence or absence of 0.5 mM-EGTA. The mitochondria were washed once in sucrose/Hepes/KOH and finally suspended in 1.5 ml of this medium. The amounts of $^{45}\text{Ca}^{2+}$ and total Ca present in the suspension of washed mitochondria and the postmitochondrial supernatant were determined as described in the Experimental section and used to estimate the amount of Ca^{2+} taken up by the mitochondria. Each set of data represent the mean $\pm$ S.E.M. of four independent experiments.

Condition	Additions to isolation medium	Ca^{2+} taken up by mitochondria (nmol/mg of protein)	Total Ca in mitochondria (nmol/mg of protein)
Control	None	3.6 ± 0.6	11.1 ± 0.2
	EGTA	0.31 ± 0.02	7.3 ± 0.4
Glucagon	None	3.5 ± 0.3	13.6 ± 1.0
	EGTA	0.23 ± 0.03	9.3 ± 0.3

Treatment of data

The values given are the means $\pm$ S.E.M. or the means and ranges when only two experiments were performed. The degrees of significance, *P*, were determined by Student's *t* test for unpaired, or where indicated, paired samples. Values of *P* > 0.05 were considered to be not significant.

Chemicals

Recrystallized insulin from pig pancreas, dissolved in sodium acetate buffer, pH 7.0, containing 0.1% (w/v) methyl *p*-hydroxybenzoate ('Novo-Actrapid') was purchased as a solution (1.6 mg dry wt. per ml) from Novo-Industri A/S.

Glucagon, adrenaline, *N*⁶*O*^{2'}-dibutyryl cyclic AMP, cycloheximide and amino acids were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; sodium deoxycholate was from E. Merck, Darmstadt, Germany; ⁴⁵CaCl₂ was from The Radiochemical Centre, Amersham, Bucks., U.K. The reagents for the determination of glucose were supplied as a diagnostic test kit from Boehringer Mannheim G.m.b.H., Mannheim, West Germany. Bovine serum albumin (fraction V; Sigma Chemical Co.) was dialysed for 20 h at 4°C against 0.9% (w/v) NaCl before use. All other reagents were of analytical grade.

Results

Transport and retention of Ca²⁺

(a) *After the administration of glucagon to rats.* Mitochondria isolated from the livers of fed rats 1 h

after the animals had received an intraperitoneal injection of glucagon exhibited an enhancement of approx. 20% in the initial rate of Ca²⁺ transport (measured at 0°C) relative to control mitochondria. In four separate experiments rates of 61.1 ± 3.8 and 51.4 ± 3.8 nmol/min per mg of protein (*P* < 0.025 by the paired *t* test) were obtained for mitochondria isolated from rats treated with glucagon and control rats respectively. Mitochondria isolated from rats treated with glucagon also showed an enhancement of at least 15-fold in the time for which the accumulated Ca²⁺ was retained at 25°C (Table 3). The effect on Ca²⁺ retention was apparent 10 min after the administration of glucagon, but was not fully developed until at least 60 min (Fig. 1). Over this period of time, the concentration of plasma glucagon-like immunoreactivity was 0.56, 0.56, 0.27 and 0.27 nM at 15, 30, 45 and 60 min respectively, compared with control values of 0.08–0.1 nM.

Effects on Ca²⁺ retention similar to those associated with the administration of glucagon to fed rats were observed after the administration of dibutyryl cyclic AMP to fed rats and glucagon to starved rats (Table 3). The actions of glucagon and dibutyryl cyclic AMP on Ca²⁺ retention in fed rats were almost completely inhibited by the co-administration of cycloheximide (Table 3). The administration to rats of this inhibitor alone produced no detectable effect on mitochondrial Ca²⁺ retention (Table 3).

(b) *After the perfusion of livers with glucagon.* Mitochondria isolated from livers perfused in the presence of glucagon (0.2–0.35 μ M) for 45–60 min

Table 3. *Effects of hormones and various agents administered to rats by intraperitoneal injection on the retention of Ca²⁺ by isolated liver mitochondria*

Administration of the agent under test 1 h before the rats were killed, isolation of liver mitochondria and measurement of Ca²⁺-retention time were performed as described in the Experimental section and in the legend of Fig. 1. The amounts of each agent administered were per 100 g body wt.: glucagon, 25 μ g; dibutyryl cyclic AMP, 750 μ g; adrenaline, 50 μ g; insulin, 50 μ g; glucose 0.75 g; cycloheximide, 250 μ g. The degrees of significance are **P* < 0.0005.

Dietary state	Agent administered to rat	Ca ²⁺ -retention time (min)
Fed	Control	0.7 $\pm$ 0.2 (14)
	Glucagon	>10.3 $\pm$ 0.5 (8)*†
	Glucagon + cycloheximide	1.1 $\pm$ 0.7 (3)
	Dibutyryl cyclic AMP	>11.5 $\pm$ 1.8 (4)*†
	Dibutyryl cyclic AMP + cycloheximide	1.5 $\pm$ 1.1 (3)
	Cycloheximide	0.5 $\pm$ 0.1 (2)
	Insulin	6.7 $\pm$ 1.2 (4)*
	Insulin + glucose	0.4 $\pm$ 0.05 (2)
	Glucose	0.3 $\pm$ 0.05 (2)
	Adrenaline	5.6 $\pm$ 1.9 (3)*
Starved	Control	1.1 $\pm$ 0.7 (6)
	Insulin	20 $\pm$ 3.9 (4)*
	Insulin + glucose	13.3 $\pm$ 7.3 (4)
	Glucagon	15.8 $\pm$ 6.3 (2)

† In some experiments, the mitochondria had not released Ca²⁺ when the assay was terminated, cf. Fig. 1(d).

Fig. 1. Effect of time of exposure of rats to glucagon on the retention of Ca^{2+} by mitochondria subsequently isolated from the liver

Glucagon ($25\mu\text{g}$ per 100g body wt.) was administered to fed rats by intraperitoneal injection (see the Experimental section) at 10 (a), 20 (b), 30 (c) and 60 min (d) before the animals were killed. Isolation of liver mitochondria from control rats ($\circ$) and rats treated with glucagon ($\bullet$), and measurement of Ca^{2+} retention at 25°C were performed as described in the Experimental section.

Table 4. Effects of perfusion of livers from fed rats with hormones and dibutyl cyclic AMP on the retention of Ca^{2+} by isolated mitochondria

Liver perfusions in the presence or absence of erythrocytes were performed as described in the Experimental section. After an equilibration period of 30 min (erythrocyte system) or 15 min (no erythrocytes) the agent(s) under test was added to the perfusate to give the concentration indicated and the perfusion continued for a further 60 (erythrocyte system) or 45 min (no erythrocytes). When present, cycloheximide was added at the same time as glucagon. Isolation of the mitochondria and measurement of Ca^{2+} retention at pH 7.3 (mitochondria isolated from livers perfused in the presence of erythrocytes) or pH 7.4 (mitochondria isolated from livers perfused in the absence of erythrocytes) were performed as described in the Experimental section. The numbers of experiments are given in parentheses. Abbreviation: n.s., not significant.

Perfusion conditions	Agent tested	Ca^{2+} -retention time (min)	P
Erythrocytes present	Control	0.9 ± 0.4 (5)	
	Glucagon ($0.2\mu\text{M}$)	4.6 ± 0.9 (6)	<0.005
	Glucagon ($0.2\mu\text{M}$) + cycloheximide ($2\mu\text{M}$)	0.6 ± 0.1 (3)	n.s.
Erythrocytes absent	Control	3.9 ± 0.4 (7)	
	Glucagon (1 nM)	9.2 ± 1.6 (5)	<0.001
	Glucagon ($0.35\mu\text{M}$)	10.6 ± 1.0 (6)	<0.0005
	Dibutyl cyclic AMP (0.16 mM)	9.4 ± 0.6 (3)	<0.0005
	Insulin ($0.67\mu\text{M}$)	3.4 ± 1.0 (3)	n.s.

Fig. 2. Effects of the perfusion of livers for different periods of time in the presence or absence of glucagon on the time for which isolated mitochondria retain Ca^{2+}

Liver perfusions were performed in the absence of erythrocytes (except for three of the control perfusions of 45 min duration) as described in the Experimental section. Recirculation was begun at 0 min, and at the times indicated by the arrows glucagon ($0.35 \mu\text{M}$ final concentration) was added and the perfusion continued for either 15 or 45 min, as indicated. Isolation of mitochondria from livers perfused in the absence of glucagon (○), in the presence of glucagon (●) or immediately after rats had been killed by decapitation (Δ), as well as the measurement of mitochondrial Ca^{2+} retention, were performed as described in the Experimental section. The data represent means $\pm$ S.E.M. for the numbers of determinations shown in parentheses.

exhibited a significant increase in the time for which Ca^{2+} was retained (Table 4). This effect was observed both in the presence and absence of erythrocytes in the perfusion medium (Table 4). An increase in Ca^{2+} retention time was also observed when livers were perfused with 1 nM -glucagon or with dibutyryl cyclic AMP (Table 4). The effects of 1 nM -glucagon, $0.35 \mu\text{M}$ -glucagon and 0.16 mM -dibutyryl cyclic AMP on mitochondrial Ca^{2+} retention were associated with increases of 87, 162 and 125% respectively, in the rate of release of glucose into the perfusate. The stimulation by glucagon of Ca^{2+} -retention time was completely abolished by the co-perfusion of cycloheximide with the hormone (Table 4), although this compound had no effect on the stimulation by glucagon of the rate of glucose release (results not shown). Perfusion of livers with cycloheximide alone had no significant effect on mitochondrial Ca^{2+} -retention time (results not shown).

The time for which mitochondria isolated from control perfused livers retained Ca^{2+} increased with increasing time of perfusion to a maximum value at about 30 min, before declining (Fig. 2). After an equilibration period of 15 min, the addition of glucagon to the perfusate for a further 15 min resulted in mitochondria with a retention time for Ca^{2+} which was close to the maximum absolute value obtained (i.e. the value after 45 min perfusion with glucagon), although little greater than the corresponding control value. After a 45 min equilibration period, the addition of glucagon for 15 min led to mitochondria with a retention time for Ca^{2+} which was 2-fold higher than that for the corresponding control mitochondria, but significantly lower than the maximum absolute value obtained in the presence of glucagon (Fig. 2).

The initial rates of Ca^{2+} transport (measured at 0°C) in mitochondria isolated from the livers of fed rats perfused for 45 min with glucagon were 57.8 ± 4.0 (mean $\pm$ range, $n = 2$) compared with $52.0 \pm 3.1 \text{ nmol/min per mg}$ of protein for mitochondria isolated from control perfused livers.

Perfusion of livers from starved rats with $0.35 \mu\text{M}$ -glucagon for 15 min after an equilibration period of 15 min resulted in mitochondria which retained Ca^{2+} for 8.5 ± 0.9 (2) min compared with 3.5 ± 0.1 (2) min for mitochondria isolated from control perfused livers of starved rats.

Endogenous mitochondrial Ca, Mg and P_i

The perfusion of livers under control conditions resulted in a decrease in the amount of Ca present in isolated mitochondria when compared with values obtained for mitochondria isolated from the livers of intact rats (Table 5). This decrease was apparent after 15 min perfusion. When $0.35 \mu\text{M}$ -glucagon was added to the perfusate at either 15 or 45 min, a significant increase in the amount of endogenous mitochondrial Ca was observed (Table 5). Similar results were obtained when livers were perfused with dibutyryl cyclic AMP (Table 5). However, this parameter was not influenced by the perfusion of livers with 1 nM -glucagon for 45 min. No significant change in the concentration of endogenous Mg was observed in mitochondria isolated from perfused livers under any of the conditions tested [18.7 ± 0.7 (12) nmol of Mg/mg of protein for control livers]. The administration of glucagon to rats by intraperitoneal injection produced no significant change in the intra-mitochondrial concentrations of either Ca (Table 5) or Mg ($28.7 \pm 1.6 \text{ nmol of Mg/mg of protein}$ for mitochondria isolated from control livers), although the concentration of the metals in these preparations of mitochondria were almost 2-fold higher than those observed in mitochondria isolated from control perfused livers.

Table 5. *Effects of glucagon, insulin and dibutyryl cyclic AMP on the amounts of endogenous Ca in mitochondria isolated from the livers of fed rats*

Administration of glucagon (75 μ g per 100g body wt.) to rats by intraperitoneal injection for a period of 1h, and subsequent isolation of liver mitochondria were performed as described in the Experimental section. Liver perfusions were performed in the absence of erythrocytes as described in the legend of Table 4 and in the Experimental section. The agents under test were added to the perfusate after a 15 min equilibration period except for glucagon, which was also added at 45 min as indicated, to give the final concentration indicated. Perfusion was then continued for the total time shown. The amounts of Ca present in isolated mitochondria were measured as described in the Experimental section. The degrees of significance are * $P < 0.025$; ** $P < 0.001$; *** $P < 0.0005$.

Condition	Total perfusion time (min)	Agent tested	Ca (nmol/mg of protein)	n
Perfused liver	15	Control	5.2 $\pm$ 0.3	3
	30	Control	4.4 $\pm$ 0.4	4
	60	Control	5.3 $\pm$ 0.3	12
	30	Glucagon (0.35 μ M)	8.2 $\pm$ 1.4*	3
	60	Glucagon (0.35 μ M)	8.1 $\pm$ 0.9**	7
	60	Glucagon (0.35 μ M) at 45 min	7.1 $\pm$ 0.4**	3
	60	Glucagon (1 nM)	5.5 $\pm$ 0.3	6
	60	Dibutyryl cyclic AMP (0.16 mM)	8.3 $\pm$ 0.8***	3
	60	Insulin (0.67 μ M)	5.2 $\pm$ 0.5	3
Whole animal		Control	9.2 $\pm$ 0.6	6
		Glucagon	9.3 $\pm$ 0.6	6

Table 6. *Effects of perfusion of livers from fed rats with hormones on the amount of endogenous P_i associated with mitochondria subsequently isolated from the liver*

Liver perfusions, the isolation of mitochondria and measurement of P_i were conducted as described in the legend of Table 5 and the Experimental section. The numbers of experiments are given in parentheses, and the degree of significance is * $P < 0.005$.

Time of perfusion with agent under test (min)	Agent	P_i (nmol/mg of protein)
45	Control	9.0 $\pm$ 0.8 (10)
	Glucagon (1 nM)	8.6 $\pm$ 0.4 (6)
	Glucagon (0.35 μ M)	12.5 $\pm$ 0.8 (9)*
	Dibutyryl cyclic AMP (0.16 mM)	9.5 $\pm$ 0.6 (3)
	Insulin (0.67 μ M)	10.9 $\pm$ 1.2 (3)
15	Control	9.5 $\pm$ 0.9 (4)
	Glucagon (0.35 μ M)	11.0 $\pm$ 1.6 (4)

Elevated concentrations of P_i were associated with mitochondria isolated from livers perfused for 45 min with 0.35 μ M-glucagon (Table 6). However, no significant increase in endogenous P_i was observed in mitochondria isolated from livers perfused for 15 min with 1 nM-glucagon or with dibutyryl cyclic AMP.

Mitochondrial respiration

The administration of glucagon to rats was associated with an increase of 21% in the State-3 (ADP-stimulated) rate of respiration with succinate as substrate, no change in the State-4 respiration rate (i.e. the rate after depletion of added ADP), and an increase in the ratio of State-3/State-4 rates (the respiratory control ratio) (Table 7). Similar results were obtained for mitochondria isolated from livers perfused for 45 min with glucagon, although the magnitude of the stimulation was less than that observed when glucagon was administered directly to rats.

Effects of insulin and adrenaline on mitochondrial Ca^{2+} retention

Previously it was shown that the administration of insulin to starved rats by intraperitoneal injection is associated with an increase in the time for which mitochondria subsequently isolated from the liver retain Ca^{2+} (Dorman *et al.*, 1975). Insulin had a similar effect on fed rats, although the magnitude of the effect is smaller (Table 3). When insulin was administered to fed rats in the presence of glucose, or when glucose alone was administered to fed rats, no increase in mitochondrial Ca^{2+} -retention time was observed (Table 3). In contrast with these results, the co-administration of glucose with insulin to

Table 7. *Effect of glucagon on the respiration rates of mitochondria isolated from the livers of fed rats*

Administration of glucagon (25 or 75 μg per 100 g body wt.) to rats by intraperitoneal injection for a period of 1 h, subsequent isolation of liver mitochondria and the measurement of rates of respiration were performed as described in the Experimental section. Liver perfusions were performed in the absence of erythrocytes as described in the legend of Table 4 and in the Experimental section. The degrees of significance, P , were determined (a) by using Student's t test for the data obtained from perfused livers and (b) by using the t test for paired samples, with the value obtained for each test set of mitochondria being paired with the value obtained for corresponding control set of mitochondria, for the data obtained for whole animals. The degrees of significance are * $P < 0.025$ and ** $P < 0.01$ with the numbers of experiments given with the parentheses.

Condition	Agent tested	Rate of O ₂ consumption (ng-atoms/min per mg of protein)		State 3/ State 4
		State 3	State 4	
Whole animal	Control (5)	155 $\pm$ 12	29 $\pm$ 2	5.5 $\pm$ 0.2
	Glucagon (5)	188 $\pm$ 15**	32 $\pm$ 2	6.0 $\pm$ 0.5
Perfused liver	Control (5)	176 $\pm$ 6	37 $\pm$ 4	5.0 $\pm$ 0.4
	1 nM-Glucagon (4)	197 $\pm$ 11	36 $\pm$ 1	5.6 $\pm$ 0.1
	0.35 μM -Glucagon (6)	195 $\pm$ 5*	39 $\pm$ 3	5.1 $\pm$ 0.3

starved rats only partly reversed the effect of insulin (Table 3).

Mitochondria isolated from the livers of either fed (Table 4) or starved rats (results not shown) perfused with insulin for 45 min exhibited retention times similar to those of mitochondria isolated from the corresponding control perfused livers. Furthermore, the concentrations of endogenous Ca (Table 5), Mg (results not shown) and P_i (Table 6) as well as the rate of State-3 respiration (results not shown) were not significantly different from the values obtained for control mitochondria.

The administration of adrenaline to rats was associated with a significant increase in the Ca^{2+} -retention time for isolated liver mitochondria, although the magnitude of the increase was not as great as that induced by glucagon or dibutyryl cyclic AMP (Table 3).

Discussion

The data are consistent with a direct effect of glucagon on the liver resulting in mitochondria which when isolated exhibit an increase in the time for which a given load of exogenous Ca^{2+} is retained. Since these effects are also induced by dibutyryl cyclic AMP, it is likely that they are mediated by changes in the intracellular concentration of cyclic AMP. The observation that the actions of both agents are completely blocked by cycloheximide is consistent with a mechanism in which cyclic AMP induces a step (or steps) which involves the nuclear DNA-directed synthesis of new protein (Pestka, 1971). However, there may be other explanations for the action of cycloheximide (Wititsuwannakul & Kim, 1977). The effect of glucagon on mitochondrial

Ca^{2+} -retention time was observed at concentrations of the hormone approaching the range of those present *in vivo*, about 3–80 pM (Unger & Orci, 1976), indicating that the effect may be a physiological one.

Both the present and previous (Dorman *et al.*, 1975) studies have shown that the administration of insulin to either fed or starved rats is associated with an increase in the ability of isolated liver mitochondria to retain Ca^{2+} . One explanation for the observation that both glucagon and insulin, when administered to rats, have similar effects on liver mitochondrial Ca^{2+} retention is that the effects of insulin are mediated by an increase in the concentration of plasma glucagon induced by hypoglycaemia (see Unger & Orci, 1976). The following observations are consistent with this proposal. (a) The perfusion of fed or starved livers with glucagon, or the administration of this hormone to fed or starved rats yields increased mitochondrial Ca^{2+} -retention times; (b) the perfusion of fed or starved livers with insulin has no effect on mitochondrial Ca^{2+} -retention time under the conditions tested; (c) the administration to fed rats of insulin in the presence of glucose, or of glucose alone, does not cause an increase in hepatic mitochondrial Ca^{2+} -retention time; (d) the administration of insulin to starved rats in the presence of glucose yields a lower hepatic mitochondrial Ca^{2+} -retention time in comparison with the effect of insulin alone.

It was previously reported that the co-administration of glucose with insulin to starved rats did not alter the effect of this hormone on mitochondrial Ca^{2+} -retention time (Dorman *et al.*, 1975). However, these earlier experiments were terminated 10 min after the addition of $^{45}\text{CaCl}_2$. At this time, mitochondria isolated from the livers of starved rats treated with insulin and glucose or with insulin alone would not have released significant amounts of Ca^{2+}

(see Table 3). Failure to obtain a complete reversal by glucose of the effects of insulin on the starved rat may be due to rapid metabolism of the glucose and/or the induction of glucagon release by other factors (Unger & Orci, 1976). It was also noted that the effect on hepatic mitochondrial Ca^{2+} retention of insulin administration to starved rats is greater in magnitude than that observed after the administration of glucagon to starved rats or insulin to fed rats. These observations may be due in part to differences in the time courses for the development of the effect and to the possible action of other hormones, e.g. glucocorticoids.

In contrast with the effects of glucagon on some mitochondrial reactions (Yamazaki, 1975; Yamazaki *et al.*, 1977; Bryla *et al.*, 1977) the onset of the effect of the hormone on mitochondrial Ca^{2+} retention in the livers of fed rats appears to be relatively slow. This is unlikely to be due to the slow entry of glucagon into the bloodstream after administration of the hormone to rats by intraperitoneal injection, since it was shown that plasma concentrations of glucagon-like immunoreactivity had reached their maximum values 45 min before maximum values for mitochondrial Ca^{2+} -retention time were observed. The data obtained with the perfused liver system are difficult to interpret in view of the marked changes in the Ca^{2+} -retention time for control mitochondria as the time of perfusion is increased. However, the results indicate that the effect obtained after 15 min perfusion with glucagon is apparently not maximal.

The effect of glucagon on mitochondrial Ca^{2+} -retention time, which was observed in both the whole-animal and perfused-liver experiments, is unlikely to be due to an increase in the mitochondrial concentration of Mg^{2+} (Sordahl, 1975), since there appears to be no significant change in the concentration of this cation. However, the effect may be caused, at least in part, by an increase in the concentration of endogenous mitochondrial ATP which has been shown to be associated with the action of glucagon on liver mitochondria (Barritt *et al.*, 1978; Bryla *et al.*, 1977) and/or an increase in the ability of the mitochondria to oxidize succinate (cf. Yamazaki, 1975; Yamazaki *et al.*, 1977; Kimura & Rasmussen, 1977). The increased initial rates of Ca^{2+} transport associated with the administration of glucagon to rats may be the result of an increase in the ability of the mitochondria to oxidize succinate and hence generate a proton gradient (cf. Yamazaki, 1975; Titheradge & Coore, 1976; Halestrap, 1977), although changes in the Ca^{2+} transporter itself (Dorman *et al.*, 1975) cannot be excluded.

The results indicate that the perfusion of livers from fed rats with glucagon results in an increase in the concentration of endogenous mitochondrial Ca when compared with mitochondria isolated from control perfused livers. However, no effect of glucagon on

this parameter was observed when the hormone was administered to rats, suggesting that the increase observed in the perfused system may be due to reversal of the loss of Ca which occurs during the initial stages of perfusion. Since the observed increases in the concentration of endogenous mitochondrial Ca do not correlate with increases in the Ca^{2+} -retention time under all conditions tested, it is concluded that they are unlikely to be due solely to the ability of the mitochondria to retain Ca^{2+} for longer periods of time.

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