Evidence that glucagon acts on the liver to decrease mitochondrial calcium stores

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1. Mitochondria isolated from rats treated with glucagon for 60 min or livers perfused in the presence of glucagon for 10 min exhibited lower rates of $^{45}\text{Ca}^{2+}$ exchange than did control mitochondria when this was measured under steady-state conditions in the presence of Mg^{2+} , ATP, P_i and $0.13\ \mu\text{M}$ - or $0.16\ \mu\text{M}$ -free Ca^{2+} at pH 7.4 and at 25°C or 37°C . Under these conditions no significant difference in the rates of Ruthenium Red-induced $^{45}\text{Ca}^{2+}$ efflux was observed. These results contrast with earlier work in which mitochondria isolated from glucagon-treated livers were shown to exhibit faster rates of Ca^{2+} uptake [Yamazaki (1975) *J. Biol. Chem.* **250**, 7924–7930] and slower rates of spontaneous Ca^{2+} efflux [Hughes & Barritt (1978) *Biochem. J.* **176**, 295–304] when these parameters were measured under different incubation conditions, including supra-physiological concentrations of free Ca^{2+} and the absence of added Mg^{2+} and ATP. 2. Perfusion of livers with glucagon before the addition of adrenaline or the Ca^{2+} -selective ionophore A23187, to release Ca^{2+} from intracellular stores, decreased the amount of Ca^{2+} released by these agents. 3. Incubation of isolated hepatocytes in the presence of glucagon at $1.3\ \text{mM}$ extracellular Ca^{2+} induced a small decrease in the plateau of the $^{45}\text{Ca}^{2+}$ -exchange curve obtained under steady-state conditions. 4. It is concluded that the actions of glucagon on liver mitochondrial Ca^{2+} transporters lead to a decrease, rather than an increase, in mitochondrial Ca^{2+} stores in the intact cell.

Mitochondria isolated from livers treated with glucagon show increases in the initial rate of Ca^{2+} uptake (Yamazaki, 1975; Hughes & Barritt, 1978; Andia-Waltenbaugh *et al.*, 1978; Yamazaki *et al.*, 1980; Andia-Waltenbaugh *et al.*, 1981) and in the time for which a load of exogenous Ca^{2+} is retained (Prpić *et al.*, 1978; Hughes & Barritt, 1978; Prpić & Bygrave, 1980; Yamazaki *et al.*, 1980; Taylor *et al.*, 1980a). Glucagon has also been shown to stimulate the uptake of Ca^{2+} by microsomal fractions derived from the smooth endoplasmic reticulum (Andia-Waltenbaugh & Friedmann, 1978; Bygrave & Tranter, 1978; Taylor *et al.*, 1979, 1980b; Andia-Waltenbaugh *et al.*, 1980). On the basis of these observations it has been suggested that one of the physiological actions of glucagon on the liver is to increase the amount of Ca^{2+} in the mitochondria (Andia-Waltenbaugh *et al.*, 1978; Barritt, 1980; Taylor *et al.*, 1980a; Yamazaki *et al.*, 1980) and smooth endoplasmic reticulum (Bygrave & Tranter,

1978; Andia-Waltenbaugh *et al.*, 1980; Barritt, 1980). However, studies of Ca^{2+} uptake by mitochondria and microsomal fractions isolated from livers treated with glucagon have generally been conducted under incubation conditions which are far removed from those likely to be present in the cell. Furthermore, these effects of glucagon on isolated organelles have not been reconciled with the apparently opposing action of the hormone in intact cells, where a release of Ca^{2+} from these organelles is observed (Friedmann & Rasmussen, 1970; Chen *et al.*, 1978; Blackmore *et al.*, 1979; Whiting & Barritt, 1982).

The aim of the present experiments was to determine whether the effects of glucagon on Ca^{2+} uptake and retention by isolated mitochondria are representative of the physiological action of glucagon on mitochondrial Ca^{2+} stores in the liver. Two experimental approaches were employed. Firstly, the effects of the treatment of livers with glucagon on Ca^{2+} transport by isolated mitochondria incubated under conditions which approach those present in

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the cell cytoplasm were investigated. Secondly, the effects of glucagon on mitochondrial Ca^{2+} stores in the perfused liver and isolated hepatocytes were studied without disruption of the cells. The results indicate that under physiological conditions the apparently diverse actions of glucagon on mitochondrial Ca^{2+} transport lead to a decrease, rather than an increase, in the quantity of Ca^{2+} in the mitochondria.

Experimental

Animals, glucagon treatment and liver-perfusion technique

Male hooded Wistar rats (Institute of Medical and Veterinary Science, Adelaide) weighing 200–300 g and fed *ad libitum* were used in all experiments. Glucagon treatment *in vivo* was by intraperitoneal injection. Glucagon (75 $\mu\text{g}/100\text{g}$ body wt.) was administered in 0.1 M- NaHCO_3 (0.5 ml). Control rats received an equal volume of NaHCO_3 .

Perfused livers were treated with glucagon essentially as described by Taylor *et al.* (1980a). Perfusion was with non-recirculating Krebs–Henseleit (1932) bicarbonate buffer containing 2.5 mM- CaCl_2 , at a flow rate of 30 ml/min. After perfusion for 10 min the right lobe was tied off and removed for preparation of control mitochondria. At 15 min after initiation of the perfusion, glucagon (0.1 μM) was added to the reservoir of perfusion medium, and the remaining lobes were perfused for a further 10 min. Glucagon-treated mitochondria were then prepared from the median and caudate lobes.

Isolation of mitochondria and measurement of mitochondrial properties

Mitochondria which sedimented between 3000 and 25000 g-min (Prpić *et al.*, 1978) were isolated as described previously (Hughes & Barritt, 1979). The mitochondrial protein concentration was measured by the method of Lowry *et al.* (1951), and rates of respiration as described by Hughes & Barritt (1978).

$^{45}\text{Ca}^{2+}$ exchange by isolated mitochondria at low concentrations of free Ca^{2+} in the presence of ATP and Mg^{2+}

Ca^{2+} exchange by isolated mitochondria at 25°C or 37°C was measured as described previously (Barritt, 1981). The reaction medium contained, in a final volume of 6 ml; 150 mM-KCl, 10 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH, 10 mM-nitrilotriacetic acid, 2 mM-potassium phosphate, 2 mM-potassium succinate, 3 mM-sodium ATP, 2 mM- MgCl_2 , 25 or 50 μM added CaCl_2 (0.13 or 0.16 μM free Ca^{2+}) and 1.5 mg of mitochondrial protein/ml. The final pH was 7.4. Tracer amounts of $^{45}\text{CaCl}_2$ [7 kBq (0.2 μCi)/ml of incubation medium] were added 15 min after the

addition of mitochondria. Free Ca^{2+} concentrations were determined as described previously (Barritt, 1981). Exponential equations and configurations of kinetically distinct compartments of exchangeable Ca^{2+} were fitted to the $^{45}\text{Ca}^{2+}$ -exchange data as described previously (Barritt, 1981).

Rates of Ruthenium Red-induced Ca^{2+} efflux were measured at 37°C in the presence of 0.16 μM free Ca^{2+} under the incubation conditions employed for $^{45}\text{Ca}^{2+}$ exchange. Ruthenium Red (70 nM) was added at 10 min after $^{45}\text{Ca}^{2+}$ and the rate of loss of $^{45}\text{Ca}^{2+}$ measured over the subsequent 4 min.

Measurement of Ca^{2+} transport by isolated mitochondria at high concentrations of free Ca^{2+} in the absence of ATP and Mg^{2+}

Initial rates of Ca^{2+} uptake at 4°C (100 μM added Ca^{2+}), the time for which a load of exogenous Ca^{2+} (150 μM) is retained at 25°C, and rates of Ruthenium Red- and EGTA-induced Ca^{2+} efflux from mitochondria loaded with exogenous Ca^{2+} (150 μM) were measured under the conditions described by Taylor *et al.* (1980a). The movement of Ca^{2+} was monitored by using either $^{45}\text{Ca}^{2+}$ or a Ca^{2+} -selective electrode (Radiometer, Copenhagen, Denmark). In each case the ratio of added Ca^{2+} to mitochondrial protein was 100 nmol/mg. The concentrations of Ruthenium Red and EGTA were 17 nM and 1.2 mM respectively.

Release of Ca^{2+} from perfused livers

The Ca^{2+} released from perfused livers by adrenaline or the Ca^{2+} -selective ionophore A23187 was measured as described by Blackmore *et al.* (1979). Glucagon treatment of perfused livers was the same as for preparation of mitochondria, but lobes were not removed. Livers were perfused for 15 min in the presence of 2.5 mM- CaCl_2 at a flow rate of 30 ml/min, then glucagon (0.1 μM) was added to the reservoir and the perfusion continued for a further 10 min. Control livers were perfused with Krebs–Henseleit bicarbonate buffer for 25 min.

At the end of the hormone treatment, the perfusion medium was changed to Krebs–Henseleit bicarbonate buffer without added CaCl_2 , and the flow rate was decreased to 14 ml/min to facilitate measurement of Ca^{2+} . After 6 min perfusion with the low- Ca^{2+} medium, adrenaline (10 μM final concn.) or ionophore A23187 (8 μM) was added to the reservoir. Samples (3.5 ml) of perfusate were collected every 15 s for determination of the Ca^{2+} concentration by atomic-absorption spectroscopy with an air/acetylene flame on a model AA6 atomic-absorption spectrometer (Varian Techtron, North Springvale, Vic., Australia). Solutions of CaCl_2 in Krebs–Henseleit bicarbonate buffer were used as Ca^{2+} standards.

$^{45}\text{Ca}^{2+}$ exchange by isolated hepatocytes

Hepatocytes were isolated by a modification of the method of Berry & Friend (1969), and rates of $^{45}\text{Ca}^{2+}$ exchange under steady-state conditions were measured as described previously (Barritt *et al.*, 1981). Cells were treated with glucagon ($0.1\mu\text{M}$) for 15 min in the presence of 1.3mM-CaCl_2 before the addition of tracer amounts of $^{45}\text{CaCl}_2$.

Treatment of experimental data

The values given are means $\pm$ S.E.M. Degrees of significance were determined by using Student's *t* test for unpaired samples, with values of $P > 0.05$ considered to be not significant.

Chemicals

Glucagon and the Ca^{2+} -selective ionophore A23187 were obtained from Calbiochem-Behring Australia Pty. Ltd., Carlingford, N.S.W., Australia, and $^{45}\text{CaCl}_2$ was from Amersham Australia Pty. Ltd., Surrey Hills, N.S.W., Australia. Other reagents were obtained from the sources described previously (Barritt *et al.*, 1981; Barritt, 1981).

Results

Isolated mitochondria

Ca^{2+} -exchange studies conducted under steady-state conditions at 0.13 or $0.16\mu\text{M}$ free Ca^{2+} and 25°C or 37°C in the presence of ATP, Mg^{2+} and potassium phosphate showed that mitochondria isolated from livers perfused for 10 min in the presence of glucagon or from the livers of rats treated with glucagon *in vivo* for 60 min exchanged less Ca^{2+} than did control mitochondria. The results obtained at $0.16\mu\text{M}$ free Ca^{2+} and 25°C are shown in Fig. 1. A decrease in the rate of $^{45}\text{Ca}^{2+}$ exchange was observed when the 15 min equilibration of mitochondria with the incubation medium was omitted, thus exposing the mitochondria to Ca^{2+} immediately before the measurement of $^{45}\text{Ca}^{2+}$ exchange (results not shown). There was no significant difference in the rate of Ruthenium Red-induced $^{45}\text{Ca}^{2+}$ efflux for control mitochondria (0.34 ± 0.003 nmol of Ca^{2+} /min per mg of protein) and mitochondria isolated from livers treated with glucagon (0.42 ± 0.004 ; $n = 5$, $P > 0.1$) under the incubation conditions described in the legend of Fig. 1.

A closed system of three kinetically distinct compartments of exchangeable Ca^{2+} (Barritt, 1981) gave a good fit to the data of Fig. 1. Inspection of the values of the kinetic constants indicated that glucagon induces a significant decrease in the flux (0.298 ± 0.006 compared with 0.322 ± 0.007 nmol/min per mg of protein, values $\pm$ S.D., $P < 0.02$) and fractional transfer rate (0.0119 ± 0.0003 compared with $0.0129 \pm 0.0003\text{min}^{-1}$, $P < 0.02$) for the trans-

Fig. 1. Effect of the perfusion of livers with glucagon on mitochondrial $^{45}\text{Ca}^{2+}$ exchange measured at low concentrations of free Ca^{2+} in the presence of Mg^{2+} and ATP at 25°C

Liver perfusions, the isolation of mitochondria from control (O) and glucagon-treated (●) liver lobes and the measurement of $^{45}\text{Ca}^{2+}$ exchange in the presence of $50\mu\text{M}$ added total Ca^{2+} ($0.16\mu\text{M}$ free Ca^{2+}) 3mM-ATP and 2mM-MgCl_2 at 25°C were conducted as described in the Experimental section. The amount of $^{45}\text{Ca}^{2+}$ associated with the mitochondria is expressed as a percentage of the initial dose of $^{45}\text{Ca}^{2+}$ added to the incubation medium. The results are the means $\pm$ S.E.M. for four experiments. The lines represent the calculated curve of best fit obtained after fitting a three-compartment closed system (see the Experimental section) to the data.

port of Ca^{2+} from the medium to the compartment of mitochondrial exchangeable Ca^{2+} with the shorter turnover time.

Mitochondria isolated from livers treated with glucagon exhibited changes similar to those reported previously by others (Yamazaki, 1975; Prpić *et al.*, 1978; Hughes & Barritt, 1978; Taylor *et al.*, 1980a), including a 2-fold increase in the initial rate of Ca^{2+} uptake measured at 4°C in the presence of $100\mu\text{M}$ initial free Ca^{2+} , decreases in the rates of Ruthenium Red- (0.38 ± 0.06 and 0.08 ± 0.01 nmol/min per mg of protein, $P < 0.01$, $n = 3$) and EGTA- (21 , 27 and 12 , 16 nmol/min per mg of protein, $n = 2$) induced Ca^{2+} efflux, and increases in the rate of ADP-stimulated O_2 utilization (233 ± 7 and 248 ± 7 ng-atoms/min per mg of protein, $P < 0.05$, $n = 30$) and the time for which Ca^{2+} (100nmol/mg of protein) is retained at 25°C (6.1 ± 0.4 and 24 ± 3 min, $P < 0.01$, $n = 14$).

Intact cells

The amount of Ca^{2+} released from perfused livers by the α -adrenergic agonist adrenaline (Blackmore *et al.*, 1979; Althaus-Salzmann *et al.*, 1980) was decreased from 0.26 to $0.12\mu\text{mol/g}$ wet wt. after pre-treatment of the livers with $0.1\mu\text{M}$ -glucagon

Fig. 2. Effects of perfusion of livers with glucagon on the subsequent release of Ca^{2+} by (a) adrenaline and (b) Ca^{2+} -selective ionophore A23187

Liver perfusions and the measurement of perfusate Ca^{2+} concentrations were performed as described in the Experimental section. (a) Adrenaline, $10\text{ }\mu\text{M}$ final concn. (filled symbols), or an equal volume of 0.9% NaCl (open circles), was added to the reservoir of perfusion medium at 6 min as indicated by the arrow. The time taken for the hormone to reach the liver was calculated to be 1 min. (b) Ionophore A23187, $8\text{ }\mu\text{M}$ final concn., dissolved in dimethyl sulphoxide (1%, v/v), was added to the perfusion medium at 6 min as indicated by the arrow. Livers were perfused in the absence of glucagon (○, ●) and in the presence of (▲) 0.5 nM - or (■) $0.1\text{ }\mu\text{M}$ -glucagon for 10 min before the perfusion medium was changed to one which contained no added Ca^{2+} or glucagon. The results are the means $\pm$ S.E.M. for three or four experiments.

(Fig. 2a). Pre-treatment with 0.5 nM -glucagon, a concentration that gives 50% stimulation of glycogenolysis (Pilkis *et al.*, 1975), also decreased the amount of Ca^{2+} released by adrenaline (Fig. 2a). When Ca^{2+} release was induced by the Ca^{2+} -selective ionophore A23187, less Ca^{2+} was released from livers pre-treated with glucagon than from control livers, although the total amount of Ca^{2+} released by ionophore A23187 was considerably less than that released by adrenaline (Fig. 2b). The slow and apparently incomplete release of Ca^{2+} from intracellular stores induced by ionophore A23187 may be due to the distribution of large quantities of the ionophore in extracellular locations (Borle & Studer, 1978) and/or a decrease in the intracellular ATP concentration (Friedmann *et al.*, 1979).

The effect of glucagon ($0.1\text{ }\mu\text{M}$) on $^{45}\text{Ca}^{2+}$ exchange by isolated hepatocytes incubated under steady-state conditions at 1.3 mM extracellular Ca^{2+} was also tested. A small but significant decrease (F-ratio test) in the plateau of the exchange curve was observed (results not shown), indicating (Barritt *et al.*, 1981) that glucagon causes a small decrease in the quantity of exchangeable Ca^{2+} in intracellular stores.

Discussion

The present results indicate that mitochondria isolated from livers treated with glucagon exhibit a

small decrease in the total amount of exchangeable Ca^{2+} when incubated under conditions resembling those of the cell cytoplasm more closely than those employed in most previous studies of the effects of glucagon on mitochondrial Ca^{2+} transport (Yamazaki, 1975; Hughes & Barritt, 1978; Prpić *et al.*, 1978; Andia-Waltenbaugh *et al.*, 1978; Yamazaki *et al.*, 1980; Taylor *et al.*, 1980a; Andia-Waltenbaugh *et al.*, 1981). The use of an extramitochondrial free Ca^{2+} concentration within the physiological range (Baker, 1978; Tsien *et al.*, 1982) and inclusion in the incubation medium of ATP (and hence ADP) and Mg^{2+} (Barritt, 1981), which have pronounced effects on the stability of isolated mitochondria and on the permeability of the mitochondrial inner membrane to Ca^{2+} (Chappell & Crofts, 1965; Sordahl, 1974; Binet & Volfin, 1975; Jacobus *et al.*, 1975; Harris, 1979), are considered to be particularly important. Brand & De Selincourt (1980) obtained results similar to those reported here using a low extramitochondrial free Ca^{2+} concentration (but cf. Yamazaki *et al.*, 1980).

The results obtained with isolated mitochondria are supported by the effects of glucagon on intracellular Ca^{2+} stores in intact liver cells. The amount of Ca^{2+} released by adrenaline from control perfused livers is 70% of the total amount of exchangeable Ca^{2+} estimated to be present in the intracellular stores by Claret-Berthon *et al.* (1977) and Barritt *et al.* (1981). Ca^{2+} released by adrena-

line is derived chiefly from the mitochondria, with some contribution from the endoplasmic reticulum (Blackmore *et al.*, 1979). The action of glucagon on the perfused liver appears to be to enhance the net outflow of Ca^{2+} from the mitochondria and endoplasmic reticulum, leading to a decrease in Ca^{2+} in these organelles, although the possibility that the hormone redistributes intracellular Ca^{2+} to stores which are not released by adrenaline or ionophore A23187 cannot be excluded. It is considered unlikely that glucagon treatment decreases adrenaline- or ionophore A23187-induced Ca^{2+} release from the perfused liver by inhibiting the outflow of Ca^{2+} across the mitochondrial inner or plasma membranes, as pre-treatment with glucagon did not affect the time taken for the release of Ca^{2+} by either agent. It is concluded that one of the physiological actions of glucagon on the liver is to decrease the quantity of exchangeable Ca^{2+} in the mitochondria.

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