An assessment of the calcium content of rat liver mitochondria *in vivo*

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The effect of systematically altering the isolation conditions on the total calcium content of mitochondria isolated from perfused rat liver was examined. We showed that, under most isolation conditions, significant redistributions of mitochondrial calcium occurred resulting in up to 5-fold changes of the total calcium content. Mitochondrial Ca^{2+} flux inhibitors such as Ruthenium Red and nupercaine were only partially effective in inhibiting such redistributions. We present evidence indicating that the total calcium content of rat liver mitochondria *in situ* may approximate $2\text{nmol}\cdot(\text{mg of protein})^{-1}$.

Most studies on Ca^{2+} homeostasis in hepatic tissue have suggested that mitochondria play a predominant role in the regulation of the cytoplasmic free Ca^{2+} concentration (Claret-Berthon *et al.*, 1977; Bygrave, 1978; Nicholls, 1978; Becker *et al.*, 1980; Nicholls & Crompton, 1980; Borle, 1981; Coll *et al.*, 1982; Joseph *et al.*, 1983). This view is supported by the finding that isolated mitochondria can buffer the extramitochondrial Ca^{2+} concentration at values close to those thought to exist in the cytoplasm (Nicholls, 1978; Becker *et al.*, 1980; Joseph *et al.*, 1983), owing to the establishment of a dynamic equilibrium between separate Ca^{2+} influx and Ca^{2+} efflux pathways (Bygrave, 1978; Carafoli & Crompton, 1978; Nicholls & Crompton, 1980; Åkerman & Nicholls, 1983).

An alternative role for the mitochondrial Ca^{2+} translocation cycle has been suggested by Denton & McCormack (1980). These authors propose that this cycle may be a means by which the matrix free Ca^{2+} concentration and hence the activity of numerous Ca^{2+} -stimulated matrix enzymes can be regulated (McCormack & Denton, 1980; Denton *et al.*, 1980). In order to define more closely the role of this translocation cycle, detailed information about the total and free mitochondrial Ca^{2+} concentrations *in vivo* is required, and an attempt to obtain these data forms the basis of the present study. The ability to manipulate precisely the external Ca^{2+} concentration in the perfused liver system (Reinhart *et al.*, 1982a), to determine accurately the free Ca^{2+} concentration in Krebs–Ringer bicarbonate media (Reinhart *et al.*, 1983) and to isolate mitochondria rapidly from an intact

liver by Percoll density-gradient centrifugation (Reinhart *et al.*, 1982b), has provided a means for closely monitoring Ca^{2+} movements during the isolation of mitochondria. Our results indicate that the value for the mitochondrial calcium content is dependent on the concentration of the circulating external Ca^{2+} , and on the presence or absence of inhibitors of mitochondrial Ca^{2+} cycling. Based on these findings we present an argument that the concentration of total calcium in rat liver mitochondria *in situ* is of the order of $2\text{nmol}\cdot(\text{mg of mitochondrial protein})^{-1}$. Some implications of this conclusion are discussed in terms of the free matrix Ca^{2+} concentration and the role of this organelle in the regulation of Ca^{2+} homeostasis.

Experimental procedures

Animals and perfusions

Male Wistar-strain albino rats, weighing between 200 and 250 g, and having free access to food, were used for all experiments. Rats were anaesthetized with sodium pentobarbital (50mg/kg body wt.), and the livers perfused with Krebs–Henseleit (1932) bicarbonate medium equilibrated with O_2/CO_2 (19:1) in the absence or in the presence of added CaCl_2 (Reinhart *et al.*, 1982a). A non-recirculating mode of perfusion was used at a flow rate of $3.5\text{ml}\cdot\text{min}^{-1}\cdot(\text{g of liver})^{-1}$ (Reinhart *et al.*, 1982c). In experiments where Ca^{2+} -free Krebs–Henseleit medium was used CaCl_2 (1.3mM) was administered by infusion syringe during the first 10 min of perfusion. At various times after 10 min the removal or re-administration of CaCl_2 into the perfusion circuit

allows the rapid manipulation of the extracellular Ca^{2+} concentration (see the Results section). All perfusions were carried out between 08:00 and 12:00h.

Liver fractionation

The liver was fractionated by a rapid Percoll density-gradient-centrifugation procedure recently developed in this laboratory (Reinhart *et al.*, 1982b). The median lobe was excised and homogenized in a medium consisting of 210mM-mannitol, 60mM-sucrose, 10mM-KCl, 10mM-sodium succinate, 0.25mM-dithiothreitol, 1mM- KH_2PO_4 , 2mM- MgSO_4 , 10 μM -EGTA and 10mM-Hepes[4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid]/KOH (pH 7.4), at 0–4°C. Where indicated in the legend to the Figures, 1.6 μM -Ruthenium Red and 0.4mM-nupercaine (cinchocaine hydrochloride) were also present in the homogenizing medium. A portion (2ml) of the resulting homogenate was layered on to a discontinuous density-gradient of Percoll made iso-osmotic with the above medium and centrifuged for 30s at 39600 g_{av} in a Sorvall RC5B refrigerated centrifuge with the SS34 rotor. In this way a fraction enriched in mitochondria (Reinhart *et al.*, 1982b) could be obtained within a total preparation time of 6min.

Assay of total calcium

Mitochondria. Immediately after isolation, the mitochondrial fraction was extracted with ice-cold 2M- HClO_4 /5mM-Hepes and centrifuged for 6min in an Eppendorf centrifuge to sediment insoluble material. Portions of the supernatant were analysed for calcium in 0.1% (w/v) KCl using an N_2O /acetylene flame (medical-grade gases) in a Varian Atomic Absorption Spectrophotometer. All solutions were prepared using deionized distilled water (Milli-Q reagent-grade water system; resistance >15M $\Omega\cdot\text{cm}^{-1}$). Calcium concentrations were determined by internal standard curve calibration, since Percoll was found to decrease the absorbance response due to calcium (P. H. Reinhart, unpublished work).

Perfusate calcium. This was measured by collecting samples from the outflow side of the liver. These were then centrifuged in an Eppendorf microfuge for 2min, and portions of the supernatant assayed for calcium as described above.

Free calcium concentration calculations

Free Ca^{2+} concentrations were calculated using an algorithm of the program (Comics) developed by Perrin & Sayce (1967). The logarithm of the total formation constants (Sillen & Martell, 1971) for the following complexes were considered (pH 7.4): $\text{PO}_4^{3-} + \text{H}^+$, 11.8; $\text{EGTA}^{4-} + \text{Ca}^{2+}$, 11.0; $\text{CO}_3^{2-} + \text{H}^+$, 10.25; $\text{EGTA}^{4-} + \text{H}^+$,

9.54; $\text{HEGTA}^{3-} + \text{H}^+$, 8.93; $\text{HPO}_4^{2-} + \text{H}^+$, 7.15; $\text{HEGTA}^{3-} + \text{Ca}^{2+}$, 5.3; $\text{EGTA}^{4-} + \text{Mg}^{2+}$, 5.2; $\text{HEGTA}^{3-} + \text{Mg}^{2+}$, 3.4; $\text{HCO}_3^- + \text{Mg}^{2+}$, 3.4; $\text{CO}_3^{2-} + \text{Ca}^{2+}$, 3.2; $\text{HPO}_4^{2-} + \text{Ca}^{2+}$, 2.77; $\text{H}_2\text{EGTA}^{2-} + \text{H}^+$, 2.73; $\text{HPO}_4^{2-} + \text{Mg}^{2+}$, 2.5; $\text{H}_2\text{PO}_4^- + \text{Ca}^{2+}$, 1.5.

Chemicals and materials

Percoll was obtained from Pharmacia Fine Chemicals AG, Uppsala, Sweden, Ruthenium Red was from Sigma Chemical Company, St. Louis, MO, U.S.A., and nupercaine from Astra Chemicals, Sydney, N.S.W., Australia. All other chemicals used were of analytical reagent grade.

Results

The effect of systematically altering the isolation conditions on the total calcium content of liver mitochondria is shown in Fig. 1. The mitochondrial calcium content varies from 2 to 10nmol·(mg of protein) $^{-1}$ depending on the Ca^{2+} content of both the liver perfusion medium and the Percoll-density-gradient solutions, and on the presence of inhibitors of mitochondrial Ca^{2+} uptake or efflux.

The highest values for the calcium content [approaching 10nmol·(mg of protein) $^{-1}$] were observed when the perfusate contained 1.3mM- Ca^{2+} , and mitochondrial Ca^{2+} flux inhibitors were absent (column 1). The addition of 0.4mM-nupercaine, an inhibitor of mitochondrial Ca^{2+} efflux (Dawson *et al.*, 1979; Dawson & Fulton, 1980), had essentially no effect (column 2), whereas the presence of either 1.6 μM -Ruthenium Red (column 3) or Ruthenium Red plus nupercaine (column 4) resulted in a 40% decrease of the mitochondrial calcium content.

An even greater effect on the calcium content was observed when the perfusate Ca^{2+} concentration was reduced from 1.3mM to either approx. 6 μM (columns 5–8) or approx. 0.1 μM (columns 9–12) 5min before homogenization of the tissue. Under these conditions mitochondrial calcium contents of between 3 and 4nmol·(mg of protein) $^{-1}$ were recorded, whereas the inclusion of Ruthenium Red decreased the content further to between 2 and 3nmol·(mg of protein) $^{-1}$ (column 7).

The reduction of the free Ca^{2+} concentration to 0.1 μM in all the density gradient solutions, as well as in the perfusate (column 13) resulted in the recovery of 2nmol of calcium·(mg of protein) $^{-1}$. Under these conditions neither Ruthenium Red nor nupercaine had any significant effect on the mitochondrial calcium content (columns 14–16).

These data suggest that large-scale Ca^{2+} redistributions may occur during the isolation of mitochondria even when using a very rapid

Fig. 1. Effect of different isolation conditions on the mitochondrial total calcium content

Livers of fed rats were perfused as described in the Experimental procedures section. At 10 min of perfusion the perfusate free Ca^{2+} concentration was adjusted to the values shown in the Figure and 5 min later the median lobe was excised and mitochondria prepared by Percoll-density-gradient centrifugation as described in the Experimental procedures section. The free Ca^{2+} concentration of the Percoll solutions was adjusted as shown in the Figure and in some experiments nupercaine (0.4 mM) or Ruthenium Red ($1.6\text{ }\mu\text{M}$) was present as indicated. For all conditions free Ca^{2+} concentrations were determined by computer analysis. Total calcium contents were determined by atomic-absorption spectroscopy, as described in the Experimental procedures section. The data represent the means of results from between three and five independent experiments performed for each condition. For the sake of clarity S.E.M. values have been omitted, but ± 0.75 for columns 1–4, ± 0.53 for columns 5–8, ± 0.28 for columns 9–12 and ± 0.14 for columns 13–16 was never exceeded.

isolation procedure. Hence further experiments were designed to examine these redistributions in more detail, with the aim of establishing conditions of minimal Ca^{2+} movements during the isolation procedure.

First, the effect of reducing the perfusate Ca^{2+} concentration from 1.3 mM to approx. $0.1\text{ }\mu\text{M}$ on the calcium content of subsequently isolated mitochondria was examined in more detail. Data in Fig. 2 show that as the perfusate Ca^{2+} concentration is decreasing from 1.3 mM to $0.1\text{ }\mu\text{M}$ the mitochondrial calcium content also decreases

Fig. 2. Effect of the perfusate Ca^{2+} concentration on the mitochondrial total calcium content

Livers of fed rats were perfused with medium containing 1.3 mM Ca^{2+} for 10 min as described in the Experimental procedures section. At 10 min the infusion of CaCl_2 was terminated and $25\text{ }\mu\text{M}$ EGTA was administered. At specified times thereafter, as indicated in the Figure, the median lobe was excised and mitochondria prepared as described for column 13 in Figure 1. The mitochondrial total calcium content ($\bullet$) and the perfusate Ca^{2+} concentration ($\circ$) was determined as described in the Experimental procedures section. The data represent the results of individual experiments ($\bullet$) or the means of three independent experiments performed ($\circ$).

rapidly, basal values of between 2 and $2.5\text{ nmol} \cdot (\text{mg of protein})^{-1}$ being reached within 3–5 min after withdrawing CaCl_2 from the perfusion medium. Half-maximal effects on either the calcium content or the perfusate Ca^{2+} concentration are observed by approx. 30 s after CaCl_2 removal. A close correlation between the perfusate Ca^{2+} concentration and the mitochondrial calcium content was observed ($r = 0.9668$). The lowest mitochondrial calcium content recorded [$2.1\text{ nmol} \cdot (\text{mg of protein})^{-1}$] was not decreased further by either increasing the time of pre-perfusion at $0.1\text{ }\mu\text{M}$ Ca^{2+} or by increasing the EGTA concentration to $100\text{ }\mu\text{M}$ (results not shown).

Secondly, the effect of low extramitochondrial Ca^{2+} concentrations on the mitochondrial calcium content was examined further by incubating isolated mitochondria with the same concentration of EGTA as that used during the isolation procedure (approx. $30\text{ }\mu\text{M}$). The data in Table 1 show that this concentration of EGTA induces only a very low rate of Ca^{2+} efflux corresponding to $1.1\text{ nmol} \cdot (\text{mg of protein})^{-1}$ during 8 min of incubation.

Discussion

The results of the present study indicate that significant Ca^{2+} movements may occur during the isolation of mitochondria, and furthermore they allow a realistic estimation of the calcium content *in vivo* to be made. Previous estimates of the total calcium content in isolated mitochondria have yielded values ranging between 5 and 25 nmol·(mg of protein)⁻¹ (Carafoli & Lehninger, 1971; Reed & Lardy, 1972; Claret-Berthon *et al.*, 1977; Borle & Studer, 1978; Hughes & Barritt, 1978; Yamazaki *et al.*, 1980; Murphy *et al.*, 1980; Taylor *et al.*, 1980; Reinhart *et al.*, 1982b; Coll *et al.*, 1982; Joseph *et al.*, 1983). The reasons for these large variations, however, have not been investigated. Those studies that have assessed the movement of Ca^{2+} during mitochondrial isolation have used either Ca^{2+} -loaded mitochondria, or examined only the ability of isolated mitochondria to maintain a given Ca^{2+} load (Claret-Berthon *et al.*, 1977; Berthon *et al.*, 1981) and hence have not determined the mitochondrial calcium content *in vivo*. Even so, previous work does suggest that Ca^{2+} can be accumulated by mitochondria during homogenization and fractionation (Slater & Cleland, 1953; Cohn *et al.*, 1967; Chen *et al.*, 1974; Hughes & Barritt, 1978). Although the inclusion of EGTA or La^{3+} in homogenization media decreases the mitochondrial calcium content (Chen *et al.*, 1974; Hughes & Barritt, 1978), the possibility of Ca^{2+} movements from the interstitial space or the outside of the plasma membrane into mitochondria, or Ca^{2+} movements during the isolation period, cannot be ruled out by such an approach. Our data are consistent with and extend reports of Ca^{2+} movements during isolation, by showing that Ca^{2+} movements leading to 5-fold changes in the mitochondrial calcium content may occur during preparation, and that mitochondrial Ca^{2+} flux inhibitors are only partially effective in minimizing these fluxes.

In attempting to prepare mitochondria with minimally disturbed total calcium contents, data obtained in the present study provide circumstantial evidence indicating that under most isolation conditions mitochondria are accumulating rather than releasing Ca^{2+} .

First, Ruthenium Red was found to lower the mitochondrial calcium content when the concentration of free Ca^{2+} in either the perfusate or the density-gradient solutions is above 0.1 μM , with maximal effects observed at 1.3 mM- Ca^{2+} (Fig. 1). Nupercaine, however, is without effect, indicating that although significant net Ca^{2+} uptake is taking place under these conditions, Ca^{2+} efflux is negligible. The minimal net loss of calcium is consistent with the relatively low V_{max} [4.7 nmol·min⁻¹·(mg of protein)⁻¹] of this activity (Coll *et*

Table 1. Effect of EGTA on the mitochondrial calcium content *in vitro*

Livers of fed rats were perfused with medium containing 1.3 mM- Ca^{2+} for 15 min, at which time mitochondria were prepared as described for column 1 in Fig. 1. Half of the mitochondrial fraction (approx. 20 mg of protein) was resuspended in 2 ml of isolation medium and the other half resuspended in 2 ml of isolation medium containing 25 μM -EGTA, incubated at 37°C for the times indicated, the mitochondria pelleted by centrifugation and a portion of the resulting supernatant (0.4 ml) assayed for the calcium content. The data represent means $\pm$ S.E.M. for three independent experiments.

Incubation time (min)	Mitochondrial calcium content [nmol·(mg of protein) ⁻¹]	
	Control	+ EGTA
0	9.85 $\pm$ 0.75	
1	9.69 $\pm$ 0.07	9.04 $\pm$ 0.09
2	9.58 $\pm$ 0.09	8.68 $\pm$ 0.011
5	9.02 $\pm$ 0.06	7.85 $\pm$ 0.13
8	8.99 $\pm$ 0.10	7.85 $\pm$ 0.12

al., 1982) coupled to the known effects of P_i and low temperatures in depressing Ca^{2+} efflux rates (Zoccarato & Nicholls, 1981). In contrast, the maximal activity of the Ca^{2+} uniporter is over 150 times greater, being limited only by the respiratory chain activity (Vinogradov & Scarpa, 1973), and is known to be operative even at sub-zero temperatures (Chance *et al.*, 1979).

Secondly, the rapidity with which a reduction of the extracellular Ca^{2+} concentration results in a lowered mitochondrial calcium content (half-maximal effects are at 30 s; Fig. 2) makes it unlikely that measured calcium content values of less than 10 nmol·(mg of protein)⁻¹ represent a loss of Ca^{2+} , especially since the rate of such a loss would be approx. 15 nmol·min⁻¹·(mg of protein)⁻¹, which is three times greater than the measured V_{max} for mitochondrial Ca^{2+} efflux (Coll *et al.*, 1982).

Thirdly, the direct addition of 30 μM -EGTA to mitochondria, resulting in an extramitochondrial free Ca^{2+} concentration well below the set point, induces only a small decrease in the total calcium content (Table 1), confirming that in the presence of P_i the rate of mitochondrial Ca^{2+} efflux is very low (Zoccarato & Nicholls, 1981). Hence it appears that mitochondrial Ca^{2+} efflux is minimal under the isolation conditions examined, whereas significant rates of Ca^{2+} uptake occur under all conditions except where the Ca^{2+} content of all solutions used to isolate the organelle are close to the mitochondrial set point.

Mitochondria isolated under these conditions contain approx. 2 nmol of calcium·(mg of pro-

tein)⁻¹. Such a low endogenous calcium content is consistent with data obtained for smooth muscle using electron probe analysis in conjunction with cryo-ultramicrotomy as an alternative technique for quantifying the composition of cellular organelles *in situ* (Shuman *et al.*, 1976; Somlyo *et al.*, 1978, 1979).

A mitochondrial calcium content of only 2 nmol·(mg of protein)⁻¹ has important ramifications as to the role mitochondria play in cellular Ca²⁺ homeostasis, since it has been shown that the ability of isolated mitochondria to buffer the ambient Ca²⁺ concentration is dependent on the total calcium content of the organelle (Joseph *et al.*, 1983). These workers showed that by experimentally altering the total mitochondrial calcium content between 1.5 and 20.5 nmol·(mg of protein)⁻¹, the Ca²⁺ setpoint could be increased by more than 3-fold. Such a change has been attributed to an increase in the matrix free Ca²⁺ concentration, in parallel with an increase in the total calcium content (Coll *et al.*, 1982). Hence a necessary condition for mitochondria to effectively buffer the cytoplasmic Ca²⁺ concentration is that the rate of mitochondrial Ca²⁺ efflux is constant (Nicholls & Åkerman, 1982; Åkerman & Nicholls, 1983). Under the conditions employed by Joseph *et al.* (1983) this does not occur until mitochondria contain greater than 10 nmol of calcium·(mg of protein)⁻¹. Hence mitochondria containing only 2 nmol of calcium·(mg of protein)⁻¹ may be expected to play little role in the regulation of cytoplasmic Ca²⁺.

However, although such a conclusion may be valid for specific conditions *in vitro*, the presence of P_i or other Ca²⁺-complexing ligands *in vivo* may significantly alter the relationship between the total calcium content and the Ca²⁺ setpoint. Hence the formation of matrix Ca²⁺ complexes *in vivo* may result in a constant rate of Ca²⁺ efflux by buffering the matrix free Ca²⁺ concentration. Indeed the addition of P_i to isolated mitochondria has recently been shown to decrease the rate of Ca²⁺ efflux, presumably by Ca²⁺-P_i complex formation (Zoccarato & Nicholls, 1981). The regulation of the cytoplasmic Ca²⁺ concentration by mitochondria containing even low concentrations of total Ca²⁺ thus remains an open question until more is known about Ca²⁺-complexing ligands *in vivo*.

A further consequence of both a low mitochondrial calcium content and the formation of matrix Ca²⁺ complexes is that the matrix free Ca²⁺ concentration *in vivo* may be much lower than the previously estimated value of 16 μM (Joseph *et al.*, 1983). The application of a null-point titration technique to isolated mitochondria *in vitro* has yielded a Ca²⁺ activity coefficient of 7 × 10⁻⁴

(Coll *et al.*, 1982), indicating that even in the absence of any added permeant anions, more than 99.9% of the total mitochondrial calcium is bound to endogenous ligands. Thus mitochondria containing 2 nmol of calcium·(mg of protein)⁻¹ may have a matrix free Ca²⁺ concentration of 1.4 μM *in vitro* and possibly even lower free Ca²⁺ concentrations *in vivo*. Consequently, the finding that a number of matrix-located enzymes are regulated by micromolar Ca²⁺ concentrations (McCormack & Denton, 1980; Denton *et al.*, 1980; Hansford, 1981; Hansford & Castro, 1981) may be of physiological significance.

We conclude that both the mitochondrial total and free Ca²⁺ concentrations are lower than previously thought and that non-mitochondrial Ca²⁺-translocation cycles may play a significant role in the maintenance of the cytoplasmic Ca²⁺ concentration, whereas the mitochondrial Ca²⁺ translocation cycle may regulate the matrix free Ca²⁺ concentration. However, under some conditions, such as during α-adrenergic agonist administration (Reinhart *et al.*, 1982a, 1984), the release of bound mitochondrial calcium may contribute to the elevation of the cytoplasmic Ca²⁺ concentration indicating that the function of the mitochondrial Ca²⁺-translocation cycle may vary, depending on the ratio of free/bound calcium on either side of the mitochondrial inner membrane. Finally these studies highlight the need to examine mitochondrial properties under conditions relating more closely to the state *in vivo*.

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