CALCIUM TRANSPORT IN ISOLATED RAT HEPATOCYTES

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1. Introduction

The accumulation of Ca^{2+} by isolated mitochondria depends on an energy driven process which includes an energy independent interaction with high and low affinity binding sites at the membrane [1,2]. Ruthenium red [3] and La^{3+} [4] are powerful inhibitors of Ca^{2+} transport. Ca^{2+} was shown to redistribute several anions across the mitochondrial membranes [5] and it has been pointed out that ion accumulation by mitochondria might control the ionic environment in the cell [2,6–8].

More recently another role for Ca^{2+} as a regulating factor in several metabolic pathways as, e.g., glycolysis [9] or gluconeogenesis [10,11], or as an intracellular mediator in hormonal action [11,12] has been suggested (for review, see [13,14]). Ca^{2+} metabolism of the liver has been followed in slices [15], in the isolated perfused organ [11], and to some extent in isolated cells [16–18]. However, changes in Ca^{2+} concentration under those conditions were found to occur rather slowly, which may be due in part to permeation problems [17,19]. We therefore studied Ca^{2+} uptake by isolated rat liver cells with methods which have been used to follow Ca^{2+} movements in isolated mitochondria [20], or recently in isolated ascites tumor cells [21].

The experiments reported here show that rat liver cells are able to accumulate Ca^{2+} within 3–4 min after addition, when substrates for mitochondrial oxidation are provided. This uptake of $Ca^{2+}$is not supported by glycolytic intermediates. Endogenous and accumulated Ca^{2+} can be released from the cells by the ionophore A23187. Sr^{2+} and Mn^{2+} are taken up by the cells under similar conditions, although Mn^{2+} uptake was found to occur at only 1/3 to 1/4 the rate of Ca^{2+} or Sr^{2+} uptake. In contrast to findings with ascites tumor cells [21], Ca^{2+} uptake was not affected by Mg^{2+}. Either ruthenium red or $LaCl_3$ (3–4 nmoles/mg protein) were sufficient to give a 50% inhibition in the rate of Ca^{2+} uptake. Inhibition of mitochondrial oxidation by antimycin A suppressed any Ca^{2+} uptake. Polarographic measurements demonstrate that the uptake of Ca^{2+} is accompanied by increased respiration as well as proton release from the cell. The ratio Ca^{2+}/H^{+} was found to be 1:1 on molar basis whereas the ratio $Ca^{2+}/(O)$ was 'superstoichiometric'. The similarities between isolated mitochondria in vitro and mitochondria in their cellular environment in vivo are discussed.

2. Materials and methods

Isolated hepatocytes were prepared from livers of male Sprague–Dawley rats (200–250 g) using the method of Zahlten et al. [22]. Collagenase (Worthington Biochemical Corp., CLS II 43 JO 43, 199 units/mg) was added to the perfusion medium to give a concentration of 2.6 mg/g liver (w/w). After digestion the liver cells were separated from the connective tissue by filtration through one layer of cheese cloth and after centrifugation subsequently washed for four times in a medium containing 118 mM NaCl, 5.9 mM KCl, 1.2 mM $MgSO_4$, 16.2 mM sodium morpholinopropane sulfonate (MOPS) buffer, pH 7.4, supplemented with 1.5 g/100 ml bovine serum albumin (Bovine albumin, Fraction V, Lot No. F 33004, Amour Pharmaceutical Co., Chicago, Ill., USA). The cells were resuspended in the same medium to give a final concentration of 6.25×10^6 cells/ml (7–9 mg protein/ml). This cell suspension was kept on ice and used for all experiments.

Ca^{2+} movements were followed either spectrophotometrically in an Aminco–Chance spectrophotometer using murexide as an indicator (wavelength 540–507 nm) [20], or polarographically by means of a Ca^{2+} sensitive electrode (Calcium Selection, Beckman Instruments Inc., Fullerton, Calif., USA). Since both Sr^{2+} and Mn^{2+} complex with murexide, the uptake of these cations was recorded spectrophotometrically using the same method as described above for Ca^{2+} [20]. Oxygen uptake was measured polarographically with a Clark-type electrode; proton movements were followed simultaneously using a pH electrode. For protein determination, an aliquot of cells was separated by centrifugation from the suspending medium and solubilized in 1% desoxycholate (w/v). Protein was determined with the biuret method using serum albumin as a standard

All incubations were at 25°C in a medium containing 6.2 mM KCl, 154 mM NaCl and 10 mM sodium morpholinopropane sulfonate buffer, pH 7.4. Other experimental conditions are as specified in the legends.

Chemicals were from the following sources: Murexide, Eastman Kodak Co.; morpholinopropane sulfonic acid, L-malic acid and sodium-D,L-β-hydroxybutyrate, Sigma Chemical Co.; L-lactic acid and L-glutamic acid, Schwarz/Mann; sodium succinate and sodium pyruvate, Boehringer Mannheim; $LaCl_3$ ultrapure (REQ) Lot No. 120573, Alfa Products; ruthenium red, Lot No. 97971, as well as rotenone, K & K Laboratories; antimycin A, Kyowa; other chemicals were analytical grade reagents, Baker Chemical Corp. and Mallinckrodt. The ionophore A23187 was a generous gift of Dr. Robert Hamill, Eli Lilly Co., Indianapolis, Ind., USA.

3. Results and discussion

Isolated rat liver cells, like ascites tumor cells [21] require the addition of an energy source to accumulate Ca^{2+}. Fig. 1 shows a comparison of Ca^{2+} uptake initiated by the addition of succinate, followed either directly with a Ca^{2+} sensitive electrode or indirectly by means of absorbancy changes due to the formation or breakdown of the Ca^{2+}–murexide complex, monitored by dual-wavelength spectroscopy. The addition of 5 mM succinate to the incubation medium causes a rapid accumulation of added Ca^{2+} by the liver cells, which

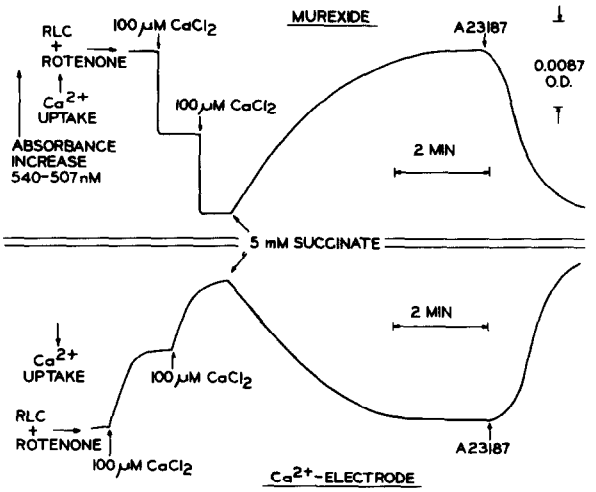

Fig. 1. Ca^{2+} uptake by isolated liver cells followed either spectrophotometrically (upper curve) or polarographically (lower curve). Rat liver cells (RLC) were incubated at 25°C in the presence of 1 μM rotenone in a medium containing 154 mM NaCl, 6.2 mM KCl and 10 mM sodium morpholinopropane sulfonate buffer, pH 7.4. For the spectrophotometric assay, murexide was added to give a concentration of 100 μM. After the addition of $CaCl_2$ (final concentration 200 μM), Ca^{2+} uptake was initiated by the addition of 5 mM succinate. Where indicated, 3.3 μg A 23187/ml were added. The total incubation was 3 ml and 9 ml for the spectrophotometric and polarographic assay, respectively. Cellular protein was 1.9 mg/ml.

is completed in about 3–4 min. The initial rate of Ca^{2+} accumulation under these conditions was 0.75 nmoles × sec^{-1} × mg $protein^{-1}$ ($n = 4$). Assuming that about 20% of the cell protein represents mitochondrial protein, this rate is comparable to the respective value determined for isolated liver mitochondria [23]. The accumulated Ca^{2+} is released by small amounts of A23187 an ionophore specific for divalent cations [24]. Since under conditions with rapid Ca^{2+} uptake (or release) the response time of the Ca^{2+} electrode may become rate limiting, most experiments reported here were done by spectroscopy. Fig. 2 demonstrates that the uptake of Ca^{2+} driven by succinate oxidation (C) is completely abolished in the presence of either antimycin A (A) an inhibitor of mitochondrial oxidation or ruthenium red (B), which is known as an inhibitor of mitochondrial Ca^{2+} transport [3]. Beside succinate, only glutamate (D) and β-hydroxybutyrate (not shown) were found to be effective in stimulating Ca^{2+} uptake by the isolated hepatocyte. L-Malate was less effective,

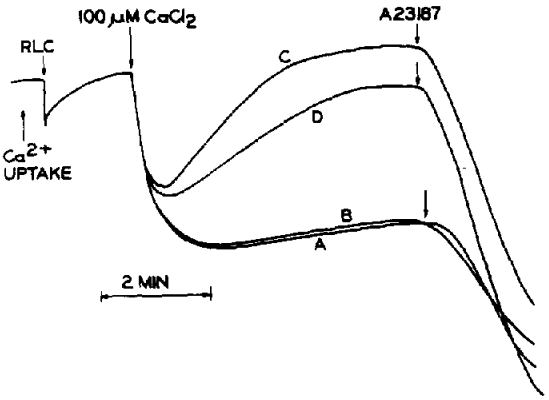

Fig. 2. Ca^{2+} uptake of isolated liver cells under various experimental conditions. The incubation medium was as given under fig. 1 (polarographic assay), except that rotenone was omitted. The cells (1.9 mg protein/ml) were incubated for 90 sec at 25°C under the following conditions: (A) 5 mM succinate + 1.1 μg/ml antimycin A; (B) 5 mM succinate + 10 μM ruthenium red; (C) 5 mM succinate; (D) 5 mM glutamate. The reaction was started by addition of 100 μM $CaCl_2$. Where indicated, 3.3 μg/ml A23187 were added (arrows).

whereas L-lactate, pyruvate or D-glucose were unable to increase Ca^{2+} uptake beyond the basal rate, when tested under comparable conditions.

To verify that the Ca^{2+} accumulation observed was not due to a contamination with mitochondria derived from broken cells, the cell suspension was centrifuged under conditions where mitochondria do not sediment (500 *g* for 1 min). This supernatant when tested under the same conditions did not support Ca^{2+} uptake. The difference in response towards different substrates may be in part due to a restricted permeability of the cell, e.g., for L-malate, which is also found in the isolated perfused liver and in liver slices [19]. On the other hand, glycolytic intermediates incubated over a rather short time period will not support mitochondrial energy conservation in isolated cells [17,21]. The oxygen consumption of rat liver cells in the presence of Ca^{2+} is shown in fig. 3. The addition of Ca^{2+} is followed by a rapid increase in respiration which coincides with the Ca^{2+} uptake. Initial rates of respiration return, as soon as the amount of Ca^{2+} added is accumulated. The Ca^{2+} uptake is accompanied by proton extrusion from the cells (lower part fig. 3). The rate of Ca^{2+} uptake/

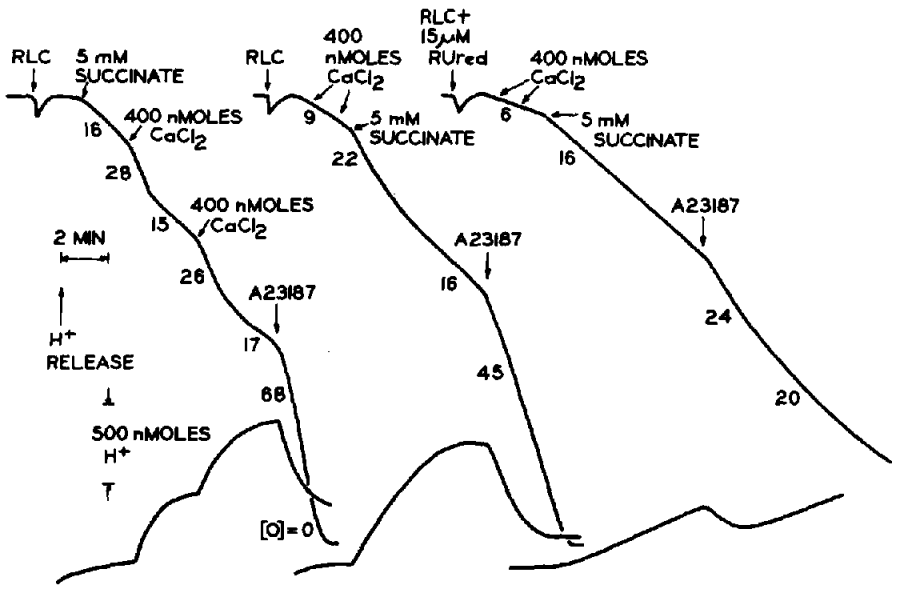

Fig. 3. The effect of Ca^{2+} on the oxygen consumption of isolated liver cells. Cells (1.7 mg protein/ml) were incubated at 25°C in the basic medium as given under fig. 1. A23187 was added to give a concentration of 1.3 μg/ml, other additions were as indicated. The numbers beside the traces represent ng atom O_2/min/mg protein utilized at the respective point. RUred = ruthenium red. Concomitant changes in hydrogen concentration are shown in the lower part of this figure. The oxygen content of the medium was taken as 0.474 μg atom O_2/ml at 25°C.

H^+ release was found to be 1:1 on a molar basis, whereas Ca^{2+} /(O) was determined as 3.52 ± 0.11 (n = 10) by means of total oxygen consumption or as 5.86 ± 0.33 (n = 10) when taking into account only the 'extra' oxygen uptake. Since in these experiments succinate was used as a substrate, the values calculated per phosphorylation site are 1.76 or 2.93, respectively. These ratios are in good agreement with data obtained with isolated mitochondria [25,26]. The 'superstoichiometric' value of about 3/site is found in mitochondria which are incubated in the absence of phosphate or other permeant anions in a saline medium containing 160 mM NaCl [27].

The addition of A23187 causes a further acceleration of respiration and is accompanied by a re-uptake of protons into the cells. This increase in oxygen consumption is due to the rapid release of Ca^{2+} from the mitochondria (and out of the cell) induced by this ionophore, which must be followed by an energy-consuming reaccumulation via the specific Ca^{2+} transporting system [2]. When ruthenium red was present, both the Ca^{2+} -induced increase in respiration as well as the concomitant proton release were abolished. The acceleration of respiration observed after A23187 addition is most likely an expression of recycling of endogenous Ca^{2+} which is continually released by the ionophore. Since the reaccumulation of Ca^{2+} is prevented by ruthenium red the system becomes depleted of Ca^{2+}, which is demonstrated by a progressive inhibition of respiration (fig. 3).

In addition to Ca^{2+}, both Sr^{2+} and Mn^{2+} are readily taken up by rat liver cells (fig. 4), although the rate of uptake of Mn^{2+} is relatively slow. This is in agreement with data obtained with liver mitochondria [20], where Mn^{2+} uptake was found to be 1/3 to 1/4 the rate of Ca^{2+} uptake, or with ascites tumor cells [21], which have been shown to accumulate Mn^{2+} at a considerable lower rate. However, the rate of uptake of all three cations tested is increased about 3–4 times in the presence of mitochondrial substrates such as succinate or glutamate, when compared to the endogenous rate.

Since Mg^{2+} was found to be a competitive inhibitor of Ca^{2+} accumulation in tumor cells [21], the effect of increasing Mg^{2+} concentrations on the Ca^{2+} uptake by isolated liver cells was investigated (fig. 5). The addition of Mg^{2+} up to concentrations of 20 mM was found to be ineffective in influencing the rate of Ca^{2+} accumulation in this system. However, the rate of Ca^{2+}

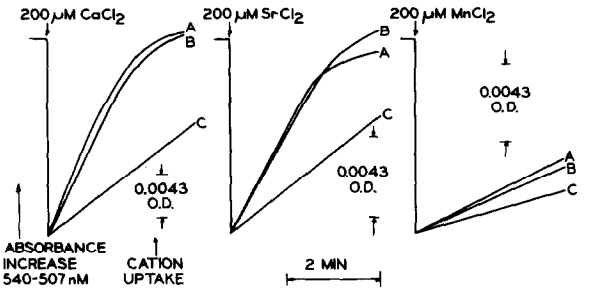

Fig. 4. Uptake of Sr^{2+} and Mn^{2+} by isolated liver cells. Cells (2.7 mg protein/ml) were incubated at 25°C in the presence of either 5 mM succinate (A), 5 mM Glutamate (B) or no substrate (C). After 2 min the reaction was started by addition of the indicated cations in a final concentration of 200 μM. The medium was as given under fig. 1 (spectrophotometric assay). Rotenone was omitted.

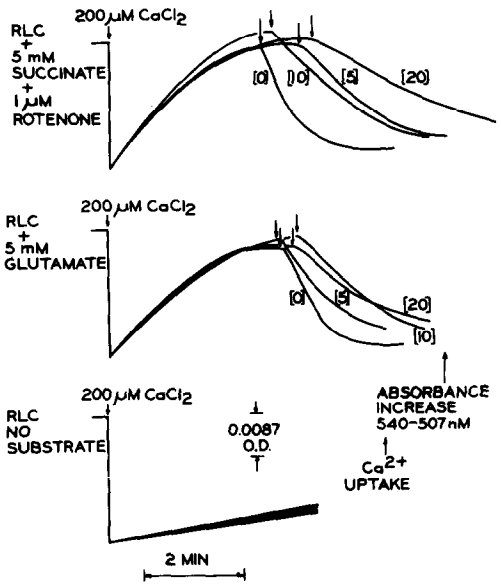

Fig. 5. Effect of varying concentrations of Mg^{2+} on Ca^{2+} uptake by isolated liver cells. Cells were washed and resuspended in a Mg^{2+} -free medium. They were incubated in the standard incubation medium (fig. 1) at 25°C in the presence or absence of substrate as shown here. $MgCl_2$ was added after 1 min. The reaction was started after 2 min by addition of 200 μM Ca^{2+}. The numbers in the brackets give Mg^{2+} concentration in mM. The arrows indicate the addition of 1.67 μg/ml A23187. Cellular protein was 2.0 mg/ml.

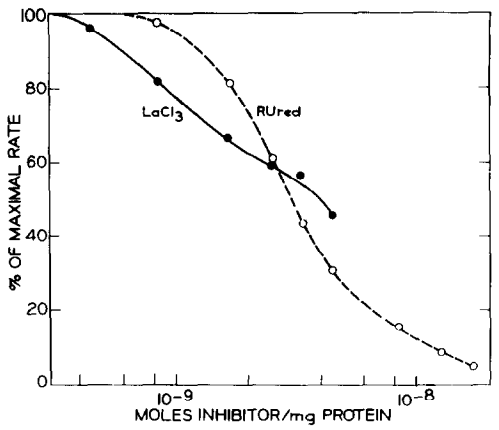

Fig. 6. Inhibition of Ca^{2+} uptake in isolated liver cells by $LaCl_3$ and ruthenium red. Cells (2.4 mg protein/ml) were incubated in the presence of 5 mM succinate and either $LaCl_3$ or ruthenium red (RUred) at the indicated concentrations. After 2 min the reaction was started by addition of 200 μM $CaCl_2$. The initial rate of Ca^{2+} uptake in the absence of inhibitor was taken as 100%. Other conditions were as given under fig. 1 (spectrophotometric assay).

release from the cells induced by A23187 was markedly decreased with increasing Mg^{2+} concentrations. This is consistent with the finding that A23187 has a higher affinity for Mg^{2+} than for Ca^{2+} [24], which in the presence of Mg^{2+} will result in a smaller amount of 'free' ionophore available to release Ca^{2+} from the cells.

In isolated mitochondria, Ca^{2+} transport is strongly inhibited by ruthenium red [3] or by lanthanides [4, 28]. The inhibition of Ca^{2+} uptake in isolated liver cells by these compounds is shown in fig. 6. No inhibition (ruthenium red) or only a slight inhibition ($LaCl_3$) was observed at concentrations under 1 nmole/mg protein. Whereas in isolated rat liver mitochondria a 50% inhibition by lanthanides was observed at concentrations as low as 0.03 nmoles/mg protein [28], 3–4 nmoles/mg protein were required to obtain 50% inhibition with isolated cells. Ruthenium red on the other hand inhibited 50% at a concentration of 3–4 nmoles/mg protein; thus, it is equally effective on intact cells and mitochondria [3].

These data suggest that the liver cell is not readily permeable to either of these inhibitors. However, it cannot be ruled out at this time that considerable amounts of these compounds may be unspecifically bound to the cell membrane, which would decrease their effectiveness. On the other hand, there is evidence that ruthenium red may not penetrate cellular membranes [29], which would locate its inhibitory action at the outer cell surface. Similar conclusions were drawn from experiments with ascites tumor cells [21].

In summary, the similarity in Ca^{2+} metabolism between isolated hepatocytes and isolated liver mitochondria supports the view that Ca^{2+} uptake by liver cells is a function of a coupled mitochondrial energy conservation. Under these circumstances, mitochondria in situ in their cellular environment behave as intact as isolated mitochondria. The data presented are in favor of the concept, that under physiological conditions, a redistribution of Ca^{2+} may control and determine the compartmentation of anionic metabolites and vice versa. In the living cell this process may be controlled by the functional state of the mitochondria.

Acknowledgements

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