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SEPARATE PATHWAYS FOR Ca^{2+} UPTAKE AND RELEASE IN LIVER MITOCHONDRIA

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1. Introduction

Evidence accumulated in recent years [1–3] has indicated that the uptake of Ca^{2+} by mitochondria is mediated by an electrophoretic process, which responds to the electrical component of the protonmotive force generated by respiration, and occurs with a charge transfer of 2. If the uptake process were to reach equilibrium against a membrane potential of about 0.18 V, one would expect a gradient of Ca^{2+} activities between the mitochondrial matrix and the medium of $\sim 10^6$. Several laboratories, however, have reported deviations from the Nernstian equilibrium in measuring energy-linked uptake of Ca^{2+} [4] or of its paramagnetic analogue Mn^{2+} [5]. Considerations of the activities of Ca^{2+}-dependent enzymes in mitochondria and in the cytoplasm [6] also suggest that the gradient of ionized Ca^{2+} concentrations between respiring mitochondria and the medium is much less than 10^6. That the process of energy-linked uptake of Ca^{2+} is not permitted to reach equilibrium is also indicated by theoretical in vivo considerations. Although the ionized Ca^{2+} concentration in the mitochondria has not yet been determined, it is very likely that its upper limit does not exceed 10^{-4} M [7,8]. This would correspond to the unrealistically low level of 10^{-10} M for the Ca^{2+} activity in the cytoplasm. A convenient way to explain the deviation from the Nernstian equilibrium is by postulating a separate Ca^{2+} releasing pathway, which should obviously be independent of the negative inside membrane potential, or even be driven by it in the direction of Ca^{2+} release. Such an independent release pathway has been demonstrated in heart mitochondria [9–11], and in mitochondria for some other tissues [12], and shown to consist of an RR-insensitive, obligatory Na^+/Ca^{2+} exchange system, [10]. In liver mitochondria, and in mitochondria from at least 2 other tissues (kidney and lung), the Na^+/Ca^{2+} exchange does not operate. For liver mitochondria, the proposal has been made [13] that the release of Ca^{2+} occurs via a reversal of the electrophoretic uptake pump. However, as in the case of heart, also in these mitochondrial types the efflux of Ca^{2+} is insensitive to RR [9–13,16]. Since in the presence of RR the influx pathway is completely blocked [14,15] Ca^{2+} efflux will in this case have to occur through a different route. To maintain the proposal that the efflux of Ca^{2+} occurs through a reversal of the electrophoretic influx pathway, it has been postulated [13] that the binding of RR to the electrophoretic uptake carrier, is dependent on the electrical potential difference ($\Delta\psi$) across the inner mitochondrial membrane. Membrane domains of lower $\Delta\psi$ would lead to the slow release of the accumulated Ca^{2+} after the addition of RR and de-energization of the membrane would lead to RR-insensitive Ca^{2+} release.

In deciding for one or two pathways for Ca^{2+} transport in liver mitochondria it is therefore critical to establish whether RR remains bound to the uptake carrier during the efflux of Ca^{2+}. The results obtained here show that the electrophoretic uptake route remains blocked by RR when Ca^{2+} release occurs in de-energized liver mitochondria. Therefore, it is concluded that separate pathways for Ca^{2+} influx and efflux exist also in liver mitochondria.

Abbreviations: BSA, bovine serum albumin; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; RR, Ruthenium Red

2. Materials and methods

Rat liver mitochondria were prepared by a conventional centrifugation procedure using 210 mM mannitol, 70 mM sucrose, 0.5 mM Na-EDTA, and 10 mM Tris–Cl (pH 7.4) as the homogenizing medium [17]. The protein concentration of the mitochondrial suspension was estimated with a biuret procedure.

The composition of the incubation media is detailed in the legends of the figures. The flow dialysis experiments were done as in [18] using ^{106}Ru Red as a tracer. In Ca^{2+} exchange experiments aliquots were withdrawn from the medium at the time intervals specified, and mitochondria collected on Millipore filters (diam. 0.45 μm; filtration time ~5 s). The filters were washed with 1 ml ice cold 140 mM mannitol, 46 mM sucrose, 6 mM Tris–Cl (pH 7.4), 33 mM $CaCl_2$, and 0.03 mM RR, (to prevent Ca^{2+} release). The filters were then analysed for ^{45}Ca.

Appropriate control experiments were carried out to rule out the possibility that the BSA added to the incubation medium removed substantial amounts of FCCP, Antimycin A, rotenone, A23187 and RR. In addition, these compounds were added to the incubation media in excess of the amounts normally employed.

All the chemicals used were of analytical grade. ^{106}Ru Red was synthesized starting from ^{106}Ru-Cl_3 [19]. A23187 was obtained from Eli Lilly and Co., Indianapolis. The solutions of Antimycin A, FCCP, rotenone, and A23187 were prepared in 95% ethanol.

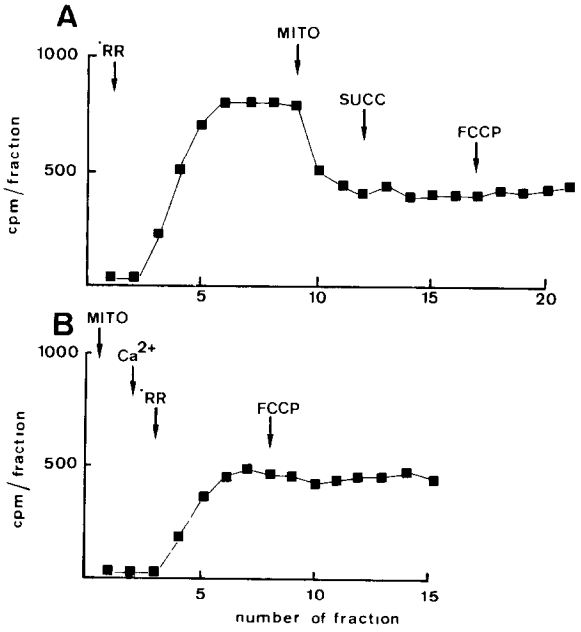

Fig.1. Binding of RR to energized (a) and de-energized (b) rat liver mitochondria. The volume of the upper chamber was 5 ml, that of the lower chamber 0.8 ml. Fractions, 1 ml, were collected. The flow rate was 0.5 ml/min. The buffer used was 120 mM KCl, 10 mM Tris–HCl (pH 7.4). The additions of RR (0.66 μM labelled with ^{106}Ru Red), mitochondria (11 mg protein), succinate (10 mM), Ca^{2+} (1.0 μmol) and FCCP (2×10^{-3} mM) were made in very small volumes. The total radioactivity originally present in the upper chamber corresponded to about 320 000 cpm. Each 1 ml fraction eluted < 0.3% of the total radioactivity.

3. Results and discussion

The flow dialysis experiment illustrated in fig.1 shows that the energization, or the de-energization, of the membrane of liver mitochondria has no influence on the amount of the RR bound. It must be noted that in the experiment described the amount of RR added was 0.3 nmol/mg protein, i.e., the minimal amount sufficient to saturate the electrophoretic uptake 'carrier', which binds RR with $K_d \leqslant 1$ μM [14,15]. It is therefore unlikely that substantial amounts of the inhibitor, in addition to the carrier, are bound non-specifically to other components of the mitochondrial membrane, which contains no other components of comparable affinity [20]. The possibility that RR could become 'redistributed' among membrane binding sites upon de-energization, i.e., that it could leave the electrophoretic uptake carrier, to become bound elsewhere in the membrane, seemed therefore highly unlikely. It was nevertheless necessary to consider it and to rule it out experimentally. The following approach was used. Energized mitochondria were allowed to accumulate a pulse of unlabelled Ca^{2+}. When the uptake was essentially complete, a pulse of $^{45}Ca^{2+}$ was administered, to raise the concentration of ionized Ca^{2+} in the medium to a level, at least equal, but probably higher, than inside the mitochondria. In the absence of inhibitors of energy transformation, additional amounts of Ca^{2+} were taken up, in a reaction which was, as expected, completely inhibited by RR. If mitochondria were de-energized (e.g., with uncouplers) immediately before the addition of the

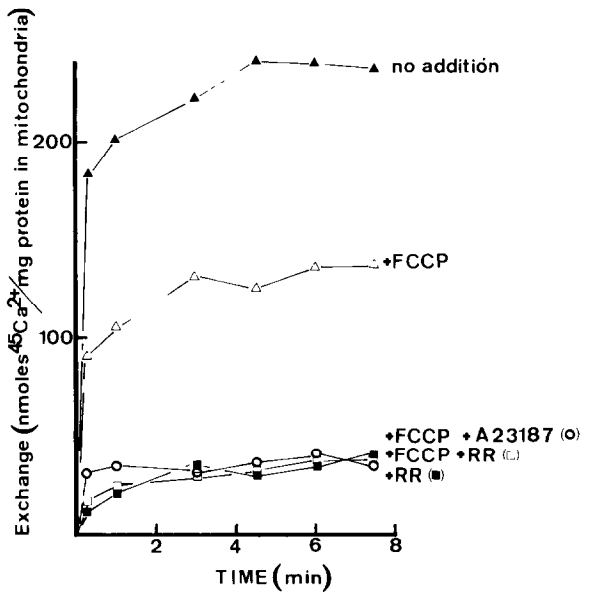

Fig.2. Ca^{2+}/Ca^{2+} exchange in liver mitochondria. Mitochondrial protein, 15 mg, was incubated for 5 min in 210 mM mannitol, 70 mM sucrose, 10 mM Tris-HCl, 0.01 mM rotenone, 0.125 mM butacaine (added to minimize non-specific, external Ca^{2+} binding) 2 mg BSA (pH 7.4), in 1 ml final vol., at 25°C. After the addition of 100 nmol/mg protein $^{45}CaCl_2$, the energy-linked uptake was initiated with 25 mM Tris–succinate (pH 7.4). After 2 min, the uptake of Ca^{2+} was essentially complete. A pulse of 100 mM $^{45}CaCl_2$ 33 mM final conc. was then added (time 0). The volume, and the concentration, of the Ca^{2+} pulse were devised to produce no major changes in the osmolarity of the medium. Aliquots, 50 μl, were withdrawn at the times indicated, filtered, and washed as in section 2. RR (0.024 mM) was added immediately before, FCCP (0.01 mM) at the same time as, and A23187 (0.05 mM) immediately after, the $^{45}Ca^{2+}$ pulse.

second pulse of $^{45}Ca^{2+}$, no energy-linked uptake of the additionally added Ca^{2+} could logically take place. Any radioactivity associated with mitochondria in this completely de-energized state could therefore only result from the exchange of the intra- and extramitochondrial Ca^{2+}. Figure 2 shows that such an exchange indeed took place, and leveled off at a point which was evidently determined by the respective magnitudes of the intra- and extramitochondrial Ca^{2+} pools, and by the proportion of the intramitochondrial Ca^{2+} pool existing in an exchangeable form. The important point in fig.2, however, is the fact that the $Ca^{2+}{}_{in}/Ca^{2+}{}_{out}$ exchange observed in the presence of uncouplers was inhibited almost completely by RR. This clearly shows:

(i) That the exchange took place via the RR-sensitive Ca^{2+} carrier;

(ii) That the RR-sensitive Ca^{2+} carrier, here operating as a Ca^{2+}/Ca^{2+} exchange system, remained fully inhibited by RR in the de-energized state.

Some essential controls, aimed at proving that the radioactivity measured on the filters in the absence of RR reflected in large part the intramitochondrial space, are also illustrated in fig.2, and in table 1. The specific Ca^{2+} ionophore A23187, which makes the intramitochondrial Ca^{2+} pool rapidly available to the external medium, was used. Unwashed filters, containing mitochondria de-energized in the absence of RR, retained the same amount of radioactivity in the absence, as well as in the presence, of A23187. By contrast, when the filters were washed, the amount of radioactivity recovered in the presence of A23187 was the same, and as low, as in the experiment in which mitochondria were exposed only to RR. The experiments described in fig.2 and table 1 thus fully support the experiment in fig.1, and add significance to it. In the de-energized state, RR remains bound to the electrophoretic Ca^{2+} carrier, and fully inhibits it. The experiment in fig.2 could be carried out also in the opposite direction, i.e., by loading mitochondria with $^{45}Ca^{2+}$ first, and by adding to them a pulse of unlabelled Ca^{2+} later. The $Ca^{2+}{}_{in}/Ca^{2+}{}_{out}$ exchange, followed in this case by the appearance of radioactivity in the extramitochondrial medium, was very strongly inhibited by RR in the absence of de-ener-

Table 1
Ca^{2+}/Ca^{2+} exchange in liver mitochondria

Conditions	nmol/mg protein
Control	757
FCCP	627
FCCP + A23187	610
RR	425
RR + FCCP	415

Experimental conditions are as in fig.2, except that the filters were not washed. Aliquots, 50 μl, were withdrawn from the medium at 30 s intervals, starting 2 min after the addition of the pulse of $^{45}Ca^{2+}$. The value for each experiment was the mean of 6–8 aliquots. The values given are the mean of 5 experiments (SD ~8%)

gizing agents. In the presence of FCCP, the exchange was still inhibited, although to a slightly lesser extent than in the energized state, by RR. On the basis of these results, it can thus be concluded that an independent pathway for Ca^{2+} release exists also in liver mitochondria. The mechanism, the regulation, and the possible activator(s) of the separate release pathway, however, are at the moment unknown.

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