EFFECT OF RUTHENIUM RED ON CALCIUM EFFLUX FROM RAT LIVER MITOCHONDRIA

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1. Introduction

It is now apparent that Ca^{2+}-uptake in mitochondria is driven by a membrane potential, negative inside, and that calcium is transported with two positive charges [1–8]. Evidence for a specific carrier is provided by the inhibitory effects on Ca^{2+}-translocation of ruthenium red and lanthanides.

The steady state distribution of calcium between mitochondria and the suspending medium most probably does not follow the Nernst equation, which suggests that separate pathways for Ca^{2+}-uptake and release may exist. EPR experiments, in which Mn^{2+} was used as paramagnetic analogue of Ca^{2+}, support this conclusion [9].

However, most evidence for a distinct pathway for Ca^{2+}-efflux has been obtained by experiments with ruthenium red. In fact this compound has been found to have different effects on Ca^{2+}-transport: while it blocks the electrophoretic uptake completely it inhibits only slightly, or not at all, the Ca^{2+}-efflux induced either by calcium chelators or by agents which collapse the membrane potential [9–18]. Furthermore it can induce by itself a release of mitochondrial accumulated calcium. This release has been ascribed to a block of calcium cycling. It would be the consequence of a complete inhibition by ruthenium red of Ca^{2+}-uptake, via the electrophoretic uniporter, whilst Ca^{2+}-efflux via a separate pathway would be unaffected [23].

Whether a high membrane potential is required for ruthenium red to inhibit Ca^{2+}-efflux, via uniporter, is still debated [9,14,19]. Also its effect on Ca^{2+}-uptake by inverted mitochondrial vescicles is not unequivocal [20,21].

Here we have investigated more extensively the inhibitory effect of ruthenium red on Ca^{2+}-efflux from rat liver mitochondria. Under our experimental conditions ruthenium red inhibited significantly the release promoted either by inhibition of the respiration, or by uncouplers or by an inward directed K^+-diffusion in valinomycin-treated mitocondria. In the last case, K_i was found to be similar for both Ca^{2+}-uptake and release.

Loss of respiratory control and a certain decrease of membrane potential were observed with >0.5 nmol ruthenium red/mg protein. It is concluded that although ruthenium red may still be considered as a useful tool in the study of Ca^{2+}-transport, considerable care should be taken in its use otherwise misleading results may ensue.

2. Materials and methods

Rat liver mitochondria were isolated as in [22] in a medium containing 0.25 M sucrose, 5 mM Tris–MOPS (pH 7.2), 1 mM EGTA. They were washed twice omitting the EGTA in the last washing. The mitochondria routinely used contained 4.6–5.5 ngions mobile Ca^{2+}/mg protein. The mobile Ca^{2+} was considered that released by mitochondria after addition of 1 μM FCCP. The experiments were routinely done at 30°C in the cell of a dual-wavelength spectro-

Abbreviations: Arsenazo III, 2-2'-(1-8 dihydroxy-3,6 bisulfo-2,7 naphtalene-bis azo) dibenzene arsonic acid; BSA, bovine serum albumin; EGTA, ethylene glycol bis-(aminoethyl)tetra-acetate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenyl-hydrazone; MOPS, morpholinpropane–sulfonic acid; $\Delta\psi$, membrane potential

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photometer, equipped with a magnetic stirrer. A Clark oxygen-electrode and a K^+-sensitive electrode were inserted into the cell through independent openings. Ca^{2+}-movements were followed spectrophotometrically using Arsenazo III as an indicator on the basis of ΔA at the wavelength pair of 590 minus 570 nm. The membrane potential was measured by following the safranine spectral shifts as in [24]. An internal calibration was carried out for each parameter in all experiments. The incubation standard medium contained 50 mM sucrose, 100 mM choline-chloride, 5 mM Tris–MOPS (pH 7.2), 100 μM KCl, 2 μM oligomycin, 6 mg protein in 3 ml final vol. Ruthenium red (BDH) was purified according to [25]. Its aqueous solution showed an A_{533} peak and its concentration was recalculated on the basis of $\epsilon = 68\,000\ cm^{-1}$. Protein concentration was assayed by the biuret method using bovine serum albumin as standard [30].

3. Results and discussion

The rat liver mitochondria routinely used in these experiments contained ~5 ngions Ca^{2+}/mg protein, which was the usual amount present in the mitochondria at the end of the isolation procedure. Addition of either antimycin or FCCP to the mitochondrial suspension led to the Ca^{2+}-release and this process was markedly inhibited by ruthenium red (fig.1).

It has been reported that the uncoupler-induced Ca^{2+}-efflux is insensitive to ruthenium red [9,11,14, 26]; the discrepancy with our observations is probably due to the different experimental conditions particularly with regard to the intramitochondrial Ca^{2+}-content. In fact the inhibition was less when Ca^{2+}-loaded mitochondria were used (not shown).

Ruthenium red also inhibited the Ca^{2+}-efflux produced by an inward-directed K^+-diffusion obtained by addition of KCl to valinomycin + antimycin-treated mitochondria. This experiment is shown in fig.2: the intramitochondrial calcium was first released following addition of antimycin. Ca^{2+}-reuptake was then driven by the membrane potential generated by an outward directed K^+-diffusion induced by addition of valinomycin. After completion of Ca^{2+}-uptake the addition of 30 mM KCl caused a rapid ejection of Ca^{2+}, most probably as a consequence of an inward directed K^+-diffusion. This efflux is inhibited by ruthenium red. Dixon plots showed that the inhibition is of a non-competitive type and an app. $K_i \sim 2 \times 10^{-8}$ M was

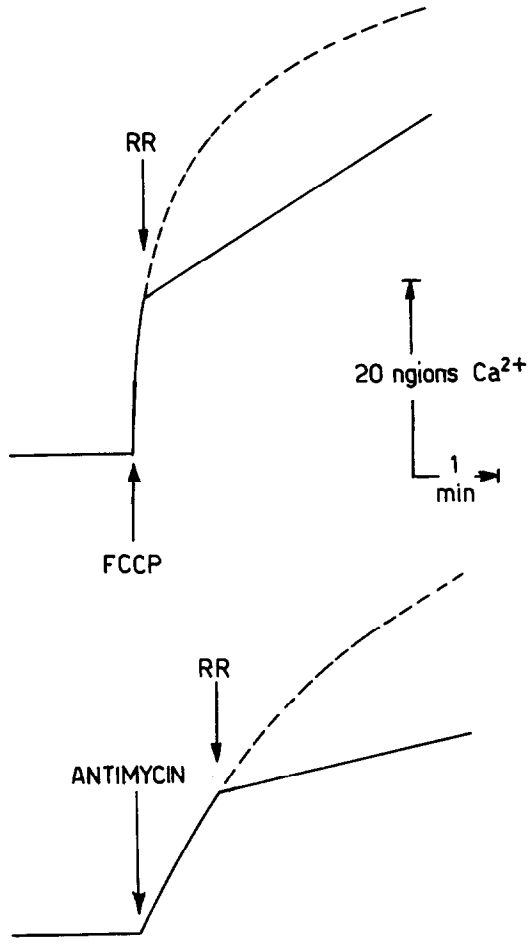

Fig.1. Time-course of Ca^{2+}-release following FCCP or antimycin additions to rat liver mitochondria. Experimental details are described in section 2. Traces express the $[Ca^{2+}]$ in the medium: 0.5 μM FCCP, 0.08 nmol antimycin and 0.25 nmol ruthenium red per mg protein were added at the arrows; 1 μM rotenone was added together with antimycin.

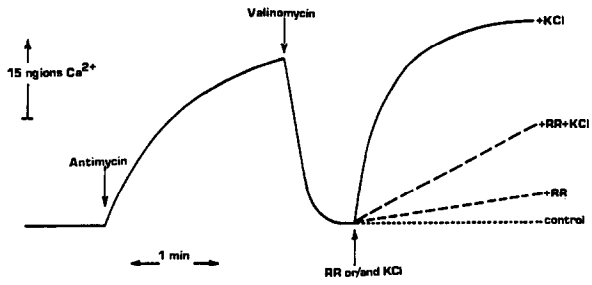

Fig.2. Time-course of Ca^{2+}-translocation in rat liver mitochondria produced by K^+-diffusion; 4 pmol valinomycin/mg protein and 25–30 mM KCl were added at the arrows. Other experimental details were as in fig.1.

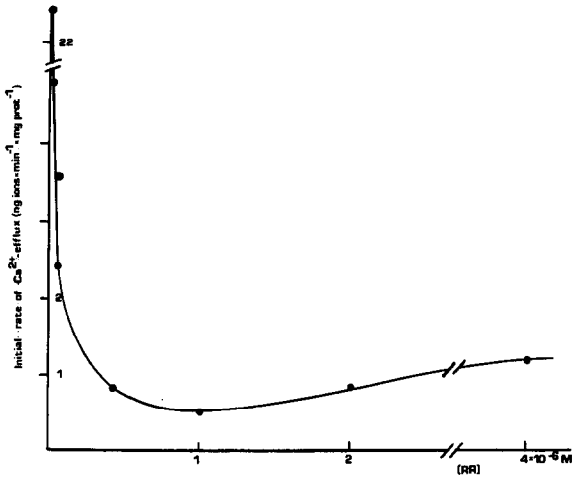

Fig.3. Effect of variable amounts of ruthenium red on Ca^{2+}-efflux induced by KCl addition to valinomycin-treated, not respiring mitochondria. Experimental conditions were as in fig.2.

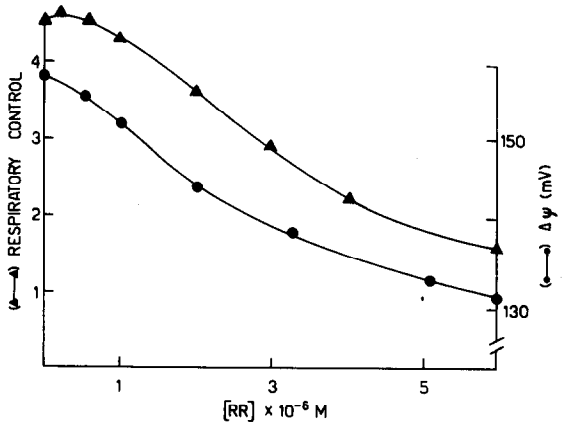

Fig.4. Effect of ruthenium red on respiratory control and membrane potential of rat liver mitochondria. Spectral shifts in safranine (12 μM) were used as indication of the membrane potential. For the measurement of oxygen consumption rates the oligomycin was omitted from the standard medium, which was instead supplemented with 5 mM K-succinate, 1 μM rotenone, 0.5 mM K-phosphate and 0.1 mM ADP.

found. This K_i value is similar to that exhibited by ruthenium red on Ca^{2+}-uptake ($K_i = 3.2 \times 10^{-8}$ M) driven either by valinomycin or by respiration [27].

A linear relationship between the amount of ruthenium red added and the decrease of the Ca^{2+}-release rate was observed up to 10^{-7} M of the dye. With higher concentrations this relationship was no longer linear (fig.3). Indeed the highest inhibition (>96%) was obtained with 10^{-6} M ruthenium red, whereas with higher amounts the inhibition was lower. The curve obtained is probably the result of two overlapping processes. An inhibitory effect on Ca^{2+}-efflux predominates at low ruthenium red concentrations, but a Ca^{2+}-release induced by the dye itself becomes evident at higher concentrations. A similar result has been observed [15] on Ca^{2+}-efflux induced by pentachlorophenol.

It has been reported that ruthenium red does not affect significantly either the respiratory control or the membrane potential [11,14,16]. We have reinvestigated this point using a larger range of ruthenium red concentrations and the results are shown in fig.4. We have confirmed that at low ruthenium red concentrations there are no significant changes in the state 3/state 4 respiratory control, but >1 μM (0.5 nmol/mg protein) a clear decrease was observed due to an increase of the oxgyen consumption rate in state 4. Same degree of respiratory control loss was found when KCl was substituted in the medium for choline chloride, however when sucrose was used, for the medium osmolarity, a much smaller effect was observed.

In parallel to the decrease in respiratory control the extent of the spectral shift of safranine, an indicator of mitochondrial membrane potential, also decreased.

The rate of ruthenium red promoted Ca^{2+}-efflux from Ca^{2+}-loaded mitochondria was found to be inhibited by ADP or BSA [14,29]. We have observed a similar effect also in liver mitochondria containing low amounts of the cation (not shown). Most probably the observed decrease of the ruthenium red-dependent Ca^{2+}-efflux is due to the stabilizing effect of ADP or BSA against membrane damage caused more by ruthenium red than by Ca^{2+}, the level of which is very low [28].

Ruthenium red is a dye which binds to different components of the membrane, but little is known about the mode and the consequences of its binding. Particularly if it causes alterations in the membrane integrity. From our results it is conceivable that some of the effects of ruthenium red on Ca^{2+}-efflux may be due to changes of membrane structure and not only as a result of its binding to the Ca^{2+}-carrier.

However, its effectiveness on inhibiting Ca^{2+}-efflux from liver mitochondria may depend very much on experimental conditions used. Therefore attention

should be taken in its use as misleading conclusions may be drawn.

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