THE EFFECT OF RUTHENIUM RED ON THE UPTAKE AND RELEASE OF Ca^{2+} BY MITOCHONDRIA

by

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Summary

Ruthenium red, an inhibitor of Ca^{2+} binding and transport by mitochondria, promotes the release of Ca^{2+} by mitochondria only if it is added to the assay medium before the accumulation of Ca^{2+} has been completed. Once essentially all of the Ca^{2+} in the medium is taken up by mitochondria, ruthenium red does not induce its release. It is proposed that ruthenium red inhibits Ca^{2+} transport by competing with Ca^{2+} for Ca^{2+} binding sites, possibly Ca^{2+} carrier molecules, on or within the inner mitochondrial membrane.

In recent studies Vasington et al. (1,2) confirmed and extended the observation of Moore (3) that ruthenium red inhibits the energy-dependent uptake of Ca^{2+} by mitochondria. They showed that ruthenium red also inhibits the energy-linked uptake of Sr^{2+} and Mn^{2+}, the K^{+}-driven translocation of Ca^{2+} and the low- and high-affinity binding of Ca^{2+} by mitochondria (1,2). In addition, ruthenium red was found to inhibit mitochondrial respiration but at slightly higher concentration than those which inhibit Ca^{2+} uptake and binding (1,2). In their earlier studies, Vasington et al. (1,2) also reported that ruthenium red neither induces the release of Ca^{2+} from Ca^{2+}-loaded mitochondria nor affects the dinitrophenol-stimulated release of Ca^{2+} by mitochondria.

In this communication we report that ruthenium red can promote the release of Ca^{2+} if it is added to the assay medium before the process of Ca^{2+} accumu-

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lation by mitochondria has been completed. On the basis of the observations reported in this paper we propose that ruthenium red inhibits Ca^{2+} transport and binding by mitochondria by competing with Ca^{2+} for Ca^{2+} binding sites, possibly Ca^{2+} carrier molecules, on or within the inner mitochondrial membrane.

MATERIALS AND METHODS

Rat liver mitochondria were isolated from Wistar strain albino rats in 0.25 M sucrose and were washed twice (4). The H^+ movement was measured with a combination electrode and a Beckman Expandomatic pH meter connected to a strip-chart recorder. Ca^{2+} uptake was measured as described previously (1) following rapid filtration of aliquots of the reaction medium through millipore membranes (0.45 μ pore size). High- and low-affinity Ca^{2+} binding was measured according to Reynafarje and Lehninger (5). Mitochondrial protein concentration was determined by a buiret reaction.

RESULTS

Effect of ruthenium red on the release of calcium from mitochondria

In earlier studies which showed that ruthenium red did not promote the efflux of mitochondrial Ca^{2+}, ruthenium red was added to the test system after Ca^{2+} accumulation by mitochondria had been completed (1,2). We now find that ruthenium red can promote the release of Ca^{2+} if it is added to the assay medium before Ca^{2+} accumulation has been completed (Figure 1). In the experiments shown in Figure 1, the uptake and release of Ca^{2+} was followed by measuring the counter release and uptake of H^+ which parallels the movement of Ca^{2+} (6). It can be seen in Figure 1A that the addition of 800 natoms of Ca^{2+} to an assay medium containing succinate as the respiratory substrate caused a typical State 4 to State 3 transition of mitochondrial respiration and an immediate release of H^+ ions into the extramitochondrial medium. In this experiment the ejection of 750 natoms of mitochondrial H^+ was accompanied by the disappearance of 780 natoms of Ca^{2+} from the extramitochondrial medium, yielding an H^+ ejected/Ca^{2+} accumulated ratio in good agreement with the value

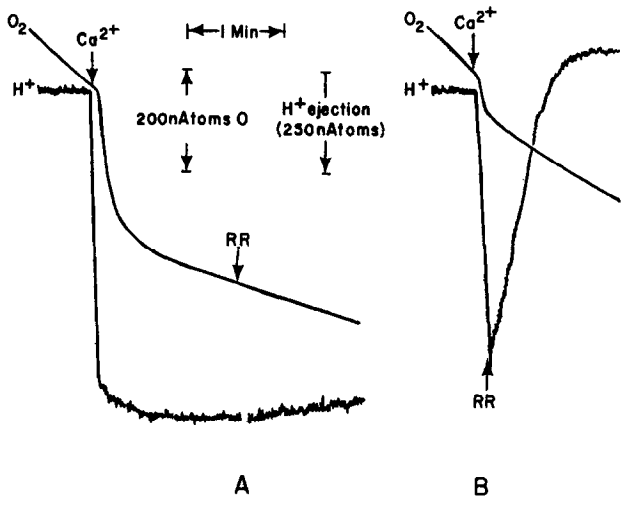

Figure 1. Effect of ruthenium red (RR) on the uptake and release of Ca^{2+} and H^+ ions. The reaction mixture contained 80 mM NaCl, 2 mM Tris-NCl (pH 7.4), 5 mM sodium succinate and 10 mg of mitochondrial protein in a final volume of 3.8 ml. Where indicated 800 natoms of Ca^{2+} (labelled with $^{45}Ca^{2+}$) and 200 nmoles of ruthenium red (20 nmoles per mg mitochondrial protein) were added. Oxygen consumption and H^+ movement were measured as described in Materials and Methods. The incubation temperature was 25°.

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of 1 obtained in the absence of a permeant anion (6). Upon the completion of Ca^{2+} uptake, the respiration rate returned to State 4 and net H^+ ejection ceased. The subsequent addition of ruthenium red caused neither a release of Ca^{2+} nor an uptake of H^+ ions (Figure 1A). When ruthenium red was added before all the Ca^{2+} in the assay medium had been taken up (Figure 1B) there was an immediate reversal of H^+ ejection, a decrease in respiration to approximately the State 4 rate and the appearance in the extramitochondrial medium of about 90 per cent of the added $^{45}Ca^{2+}$ (data for $^{45}Ca^{2+}$ uptake not shown).

The amount of H^+ ions reabsorbed by mitochondria following the addition of ruthenium red varied somewhat from experiment to experiment, ranging from approximately 30 to 100 per cent of the amount of H^+ ions ejected. This variability may be due primarily to the time period which elapsed between the addition of Ca^{2+} and ruthenium red. In any case, the uptake of H^+ promoted by the addition of ruthenium red was associated with a stoichiometric release of mitochondrial Ca^{2+} as shown by a final H^+ ejected/Ca^{2+} accumulated ratio of

TABLE I

EFFECT OF RUTHENIUM RED ON THE RELEASE AND UPTAKE OF Ca^{2+} AND H^+ IONS

The reaction mixture and conditions of incubation were the same as described in Figure 1, except that 5 mg of mitochondrial protein were used. Ruthenium red (RR) was added to the reaction mixture after $^{45}Ca^{2+}$ (800 natoms) but before all of it was taken up by mitochondria as shown in Figure 1. The final amount of Ca^{2+} accumulated and H^+ ions ejected following the addition of ruthenium red was determined after the final steady-state levels were reached.

	H^+ ejected (natoms)	Ca^{2+} accumulated (natoms)	H^+ ejected/ Ca^{2+} accumulated
No addition	380	400	0.95
RR (10 nmoles/mg protein)	150	115	1.3
RR (10 nmoles/mg protein)	265	330	0.80
RR (10 nmoles/mg protein)	190	180	1.05

approximately 1 (Table I). In the experiments shown in Table I, the amount of H^+ ejected and Ca^{2+} accumulated was measured at the final steady state level reached after the addition of ruthenium red.

To determine whether the effect of ruthenium red was a specific effect of the ruthenium red complex or of ruthenium itself, two other ruthenium compounds, K_2RuCl_6 and $RuNOCl_3$, were tested. Neither compound caused the release of Ca^{2+} nor the reabsorption of H^+ by mitochondria. In the range of 5 - 50 μM, a concentration range in which ruthenium red exerts its maximal inhibitory effects, $RuNOCl_3$ has no effect on either mitochondrial respiration or the energy-dependent uptake of Ca^{2+}. Vasington et al. (1,2) had shown earlier that K_2RuCl_6 does not inhibit Ca^{2+}-stimulated respiration and Ca^{2+} uptake by mitochondria. It is shown in Table II that K_2RuCl_6 also has no effect

on the high- and low-affinity binding of Ca^{2+} by mitochondria. As shown in Table II, ruthenium red inhibits both types of Ca^{2+} binding, confirming our earlier findings (1,2).

Despite its lack of effect on Ca^{2+}-stimulated respiration and Ca^{2+} transport and binding by mitochondria, K_2RuCl_6 inhibits State 4 respiration as effectively as does ruthenium red (1,2). However, it appears that these two compounds inhibit respiration by different mechanisms. Inorganic phosphate only partially overcomes the ruthenium red inhibition of respiration in an uncompetitive nature, whereas it almost completely overcomes K_2RuCl_6 inhibition in competitive manner (7).

It has been proposed that Ca^{2+} transport across the mitochondrial membrane involves a Ca^{2+} carrier within the inner mitochondrial membrane (5,8-11). On the basis of the observations reported in this paper, we propose that ruthenium red competes with the Ca^{2+} carrier, and possibly other Ca^{2+} binding sites, causing a release of bound Ca^{2+} and a concurrent reabsorption of H^+ ions. Since in many experiments essentially all of the Ca^{2+} taken up by mitochondria was released upon addition of ruthenium red, any Ca^{2+} that had been transported completely across the mitochondrial membrane during the early phase of H^+ ejection must have been either free in solution or temporarily bound to sites which were accessible to ruthenium red, accounting for its release. If such temporary binding sites do indeed exist within the mitochondrion, the inability of ruthenium to induce the release of accumulated Ca^{2+} once all of the Ca^{2+} in the medium had been taken up suggests that these sites were no longer accessible to ruthenium red. Possibly the accumulated Ca^{2+} had been transported from the temporary binding sites to other sites that are not available to ruthenium red; another possibility is that the binding of Ca^{2+} caused a conformational change of the temporary binding sites, rendering them inaccessible to ruthenium red.

The inhibitory action of ruthenium red on Ca^{2+} transport appears to be a specific property of the entire ruthenium red molecule since neither K_2RuCl_6

TABLE II

Effect of ruthenium red and of K_2RuCl_6 on the high- and low-affinity binding of calcium by rat liver mitochondria. The system contained: 250 mM sucrose, 10 mM Tris-Cl, pH 6.6, 1 x 10^{-6} M rotenone, 2.5 ug Antimycin A, and 5 mg mitochondrial protein in a volume of 2 ml. Incubation was carried out at 0° for two minutes.

	High-affinity binding nmoles Ca^{2+}/mg prot.		Inhibition %	Low-affinity binding nmoles Ca^{2+}/mg prot.		Inhibition %
	Ca^{2+} added	Ca^{2+} bound		Ca^{2+} added	Ca^{2+} bound	
No addition	4.0	3.5	-	80.0	18.0	-
	8.0	5.0	-	160.0	22.5	-
Ruthenium red (5 nmoles/mg protein)	4.0	1.0	71	80.0	9.5	47
	8.0	1.8	65	160.0	15.5	33
K_2RuCl_6 (10 nmoles/mg protein)	4.0	3.0	14	80.0	18.5	0
	8.0	55.5	0	160.0	22.0	0

nor $RuNOCl_3$ have any effect on Ca^{2+} binding, transport, and release by mitochondria. Moore (3) showed earlier that $RuCl_3$ also has no effect on Ca^{2+} transport by mitochondria (3).

Since ruthenium red has been used as a stain for muco- and glycoproteins, the high sensitivity of the Ca^{2+} transport process to ruthenium red suggests that such compounds may be involved in the Ca^{2+} transport system (1-3). This view is supported by the recent isolation by Sottocasa et al. (12) of a mitochondrial glycoprotein that has a high affinity for Ca^{2+} (13,14).

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