THE INHIBITION OF CALCIUM UPTAKE AND RELEASE BY RAT LIVER MITOCHONDRIA BY RUTHENIUM RED

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1. Introduction

The mucopolysaccharide stain, Ruthenium Red, was shown by Moore [1] to inhibit energy-dependent calcium transport without altering respiration or energy transfer in isolated mitochondria. The studies of Vasington et al. [2] confirmed these observations and further demonstrated that high concentrations of Ruthenium Red inhibit respiration. Currently there seems to be general agreement regarding the inhibitory effect of Ruthenium Red on mitochondrial calcium uptake but some degree of disagreement over the effect(s) of Ruthenium Red on the efflux of calcium from mitochondria. Rossi et al. [3] demonstrated that Ruthenium Red does not promote the release of calcium from mitochondria when added after the calcium uptake is complete. Other laboratories have presented evidence indicating that Ruthenium Red indeed causes the release of calcium from mitochondria pre-loaded with calcium [4,5]. Also, it has been reported that Ruthenium Red has no effect on uncoupler-induced discharge of calcium from pre-loaded mitochondria [3,5]. Hence, the previous studies of the effects of Ruthenium Red on mitochondrial calcium metabolism suggest that influx but not efflux of calcium is sensitive to this compound.

In the present study it was demonstrated that Ruthenium Red inhibits the release and the re-accumulation of endogenous calcium in rat liver mitochondria. Further, evidence is presented indicating that, under certain experimental conditions. Ruthenium Red does not promote the release of calcium from mitochondria pre-loaded with calcium and, in addition, Ruthenium Red prevents the release of accumulated calcium which is mediated by the addition of the permeant anion, phosphate.

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2. Materials and methods

Rat liver mitochondria were isolated from male Sprague-Dawley rats (300–400 g) using a slight modification of the procedure of Schneider and Hogeboom [6]. The homogenization medium contained 75 mM sucrose, 225 mM mannitol, and 0.1 M EGTA. The composition of the wash solution was identical to that of the homogenization medium except the chelator EGTA was omitted. Mitochondria were stored at 4°C for no more than 2 h prior to and during their use in the experiments. Protein was estimated using a biuret procedure [7].

Mitochondrial incubations were performed in a glass reaction chamber with constant stirring in a reaction medium of 310 mM sucrose and 20 mM Tris–chloride, pH 7.0. Other additions to the various incubations are described in the figure legends.

The procedure for measuring the uptake of exogenous calcium by the rat liver mitochondria was described earlier [8]. Calcium uptake is expressed as nmol ^{45}Ca/mg protein and was calculated from the known specific radioactivity of the ^{45}Ca used in the incubation. In the experiments in which alterations in the endogenous calcium levels were monitored the mitochondria were labeled with ^{45}Ca during the isola-

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tion procedure with a very high specific radioactivity ^{45}Ca (i.e., about 16 Ci/mmol) but a very low concentration of ^{45}Ca (i.e., about 60 nM). Sampling and sample processing were performed as described earlier [8].

An aqueous solution of Ruthenium Red (Ventron Corp.) was used without further purification. ^{45}Ca-cium chloride was obtained from New England Nuclear Corporation. *p*-Trifluoromethoxyphenyl hydrozone of carbonyl cyanide (FCCP) was the generous gift of Dr P. G. Heytler of the E. I. DuPont de Nemours Co. All other reagents were of the highest quality available and were purchased from commercial suppliers.

3. Results and discussion

During the mitochondrial isolation procedure essentially carrier free ^{45}Ca was included in the first of 3 wash steps. The rationale for attempting this procedure was to develop a method for labeling with ^{45}Ca the endogenous pool(s) of calcium associated with the mitochondrial membranes without significantly altering the total amount of calcium in the isolated mitochondria. These ^{45}Ca-labeled mitochondria were utilized in the experiments to ascertain the effects of Ruthenium Red on endogenous mitochondrial calcium movements. The data shown in fig.1 demonstrate that the addition of the respiratory chain inhibitor antimycin A to ^{45}Ca-labeled mitochondria (at addition point A) resulted in nearly a complete loss of ^{45}Ca from the mitochondria. Whereas antimycin A treatment resulted in approx. 90% loss in the ^{45}Ca of the liver mitochondria, measurement of the total calcium in the mitochondria before and after antimycin A treatment indicated a loss of approx. 50% of the calcium during de-energization (data not shown). Re-energization of the antimycin A-treated mitochondria with *N,N,N',N'*-tetramethyl-phenylenediamine (TMPD) (at addition point B) resulted in a partial re-accumulation of the released ^{45}Ca. Following re-energization with TMPD the addition of inorganic phosphate (at addition point C) resulted in the uptake of the remainder of ^{45}Ca, originally released by the mitochondria upon antimycin A addition. When Ruthenium Red was added to the incubation just prior to the addition of antimycin A (addition at point A)

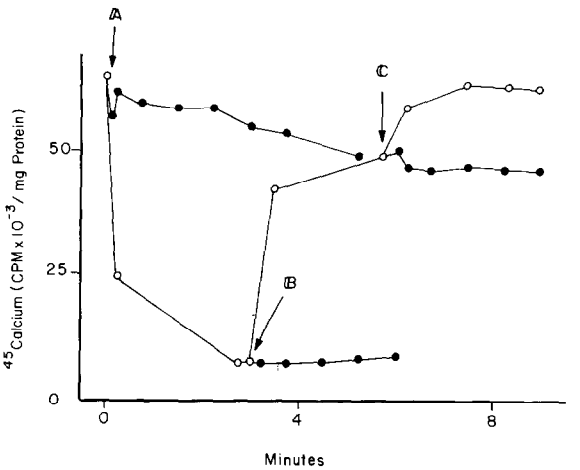

Fig.1. The effect of Ruthenium Red on the release and the re-accumulation of ^{45}Ca using mitochondria previously labeled with ^{45}Ca. Rat liver mitochondria (1.38 mg protein/ml) pre-labeled with ^{45}Ca as described in Materials and methods were incubated at room temperature in a buffer containing sucrose 310 mM, and Tris–chloride 20 mM (pH 7.0). Antimycin A 1.82 μg/mg protein, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) 100 μM, and potassium phosphate 1 mM were added at points A, B and C, respectively. Ascorbate (2.5 mM) was present in the incubations prior to addition of mitochondria. Ruthenium Red (12.5 μM) was included just prior to the various additions made at points A, B and C. The symbol used in the figure following the addition of Ruthenium Red to the mitochondria is a closed circle (•).

there occurred nearly a complete prevention of the loss of ^{45}Ca.

Similarly, when Ruthenium Red was added to antimycin A-treated mitochondria prior to the addition of TMPD (addition at point B) the energy-dependent re-accumulation of the ^{45}Ca which had been released by antimycin A treatment was completely prevented. These results clearly indicate that both the release and re-accumulation of endogenous mitochondrial calcium caused by manipulation of the energetic state of the mitochondria were inhibited by Ruthenium Red.

Another point of interest is the ability of Ruthenium Red to inhibit the phosphate-mediated enhancement of ^{45}Ca accumulation by the mitochondria. In a previous study [8], the fluorescence of the divalent metal cation chelate-probe chlorotetracycline was correlated with the movements of endogenous and

exogenous calcium during changes in the energetic state of the mitochondrial membranes. Evidence was presented indicating that treatment of mitochondria with antimycin A resulted in a release of calcium from both the membrane and the matrix compartments. Re-energization of the mitochondria in the presence of phosphate resulted in a re-accumulation of calcium primarily into the matrix compartment. In view of this observation and the results shown in fig.1, it seems apparent that Ruthenium Red is capable of inhibiting the anion-dependent translocation of calcium from the membrane into the matrix compartment.

Recently Schwerzmann et al. [9] presented evidence indicating that Ruthenium Red could replace TMPD in the transfer of electrons to cytochrome *c* during the oxidation of ascorbate. In view of this observation it could be suggested that in the experiment presented in fig.1, Ruthenium Red merely overcame the inhibition of electron transfer by antimycin A and that there occurred essentially no de-energization of the mitochondria membranes as a result of antimycin A addition. The result of such a situation would be little or no loss of ^{45}Ca from the mitochondria. However, addition of Ruthenium Red at point B in the figure should have exactly mimicked the effect of TMPD; that this was not the case was clearly observed. Further, the omission of ascorbate from the incubation medium should have resulted in the release of ^{45}Ca from the mitochondria by antimycin A even in the presence of Ruthenium Red. That this was not the case is indicated in the data depicted in fig.2. Even in the absence of ascorbate, Ruthenium Red markedly inhibited the release of ^{45}Ca promoted by antimycin A. The addition of the uncoupler *p*-trifluoromethoxyphenylhydrozone of carbonyl cyanide (FCCP) also resulted in a release of ^{45}Ca and this uncoupler-mediated release was also Ruthenium Red sensitive. Unless Ruthenium Red directly interfered with the interaction of FCCP or antimycin A with the mitochondrial membranes, the nearly complete inhibition by Ruthenium Red of ^{45}Ca release caused by these two compounds suggests that (a) the mechanism(s) for release and re-accumulation of endogenous mitochondrial calcium were the same, or (b) the mechanism(s) for release and re-accumulation may be different but both were highly sensitive to inhibition by Ruthenium Red. The slight decrease in

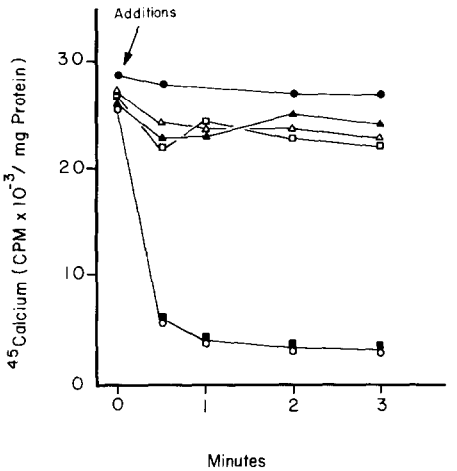

Fig.2. The effect of Ruthenium Red on the antimycin A and uncoupler-mediated release of ^{45}Ca from pre-labeled mitochondria. Rat liver mitochondria were labeled with ^{45}Ca using the procedure described in Materials and methods and were incubated under conditions identical to those described in the legend to fig.1. The mitochondrial protein concentration in the incubations was 1.13 mg/ml medium. Antimycin A, 2.2 μg/mg protein (●–●); FCCP, 0.025 μM (■–■); Ruthenium Red, 12.5 μM (▲–▲); antimycin 2.2 μg plus Ruthenium Red 12.5 μM (△–△); FCCP 0.025 μM plus Ruthenium Red 12.5 μM (□–□) were added to the incubation at the points indicated in the figure. The control experiment is represented by closed circles (●).

the ^{45}Ca content of the mitochondria upon addition of Ruthenium Red (see fig.2) may have been due to the replacement of ^{45}Ca on the membrane-binding sites by Ruthenium Red. It was observed in an experiment not shown that up to 20% of the ^{45}Ca was released from the mitochondria upon the addition of very high concentrations of Ruthenium Red.

If rat liver mitochondria were allowed to accumulate ^{45}Ca in the presence of an oxidizable substrate but in the absence of highly permeant anion such as phosphate or acetate, approx. 30 nmol ^{45}Ca/mg protein were taken up in the first minute following initiation of the reaction (see fig.3). Inclusion of Ruthenium Red in the incubation medium prior to the addition of mitochondria prevented the uptake of ^{45}Ca completely. Following the attainment of a steady state, i.e., after about 3 min, the addition of phosphate resulted in the rapid release of the accumulated ^{45}Ca. Addition of Ruthenium Red prior to phosphate addi-

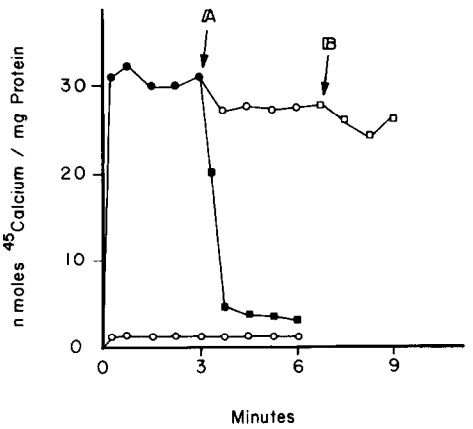

Fig.3. The effect of Ruthenium Red and phosphate on ^{45}Ca content of pre-loaded rat liver mitochondria. Mitochondria (1.42 mg protein/ml) were incubated with 50 μM calcium under the incubation conditions described in the legend for fig.1. Following 3 min incubation of mitochondria with ^{45}Ca, at addition point A, either 2.5 mM potassium phosphate (■–■) or Ruthenium Red (○–○) were added. Following the addition of Ruthenium Red, at point B, 2.5 mM potassium phosphate was added (□–□). The closed circles represent the uptake of ^{45}Ca by mitochondria in the absence of Ruthenium Red while open circles represent the uptake of calcium by mitochondria in the presence of Ruthenium Red. Succinate (2.5 mM) was present in all the incubations prior to the addition of mitochondria.

tion (at point A) largely prevented this loss of ^{45}Ca mediated by phosphate (phosphate added at point B).

It is known that addition of phosphate to mitochondria in the absence of magnesium and adenine nucleotide which have been allowed to accumulate calcium results in mitochondrial swelling and the release of accumulated calcium from the mitochondria [10]. Further, it has been indicated in various studies that calcium uptake by mitochondria in the absence of phosphate is primarily a membrane-loading without actual translocation of the calcium into the mitochondrial matrix [10,11]. Bearing these observations in mind, the inhibitory effect of Ruthenium Red on the phosphate-mediated release of calcium might be interpreted as a prevention of either the release of ^{45}Ca from the membrane and/or an inhibition of phosphate transport. However, Vasington et al. [2], monitoring large amplitude swelling of mitochondria in ammonium phosphate as an indicator of phosphate transport, demonstrated that Ruthenium Red addition did not cause impairment of phosphate transport. Hence, it is suggested that in the present experiment Ruthenium Red probably inhibited the release of ^{45}Ca from the membrane rather than the transport of phosphate.

Our observation that Ruthenium Red did not promote the release of ^{45}Ca from mitochondria which have been loaded with ^{45}Ca, while in agreement with the results of Rossi et al. [3], is in contradiction with the findings of Sordhal [4] and Puskin et al. [5]. This contradiction could be attributed to differences in the incubation conditions e.g., both Sordhal [4] and Puskin et al. [5] conducted their calcium loading of the mitochondria in the presence of a permeant anion. However, Puskin et al. [5] mentioned that their Ruthenium Red-induced release of calcium from pre-loaded mitochondria occurred even in the absence of a permeant anion. The experiments of Rossi et al. [3] and our experiments (see fig.3) were performed using mitochondria loaded with calcium in the absence of a permeant anion.

At least two reports [2,5] have indicated that uncoupler-mediated release of calcium from mitochondria loaded with calcium is insensitive to Ruthenium Red. Based upon this observation it was suggested that separate mechanisms are operative in the uptake and release of calcium from mitochondria. Our data concerning the effect of Ruthenium Red on the release of endogenous calcium by uncoupler indicates that Ruthenium Red can indeed prevent the efflux of calcium from mitochondria. Whether there is a significant difference between the release mechanism(s) for endogenous calcium and for exogenous calcium which has been accumulated by mitochondrial membranes remains to be investigated.

4. Summary

Evidence is presented indicating that Ruthenium Red, an inhibitor of energized calcium uptake in mitochondrial systems, is capable of preventing the release and the energy-linked re-uptake of endogenous mitochondrial calcium. Further, it is demonstrated that Ruthenium Red does not itself promote the release of calcium from mitochondrial membranes which have been previously loaded with calcium under

limited loading conditions (i.e., in the absence of permeant anions). Ruthenium Red also prevented the phosphate-mediated release of calcium from calcium-loaded mitochondria.

Acknowledgements

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