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# INHIBITION OF RUTHENIUM RED-INDUCED $Ca^{2+}$ EFFLUX FROM LIVER MITOCHONDRIA BY THE ANTIBIOTIC X-537A

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SUMMARY: It has been reported (Becker, G.L., Fiskum, G. and Lehninger, A.L. (1980) J.Biol.Chem.255,9009-9012) that respiring rat liver mitochondria suspended in KCl medium containing ATP, $Mg^{2+}$ and phosphate, maintain a steady state extramitochondrial free $Ca^{2+}$ concentration of about 0.5 μM. The results reported here show that the addition of the antibiotic X-537A, at concentrations far below those required for ionophorous activity, caused a perturbation in this steady state, lowering the extramitochondrial free $Ca^{2+}$ concentration by about 0.20 μM. This shift in steady state was clarified by a study of X-537A inhibition of the $Ca^{2+}$ efflux induced by ruthenium red; a half-maximum effect was observed at approximately 25 nM X-537A. No effect on $Ca^{2+}$ transport through the influx uniporter was observed. The possibility of a generalized stabilizing action of the antibiotic on the mitochondrial membrane seems to be ruled out by its effectiveness at very low concentrations. 

Experimental evidence indicates that the steady state distribution of free $Ca^{2+}$ concentration between intra and extramitochondrial space is kinetically regulated by the simultaneous operation of two independent pathways (cf.(1-3)). $Ca^{2+}$ influx is mediated by a ruthenium red-sensitive uniporter driven by the transmembrane potential (4,5). $Ca^{2+}$ efflux takes place through a ruthenium red-insensitive antiporter which catalyzes a $Ca^{2+}/2Na^{+}$ exchange in mitochondria from excitable tissues (6) and a sodium-independent, possibly a $Ca^{2+}/2H^{+}$ antiporter in liver mitochondria (7) (but see (8)). Therefore, the steady state depends on the relative rates of $Ca^{2+}$ transport through the influx and efflux

Abbreviations: BSA, Bovine serum albumin; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N'-tetra acetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid; DNP, 2,4-dinitrophenol; suc, succinate.

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pathways and can be altered by the stimulation or inhibition of one or both pathways (3). One strong evidence supporting this model comes from the fact that inhibition of the $Ca^{2+}$ influx pathway by addition of ruthenium red after $Ca^{2+}$ accumulation reaches a steady state triggers a net release of $Ca^{2+}$ from the mitochondria into the medium (cf.(1-3)). This communication reports experiments showing that the antibiotic X-537A,at concentrations far below those required for ionophorous activity (9,10)specifically inhibits the ruthenium red-insensitive $Ca^{2+}$ efflux from liver mitochondria but has no effect on $Ca^{2+}$ transport through the influx uniporter. A preliminary report of some of these findings has been presented (11).

## EXPERIMENTAL PROCEDURES

Liver mitochondria were isolated by the method of Schneider (12), and heart mitochondria by the method of Vercesi *et al* (13) from overnight fasted male Wistar-strain rats weighing approximately 250 g. All the incubations were carried out at 25°C, in a medium containing 65 mM KCl, 125 mM sucrose, 3.0 mM Hepes pH 7.2, 0.25 mM Pi, 0.05% BSA, 3.0 mM ATP and 4.0 mM $Mg^{2+}$. Changes in free $Ca^{2+}$ concentration in the suspending medium were followed using a $Ca^{2+}$-selective electrode (Radiometer, F2112 Calcium selectrode), calibrated by the addition of $Ca^{2+}$/EGTA buffers to the reaction medium alone (14,15). Changes in the mitochondrial membrane potential were monitored in a dual-wavelength double beam spectrophotometer (Perkin-Elmer model 356), employing 10 μM safranin as the indicating dye at the wavelength pair 533-511 nm (16). Oxygen consumption was measured using a Clark electrode connected to a Gilson oxygraph. The antibiotic X-537A (sodium salt), from Hoffmann-La Roche, was dissolved in water at a stock concentration of 0.2 mM.

## RESULTS

The experiment in Fig.1 shows that addition of rat liver mitochondria to aerobic medium containing succinate as respiratory substrate and a free $Ca^{2+}$ concentration of about 2.0 μM resulted in $Ca^{2+}$ uptake with a decrease in free $Ca^{2+}$ concentration. In agreement with the results of Becker *et al* (15), a steady state level of about 0.50 μM was reached, and maintained constant during the course of the experiment (trace A). However, the addition of the antibiotic X-537A at the point shown at a concentration of

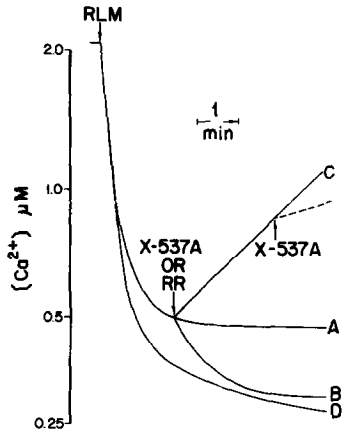


Figure 1. Effect of the antibiotic X-537A and ruthenium red on mitochondrial $Ca^{2+}$ fluxes. To 1.0 ml of the reaction medium described under "Experimental Procedures", were added 5.0 μM rotenone, 2.0 μg oligomycin, 2.0 mM succinate and 2.0 mg mitochondrial protein. Ruthenium red (0.7 μM, trace C) and X-537A (125 pmol/mg protein, trace B) were added where shown. Trace A is the control and in trace D mitochondria were added to the medium already containing the antibiotic.

125 pmols $mg^{-1}$ protein (125 nM), caused a lowering of the medium free $Ca^{2+}$ concentration to approximately 0.30 μM (trace B). If ruthenium red instead of X-537A was added at the same point, a net release of $Ca^{2+}$ from mitochondria occurred, at a rate of about 0.85 nmol $mg^{-1}min^{-1}$ (trace C). This rate of $Ca^{2+}$efflux was markedly decreased by the addition of X-537A (dotted line). Trace D shows that when mitochondria were added to the medium already containing the antibiotic, the steady state extramitochondrial free $Ca^{2+}$ concentration attained was the same as that observed when X-537A was added after the steady state had been reached.

It has been reported that the antibiotic X-537A selectively inhibits the oxidation of glutamate and isocitrate in liver mitochondria by binding to a site for bivalent cations that is associated with the inner mitochondrial membrane (9,10). The dose-response relationships for the inhibition of $Ca^{2+}$ efflux and of glutamate oxidation by X-537A were identical (Fig.2). This data are consistent with the existence of a common binding site

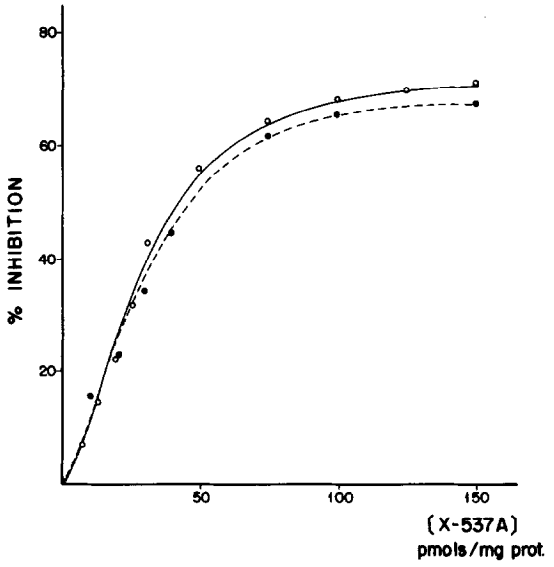


Figure 2. Dose-response relationship between the antibiotic X-537A and glutamate oxidation (solid line) or ruthenium red-induced $Ca^{2+}$-efflux (dotted line). Mitochondria (2.0 mg of protein) were added to 2.0 ml of the reaction medium containing 5.0 mM glutamate or 5.0 μM rotenone, 2.0 mM succinate and 0.7 μM ruthenium red. X-537A was present at the concentrations shown.

responsible for both effects. Half-maximum inhibition of both phenomena was obtained at approximately 25 nM X-537A. An inhibition of 70% was observed at a concentration of 125 nM (125 pmols $mg^{-1}$).

Since a recent report (17) indicated the existence of a relationship between membrane potential ($\Delta\psi$), steady state extramitochondrial $Ca^{2+}$ concentration and the rate of ruthenium red-induced $Ca^{2+}$ efflux from liver mitochondria, experiments were performed to study the effect of X-537A on $\Delta\psi$. Fig.3 shows that the antibiotic induced a slight increase in $\Delta\psi$ with a maximum effect (about 15 mV) at a concentration of 250 nM. This effect decreased with concentrations of X-537A higher then 400 nM, and was almost absent at a concentration of 1 μM (not shown). It should be emphasized that the effect of X-537A on $\Delta\psi$ was independent of changes in the rate of $Ca^{2+}$ cycling across the membrane, since the same result was observed in a $Ca^{2+}$-free medium (not shown). The experiment of Fig.4 shows that the antibiotic X-537A had no effect

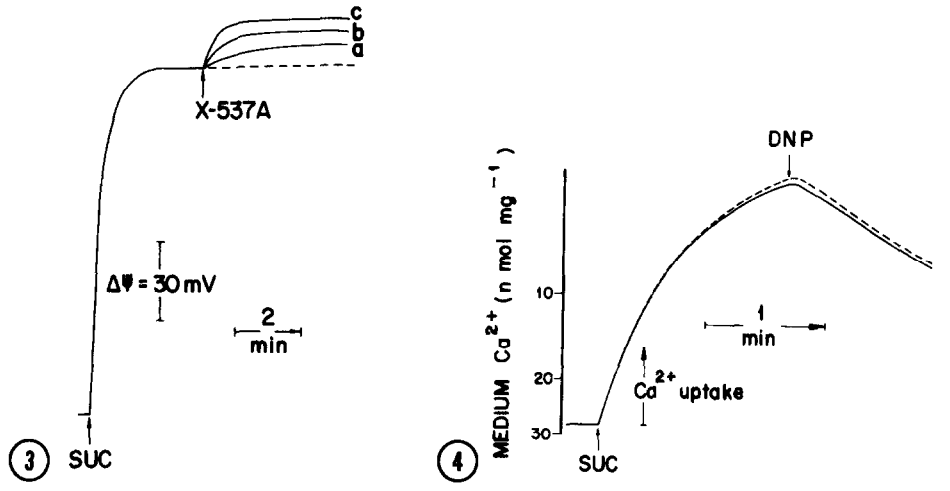


Figure 3. Effect of the antibiotic X-537A on mitochondrial membrane potential ($\Delta\psi$). Mitochondria (2.0 mg of protein) were added to 2.0 ml of the reaction medium containing 10 µM safranin, 5.0 µM rotenone and 2.0 mM succinate. After $\Delta\psi$ reached a constant value, X-537A was added at different concentrations (a) 62.5 nM, (b) 125 nM, (c) 250 nM.

Figure 4. The $Ca^{2+}$ flux through the uniporter is not affected by the antibiotic X-537A. To 1.0 ml of the reaction medium containing 5.0 µM rotenone and 2.0 mM succinate, was added 1.0 mg mitochondrial protein. DNP (50 µM) was added where shown, in the absence (solid line) or in the presence (dotted line) of 125 pmol X-537A/mg protein.

on the rate of $Ca^{2+}$ movements through the uniporter. In this experiment, de-energized mitochondria were preincubated in the presence of $Ca^{2+}$ and $Ca^{2+}$ influx was started by the addition of succinate. After the completion of $Ca^{2+}$uptake, DNP was added to induce $Ca^{2+}$ efflux by reversal of the uniporter. The dotted line shows the experiment in the presence of X-537A. The $Na^{+}$-induced $Ca^{2+}$ efflux in rat heart mitochondria was not inhibited by these low concentrations of X-537A (not shown).

## DISCUSSION

The results described in this communication are consistent with the current model of two separate routes for $Ca^{2+}$ transport across the inner mitochondrial membrane (cf.(1-3)). According to this model the steady state distribution of $Ca^{2+}$between the intra and extramitochondrial space is determined by a continuous $Ca^{2+}$

cycling. Indeed, the inhibition of the ruthenium red-induced $Ca^{2+}$ efflux by the antibiotic X-537A caused a perturbation in the steady state, lowering the extramitochondrial free $Ca^{2+}$ concentration by about 0.20 μM. This effect appears to be unrelated to enhanced mitochondrial stability, since the experiments were carried out in a medium containing BSA, low concentrations of $Ca^{2+}$ and phosphate and physiological concentrations of the membrane stabilizers $Mg^{2+}$ and ATP (15). However, the slight increase in $\Delta\psi$ observed in Fig.3 may reflect a specific decrease in the proton conductance of the inner mitochondrial membrane. A direct relationship between the effects of X-537A on $Ca^{2+}$ efflux and on the transmembrane potential observed in Fig.3 is unlikely, since the results of Bernardi and Azzone (17) indicate that this slight increase in membrane potential would be expected to cause an increase in the rate of ruthenium red-induced $Ca^{2+}$ efflux rather than the inhibition observed here. Moreover, other results indicate that the rate of ruthenium red-induced $Ca^{2+}$ efflux is not affected by changes in membrane potentials (8,18).

With respect to the mechanism of this inhibition, it is important to recall earlier reports (9,10), that showed an interaction between low concentration of the antibiotic X-537A and a membrane binding site for bivalent cations. The experiments here favor the notion of a close relationship between this specific binding site for the antibiotic X-537A, located in the inner mitochondrial membrane, and the pathway for ruthenium red-induced $Ca^{2+}$ efflux. This pathway might be a passive $Ca^{2+}/2H^{+}$ antiporter that binds X-537A (cf. however 8), or the antibiotic may be a specific inhibitor of either independent $H^{+}$ or $Ca^{2+}$ fluxes across the membrane. As the binding site for X-537A has been localized specifically at the site for reduction of $NADP^{+}$ by glutamate or isocitrate in liver mitochondria (9,10), we are currently trying

to determine whether this site is involved in the mechanism responsible for $Ca^{2+}$ efflux stimulated by the oxidized state of mitochondrial NADP (19-21), The use of X-537A at low concentrations will certainly be a valuable tool in the study of the mechanism of $Ca^{2+}$ efflux from mitochondria.

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