Vol. 105, No. 2, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 30, 1982 Pages 432-438

# RUTHENIUM RED-INDUCED LOSS OF MATRIX $K^+$ FROM UNCOUPLED HEART MITOCHONDRIA

Dennis W. Jung and Gerald P. Brierley

Department of Physiological Chemistry, Ohio State University
Columbus, Ohio 43210

Received February 4, 1982

Summary - Ruthenium red induces the loss of endogenous $K^+$ from isolated beef heart mitochondria treated with an uncoupler. This induction of $K^+$ loss occurs at the same ruthenium red titer as the inhibition of the $Ca^{2+}$-uniporter. This raises the possibility that ruthenium red may alter the $Ca^{2+}$-uniporter in such a way as to produce a $K^+$-conducting channel.

Heart mitochondria suspended in KCl do not lose endogenous $K^+$ when the membrane potential ($\Delta\Psi$) is dissipated by addition of an uncoupler unless other conditions are also satisfied (1). These conditions include: (a) that the mitochondrial NAD(P)H pool must be oxidized and (b) that either ruthenium red or a $Ca^{2+}$-chelator is present (1). Each of these later conditions strongly suggests that there is a relationship between retention of $K^+$ and the uptake or loss of $Ca^{2+}$ by the mitochondrion. Mitcochondrial $Ca^{2+}$ levels appear to reflect a balance between electrophoretic uptake via a highly active, ruthenium red-sensitive uniporter and an electroneutral efflux reaction (see 2-5 for reviews). The present communication examines the ruthenium red-induced efflux of $K^+$ in more detail and reports that the pathway for $K^+$-loss is opened at virtually the same titer as the inhibition of $Ca^{2+}$-uptake. This raises the possibility that the $Ca^{2+}$-uniporter can be modified by ruthenium red in such a way as to produce a channel for $K^+$ conduction.

## RESULTS

The loss of $K^+$ from uncoupled heart mitochondria induced by ruthenium red, like that induced by EGTA and other $Ca^{2+}$-chelators, is strongly inhibited

0006-291X/82/060432-07$01.00/0

Table I - Effect of Rotenone, $Ca^{2+}$, and Acetoacetate on the Efflux of $^{42}K^+$ from Uncoupled Heart Mitochondria Induced by Ruthenium Red and by $Ca^{2+}$-Chelators

| | $^{42}K^+$ Lost from Matrix (%) | | |
|---|---|---|---|
| | No Addition | Ruthenium Red | EDTA+ EGTA |
| Endogenous substrate | 10 | 12 | 15 |
| " " + CCP | 18 | 86 | 77 |
| " " + CCP + rotenone | - | 10 | 14 |
| " " + CCP + antimycin | - | 12 | 13 |
| Succinate + CCP + rotenone | - | 13 | 15 |
| Succinate + CCP + rotenone + acetoacetate | - | 19 | 14 |
| Endogenous + CCP + $Ca^{2+}$ | - | 78 | 13 |

Beef heart mitochondria were prepared using Nagarse in the presence of EGTA (6) and equilibrated with $^{42}K^+$ as previously described (1,7). The $^{42}K^+$-labeled mitochondria were suspended (1 mg/ml) in a medium of KCl (100 mM), N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES, 4mM, pH 7.0), and where indicated, ruthenium red (0.19μM), m-chlorocarbonylcyanide-phenylhydrazone (CCP, 1.5 μM), Tris EDTA (25μM), Tris EGTA (25μM), Tris succinate (4mM), rotenone (3μg/ml), antimycin (2μg/ml), $CaCl_2$ (100μM), and Tris acetoacetate (5mM). Reactions were started by addition of mitochondria to tubes with all other reagents present. After 3 min at 24° the mitochondria were sedimented at 18,000 rpm in a Sorvall SE-12 rotor and the supernatants were decanted for the determination of radioactivity. Radioactivities of the total suspension and of the supernatant of a zero-time control were also determined and the per cent loss was calculated as previously described (7).

by rotenone in the presence or absence of succinate respiration (Table I). A similar inhibition is produced by antimycin (Table I). These inhibitors prevent the extensive oxidation of NAD(P)H seen in uncoupled mitochondria (1) and it appears that oxidation of the pyridine nucleotides is necessary for the induction of $K^+$ loss by either ruthenium red or $Ca^{2+}$-chelators. Acetoacetate in the presence of rotenone oxidizes about 70% of the mitochondrial NADH but leaves NADPH reduced (1). Addition of acetoacetate does not overcome the rotenone inhibition of $^{42}K^+$-efflux induced by either ruthenium red or $Ca^{2+}$-chelators (Table I). This result supports our previous conclusion (1) that $K^+$-efflux may be regulated by NADPH levels in the matrix. The efflux of $K^+$ from uncoupled mitochondria induced by ruthenium red differs from that produced by $Ca^{2+}$-chelators in that the former is not prevented by exogenous $Ca^{2+}$ (Table I).

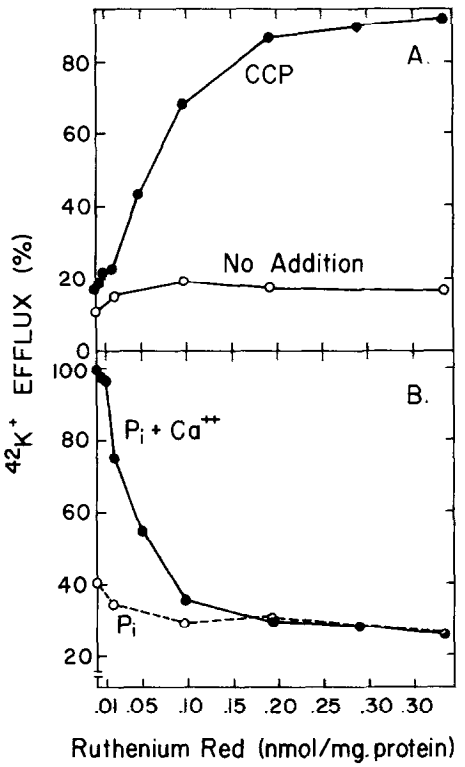


Fig. 1 - The effect of ruthenium red concentration on the efflux of $^{42}K^+$ from uncoupled heart mitochondria (A) and on the release of $^{42}K^+$ induced by $Ca^{2+}$ and $P_i$ accumulation (B). The experimental conditions were as those described in Table I. In (A), CCP was present at 1.5 μM where indicated. In (B), uncoupler was omitted and $CaCl_2$ (100μM) and $K^+$ $P_i$ (4mM) were present. The mitochondria were respiring with endogenous substrate in the absence of rotenone. Ruthenium red was used as received from K and K Labs (Plainview, N.Y.) with the concentration of stock solutions determined using $E_{mM}$ of 61.5 at 532 nm (8,9). Identical results were obtained with ruthenium red from Sigma recrystalized as described by Fletcher et al. (9).

The ruthenium red titration shown in Fig. 1A reveals that the loss of matrix $^{42}K^+$ to a KCl medium is stimulated half-maximally by about 70 pmol of ruthenium red per mg of protein. This compares favorably with estimates of 30 and 50 pmol·mg$^{-1}$ for inhibition of $Ca^{2+}$-uniport in liver (8) and brain mitochondria (10). Such titrations may not be strictly comparable, however, due to the uncertain purity of ruthenium red solutions (8,9), the tendency of the reagent to adsorb to reaction vessel surfaces (8), and other considerations. In order to compare the titer of ruthenium red required to block the $Ca^{2+}$ uniporter with the titer necessary to induce release of $K^+$ in uncoupled mitochondria, a reaction dependent on $Ca^{2+}$ uptake was assayed for ruthenium

red sensitivity (Fig. 1 B). In this reaction the respiration-dependent accumulation of $Ca^{2+}$ and $P_i$ in the absence of rotenone results in a non-specific increase in membrane permeability (2) characterized by loss of matrix $^{42}K^+$ to a KCl medium, net loss of $K^+$ to a $K^+$-free medium, loss of matrix $Mg^{2+}$, and mitochondrial swelling in KCl (Fig. 2). The permeability increase reflected by loss of $^{42}K^+$ to a KCl medium is prevented when $Ca^{2+}$-uptake is blocked by ruthenium red and the titration curve for this inhibition shows a $K_i$ of about 40 $pmol \cdot mg^{-1}$ (Fig. 1B). Since this titration was carried out on the same preparation of mitochondria as that for Fig. 1A, and used the same reagents, volumes of suspending media and incubation tubes, it is clear that the two processes are affected by very similar, if not identical, levels of ruthenium red. Ruthenium red has recently been shown to inhibit $Na^+$ uptake by brain mitochondria, but this effect requires a threefold excess of the reagent over the uniport titer (10).

## DISCUSSION

The titration experiments shown in Fig. 1 clearly suggest that when ruthenium red binds to the $Ca^{2+}$-uniporter it not only inhibits uptake of $Ca^{2+}$ but also opens a pathway for $K^+$ loss when mitochondria are uncoupled and NAD(P)H is oxidized (Table I). The mechanism for this dual activity is uncertain, but the following possibilities may be considered:

(a) Non-specific changes in permeability - Efflux of $K^+$ from uncoupled heart mitochondria in the presence of either ruthenium red or EGTA is not accompanied by swelling (Fig. 2) or by loss of endogenous $Mg^{2+}$ (1). These results would argue that non-specific permeability changes, such as those produced by $Ca^{2+}$ and $P_i$ uptake under conditions of NAD(P)H oxidation (Fig. 2), are not the primary factor in the extensive loss of matrix $K^+$ which is observed under these conditions.

(b) $Ca^{2+}$-cycling through the uniporter - The fact that both chelation of external $Ca^{2+}$ and inhibition of uniport activity by ruthenium red induce the efflux of $K^+$ suggests that uptake of endogenous $Ca^{2+}$ through the uniporter might somehow prevent the loss of $K^+$. The ability of trace levels of $Ca^{2+}$

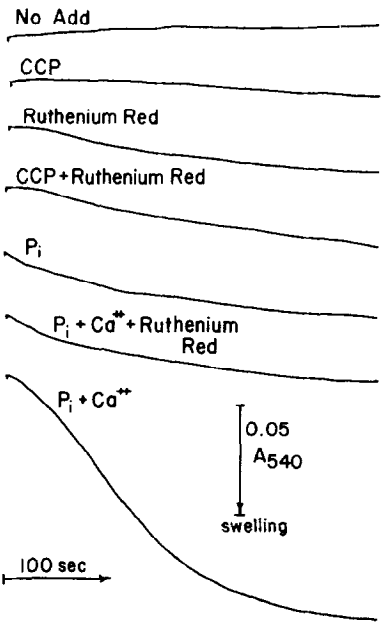


Fig. 2 - Effects of CCP, ruthenium red, $P_i$ and $Ca^{2+}$ on swelling of heart mitochondria. Mitochondria (2.5 mg) were suspended in 3 ml of KCl (100 mM), TES (4mM, pH 7.0) at 24°. Swelling was recorded by the change in absorbance at 540 nm in an Aminco DW-2 spectrophotometer with air as the reference. The reactions were started by addition of mitochondria. The concentration of additives was as given in Table I.

to inhibit $K^+/H^+$ and $K^+/K^+$ exchange activity would support this possibility (11). However, the ruthenium red-dependent loss of $K^+$ occurs only in the presence of sufficient uncoupler (1.5 μM CCP) to eliminate $Ca^{2+}$ uptake or cause rapid net loss of accumulated $Ca^{2+}$ from these mitochondria when measured under usual conditions in the presence of antipyrylazo III (12). In view of the amount of uncoupler present it appears unlikely that $Ca^{2+}$ uptake through the uniport could have a significant impact on the maintenance of matrix $K^+$.

(c) Ruthenium red alters the $Ca^{2+}$-uniporter to produce a $K^+$-conducting pathway - The mechanism by which ruthenium red prevents $Ca^{2+}$-uniport activity remains to be clarified (2-5,8). It is presently thought to react with a glycoprotein component of the uniporter which may function as a $Ca^{2+}$-recognition site (5). Reaction with ruthenium red may alter the structure of this recognition site so as to prevent the high-affinity interaction with $Ca^{2+}$, leaving the subunits of the uniporter which span the

membrane unaffected and available to support $K^+$ efflux. $Ca^{2+}$ binding to the uniporter by itself may be sufficient to block $K^+$ efflux since removal of $Ca^{2+}$ by nonpenetrant chelators also induces $K^+$ efflux in the presence of uncoupler (Table I). In this regard it should be noted that ruthenium red blocks the influx of $Ca^{2+}$ under virtually all conditions, but inhibits $Ca^{2+}$-efflux to a limited and variable extent even when the loss of $Ca^{2+}$ results from uncoupler-induced loss of $\Delta\Psi$ and could be presumed to occur by reversal of the uniport pathway (13-17).

The ruthenium red-induced release of $K^+$ from uncoupled mitochondria requires that NAD(P)H also be oxidized (Table I). This suggests that the $Ca^{2+}$-uniporter may be represented as an asymmetric channel regulated either directly or indirectly from the matrix side by the pyridine nucleotide redox state and that $K^+$-efflux will result only when both interior and exterior surfaces of the uniporter are modified. In this regard, it should be recalled that the uncoupler-induced efflux of $Ca^{2+}$ is also inhibited when the pyridine nucleotides are reduced (17).

The present results strongly suggest that, although ruthenium red inhibits $Ca^{2+}$-uniport activity, it does not do so by physically obstructing the ion channel through the membrane and that it does not inhibit ion efflux. This finding requires a re-evaluation of much of the available evidence which has been interpreted as support for a separate $Ca^{2+}$-efflux pathway in the mitochondrion (2-5), since the ruthenium red insensitivity of the efflux reaction has been one of the main arguments for a separate pathway.

Acknowledgments - These studies were supported in part by U.S. Public Health Services Grant HL09364.

REFERENCES

1. Jung, D.W. and Brierley, G.P. (1981) J. Biol. Chem. 256, 10490-10496.
2. Nicholls, D.G. and Crompton, M. (1980) FEBS Lett. 111, 261-268.
3. Carafoli, E. (1979) FEBS Lett. 104, 1-5.
4. Saris, N.E.L. and Akerman, K.E.O. (1980) Curr. Top. Bioenerget. 10, 103-179.
5. Carafoli, E. (1981) in Lee, C.P., Schatz, G., and Dallner, G. (Eds.) Mitochondria and Microsomes, Addison-Wesley Publ. Co., Reading, Mass., p. 357-374.

6. Jung, D.W., Chavez, E., and Brierley, G.P. (1977) Arch. Biochem. Biophys. 183, 452-459.
7. Chavez, E., Jung, D.W. and Brierley, G.P. (1977) Arch. Biochem. Biophys. 183, 460-470.
8. Reed, K.C. and Byqrave, F.L. (1974) Biochem. J. 140, 143-155.
9. Fletcher, J.M., Greenfield, B.F., Hardy, C.J., Scargill, D. and Woodhead, J.L. (1961) J. Chem. Soc. London, 2000-2006.
10. Barnard, P.A. and Cockrell, R.S. (1982) Biochim. Biophys. Acta 679, 68-74.
11. Shi, G-T., Jung, D.W., Garlid, K.D., and Brierley, G.P. (1980) J. Biol. Chem. 255, 10306-10311.
12. Scarpa, A., Brinley, F.J., Jr., and Dubyak, G. (1978) Biochemistry 17, 1378-1386.
13. Pozzan, T., Bragadin, M. and Azzone, G.F. (1977) Biochemistry 16, 5618-5625.
14. Fiskum, G. and Cockrell, R.S. (1978) FEBS Lett. 92, 125-128.
15. Caroni, P., Schwerzmann, K., and Carafoli, E. (1978) FEBS Lett. 96, 339-342.
16. Puskin, J.S., Gunter, T.E., Gunter, K.K., and Russell, P.R. (1976) Biochemistry 15, 3834-3842.
17. Hunter, D.R. and Haworth, R.A. (1979) Arch. Biochem. Biophys. 195, 468-477.