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RESTORATION OF MEMBRANE POTENTIAL IN MITOCHONDRIA DEENERGIZED WITH CARBONYL CYANIDE *p*-TRIFLUOROMETHOXYPHENYLHYDRAZONE (FCCP)

A. TONINELLO and N. SILIPRANDI

Institute of Biological Chemistry, University of Padua and CNR Unit for the Study of Mitochondrial Physiology, 35100 Padova (Italy)

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The membrane potential ($\Delta\psi$) of rat liver mitochondria dropped upon addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) but was gradually and fully restored to the original value by the subsequent addition of dithioerythritol. Concomitantly, Ca^{2+} released from mitochondria was reaccumulated and the oxidative phosphorylation process completely recoupled. Neither of these effects has been observed with dinitro-*o*-cresol or 2,4-dinitrophenol, uncouplers which, unlike FCCP, do not react with thiols. $\Delta\psi$ abolished by FCCP was also restored, though incompletely, by albumin; a prompt and complete restoration was however achieved upon subsequent addition of dithioerythritol. Dithioerythritol also completely and rapidly restored the $\Delta\psi$ decreased by addition of diazene dicarboxylic acid bisdimethylamide (diamide).

Introduction

The ability of thiols to restore energy-linked processes partially or completely lost during aging [1] or by treatment with diamide [2] is now well established. It suggests that the deenergized state results in both cases from thiol oxidation to disulfide which is a reversible process [3].

Blockage of certain SH groups may also have the same effect. Thus, Kaback et al. [4] found that the uncoupling effect of CCCP, a known SH reagent, on isolated bacterial vesicles is prevented as well as reversed by sulfhydryl compounds. However, the possibility of restoring the membrane potential ($\Delta\psi$) in mitochondria treated with carbonyl cyanide phenylhydrazones has not as yet been investigated. The only method known at present of reversing the uncoupler action of this substance is, to our knowledge, its removal by binding to albumin [5,6].

We have therefore studied the effect of dithiols (dithioerythritol or dithiothreitol) on $\Delta\psi$ in mitochondria deenergized either with FCCP (closely related to CCCP) or dinitro-*o*-cresol which is not a thiol reagent. The results reported in the present paper show that dithioerythritol restores both $\Delta\psi$ and the ability to reaccumulate Ca^{2+} lost by adding FCCP. In contrast, dithioerythritol has no effect in reversing the uncoupling action of dinitro-*o*-cresol.

Materials and Methods

Rat liver mitochondria were isolated in 0.25 M sucrose and 2 mM Hepes (pH 7.4) according to the method of Schneider [7]. Protein concentration was determined by the biuret method [8]. Membrane potential ($\Delta\psi$) was measured by an electrode specific for TPP^+ prepared in our laboratory according to the method of Kamo et al. [9] with a

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; diamide, diazene dicarboxylic acid bisdimethylamide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPP^+, tetraphenylphosphonium.

calomel electrode (Radiometer K 401) as the response electrode. The electrode potential is linear with respect to the logarithm of the TPP^+ activity with a slope of 59 mV in agreement with the Nernst equation. Ca^{2+} movements were estimated by atomic absorption spectroscopy of the supernatant [10]. Mitochondrial incubations were carried out at 20°C with 1 mg mitochondrial protein/ml in the following standard medium: 200 mM sucrose, 10 mM Hepes (pH 6.8), 5 mM sodium succinate, 1.25 μM rotenone, 1 μM TPP^+. The final Ca^{2+} concentration was brought to 10 μM after determination of the Ca^{2+} content of the medium. Bovine serum albumin (fatty acid free) and dithioerythritol were purchased from Sigma Chemical Co. FCCP was purchased from Boehringer Mannheim GmbH. Diamide was obtained from Calbiochem. Dinitro-*o*-cresol was a gift from Professor P. Buffa, University of Modena.

Results

Rat liver mitochondria incubated in the presence of Ca^{2+} (10 μM) and succinate (5 mM) quickly acquire a membrane potential ($\Delta\psi$) of approx. 200 mV and concomitantly accumulate external Ca^{2+} [11]. We have found that FCCP causes an immediate fall of $\Delta\psi$ which is, however, gradually restored to the original value by the subsequent addition of dithioerythritol; concomitantly, Ca^{2+} released from the mitochondria is completely reaccumulated (Fig. 1). At the Ca^{2+} concentration used in the present experiments (from 10 to 50 μM) Ca^{2+} begins to escape when the $\Delta\psi$ decreases to about 130 mV; likewise, Ca^{2+} reuptake starts when the potential rises again above this value with dithioerythritol and is completed much earlier than it would have been if $\Delta\psi$ had attained the maximum value. Addition of dithioerythritol prior to FCCP blocks the effect of the uncoupler (results not reported). This result is to be expected from the report by Heytler [12] and Drobnica and Šturdík [13] that thiols react with FCCP. The reaction between FCCP and dithioerythritol in free solution occurs rapidly as can be deduced from the shift of the absorption maximum (not shown). However, the effect of dithioerythritol in restoring $\Delta\psi$ to mitochondria deenergized with FCCP is rather slow (Fig. 1). Such

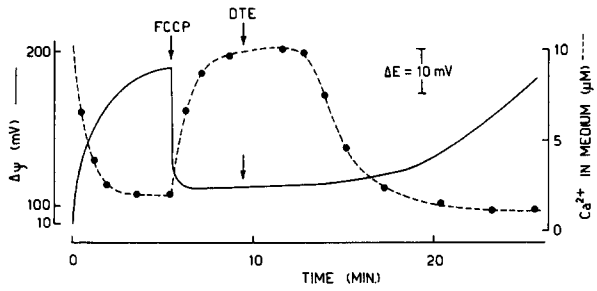

Fig. 1. Restoration by dithioerythritol of $\Delta\psi$ and Ca^{2+} uptake ability to mitochondria deenergized with FCCP. Rat liver mitochondria (1 mg/ml) were suspended in the standard medium described in Materials and Methods. Where indicated 100 nM FCCP and 2 mM dithioerythritol (DTE) were added. $\Delta\psi$ measurement (continuous line) and Ca^{2+} movements were determined in the same vessel.

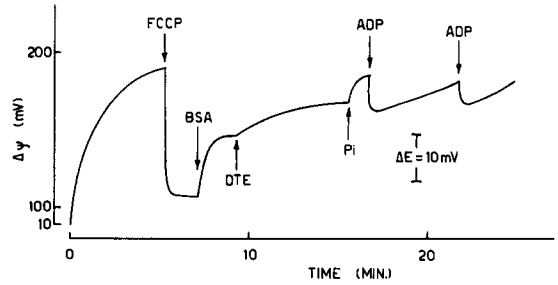

Fig. 2. Restoration of $\Delta\psi$ and oxidative phosphorylation by albumin and dithioerythritol to mitochondria deenergized with FCCP. Experimental conditions as in Fig. 1. Where indicated 100 nM FCCP, 16 μM bovine serum albumin (BSA), 2 mM dithioerythritol (DTE), 2 mM sodium phosphate (P_i) and 0.2 mM ADP were added.

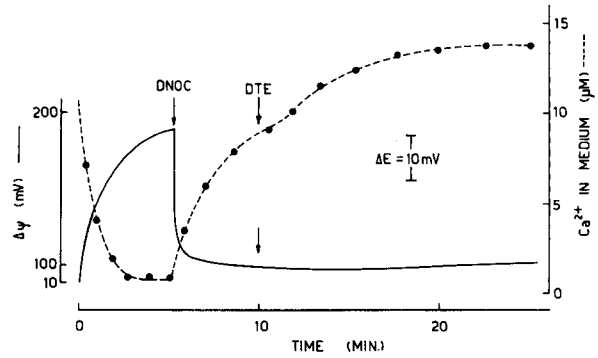

Fig. 3. Inability of dithioerythritol to restore $\Delta\psi$ to mitochondria deenergized with dinitro-*o*-cresol. Experimental conditions as in Fig. 1. Where indicated 5 μM dinitro-*o*-cresol (DNOC) and 2 mM dithioerythritol (DTE) were added.

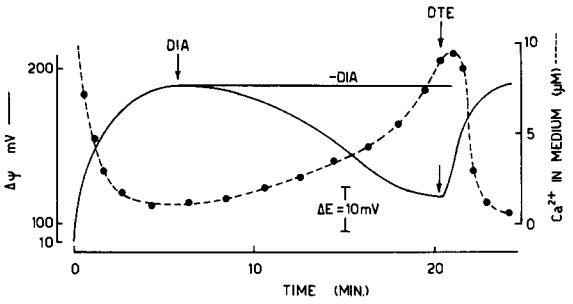

Fig. 4. Effect of dithioerythritol on $\Delta\psi$ and Ca^{2+} uptake in mitochondria treated with diamide. Experimental conditions as in Fig. 1. Where indicated 0.4 mM diamide (DIA) and 2 mM dithioerythritol (DTE) were added.

sluggishness might be due either to the poor accessibility of dithioerythritol to FCCP within mitochondria, or to the time required for reversing the alterations induced by the uncoupler.

As shown in Fig. 2, $\Delta\psi$ abolished by FCCP is restored, although not completely, by albumin (the molarity of the added albumin was 150-fold that of FCCP). However, restoration both of $\Delta\psi$ and of oxidative phosphorylation is achieved upon subsequent addition of dithioerythritol. The fall of $\Delta\psi$ subsequent to ADP addition and the gradual restoration to the previous value are both due to the energy utilization required for phosphorylation [9].

Uncouplers which, like dinitro-*o*-cresol or 2,4-dinitrophenol, do not react with thiols behave in a different way. As shown in Fig. 3, $\Delta\psi$ collapsed by addition of dinitro-*o*-cresol is not restored by dithioerythritol, which is also unable to promote the reaccumulation of Ca^{2+}.

Diamide, a thiol-oxidizing agent [14], exhibits some analogy with FCCP (Fig. 4). When added to energized mitochondria after a time lag the $\Delta\psi$ gradually decreases. When the potential is completely dissipated, addition of dithioerythritol results in a rapid and complete restoration. Concurrently, Ca^{2+} is released and reaccumulated, respectively.

Discussion

In agreement with the results obtained by Kaback et al. [4] on isolated bacterial vesicles, the findings reported here show that the uncoupling action of FCCP can be fully reversed by dithioerythritol or other thiols. These results contrast with the current opinion, essentially based on the results of Heytler [12], that the uncoupling action of carbonyl cyanide phenylhydrazones may be prevented but not reversed by thiols. It is possible that because of the rather delayed effect of dithioerythritol in restoring $\Delta\psi$ collapsed by FCCP or by other analogous uncouplers, this finding could have escaped observation.

In the light of the present results, the action of FCCP might be due to its combining with certain mitochondrial thiols to modify the permeability properties of the membrane in such a way as to allow protons to diffuse unspecifically in a futile cycle. The reconstitution of mitochondrial thiols by dithioerythritol would result in the restoration of $\Delta\psi$ and related processes, like Ca^{2+} uptake and oxidative phosphorylation. A direct involvement of sulfhydryl groups in the uncoupling action of carbonyl cyanide phenylhydrazones is implied in the demonstration by Kaback et al. [4] that FCCP inhibits the reactivity of bacterial membranes towards *N*-ethylmaleimide. It is also in accordance with the results obtained with diamide (Fig. 4). The full and prompt reversibility of the uncoupling action of diamide by dithioerythritol suggests that diamide exerts its effect by reacting specifically with mitochondrial thiols rather than by unspecifically damaging mitochondria. Whereas the uncoupling action of FCCP is rapid and its reversion by dithioerythritol slow, the uncoupling action of diamide is slow and its reversion rapid. This difference may be due to the hydrophilicity of diamide and dithioerythritol on the one hand and the hydrophobicity of FCCP on the other. In consequence, there will be profound differences between the two reagents in the rate of diffusion within mitochondrial membranes.

As previously reported by Weinbach and Garbus [6], the efficacy of albumin in restoring respiratory control in uncoupled mitochondria is a consequence of its ability to bind the uncoupling agents and hence, by removing them from the medium, to allow their outward diffusion from uncoupled mitochondria. However, in the light of the present results, albumin cannot restore completely the $\Delta\psi$ collapsed by either FCCP or di-

nitro-*o*-cresol. This is probably due to the inaccessibility of albumin to the uncoupler tightly bound within the membrane. In contrast, the combined action of albumin and dithioerythritol results in a prompt and complete restoration of $\Delta\psi$ to mitochondria treated with FCCP, but not with dinitro-*o*-cresol. Evidently, the interaction of dithioerythritol with the uncoupler bound to mitochondrial membranes is facilitated by albumin or vice versa.

The present results, though indicating an involvement of thiols in the uncoupling process, do not disprove the generally accepted concept that uncouplers act as proton translocators across the mitochondrial membrane. As proposed by Kaback et al. [4], an increased proton conductivity induced by FCCP could be a more important factor than its ability to combine with sulfhydryl groups. Nevertheless, it is possible that blockage of certain SH groups could alone result in uncoupling as suggested by our results with diamide, a hydrophilic compound which could not act as proton translocator (Fig. 4). The more rapid action of FCCP or other analogous phenylhydrazones may be due to their ability to penetrate rapidly the mitochondrial membrane and to affect more specifically the membrane-bound thiols critical for the permeability properties of the membrane.

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