THE EFFECT OF CA^{2+} ON THE OXIDATION OF β-HYDROXYBUTYRIC ACID BY HEART MITOCHONDRIA

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Summary: The respiration of rat heart mitochondria incubated with EGTA fails to respond to the addition of uncouplers when β-hydroxybutyric acid is the substrate. By contrast,the addition of ADP and phosphate is followed by the normal State 4/State 3 transition. The phenomenon is due to the complete loss of Ca^{2+} from mitochondria induced by uncouplers and EGTA, and can indeed be duplicated by incubation in the presence of the specific Ca^{2+} ionophore A23187 and ruthenium red (the latter prevents the re-uptake of the lost Ca^{2+}). Since the loss of Ca^{2+} has no effect on the oxidation of other NAD-dependent substrates, it is concluded that Ca^{2+} is essential for the interaction of β-hydroxybutyric acid dehydrogenase with a specific intramembrane NAD^+/NADH pool.

INTRODUCTION

Ca^{2+} has been shown to influence the oxidation of various substrates by mitochondria: it stimulates the oxidation of α-glycerophosphate, due to an allosteric activation of α-glycerophosphate dehydrogenase (1). It stimulates the oxidation of pyruvate by activating a specific phosphatase responsible for the transformation of pyruvate dehydrogenase into its active form (2). It stimulates the oxidation of succinate by uncoupled mitochondria by an unknown mechanism (3) and the oxidation of exogenous NADH in partially swollen mitochondria by promoting the transfer of NADH across the inner membrane (4). The role of Ca^{2+} in promoting the transfer of pyridine nucleotides across the inner membrane is probably of central importance: under certain experimental conditions, Ca^{2+} has been shown to induce leakage of endogenous pyridine nucleotides, thus inducing an inhibition of respiration (5, 6). The interaction of cytochrome C with the inner membrane, and thus with cytochrome oxidase, has also been

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List of abbreviations: DNP: 2,4,dinitrophenol; β-OH-butyrate: beta-hydroxy-butyrate (Na-salt); RR: ruthenium red; FCCP: carbonyl cyanide p-trifluoromethoxyphenylhydrazone; EGTA: ethylene glycol bis-(aminc ethylether N,N^1-tetraacetic acid (Tris-salt).

shown to be influenced by Ca^{2+}, at least under in vitro conditions (7). Lastly, at least one of the inner membrane substrate permeases, that for α-oxoglutarate, seems to be controlled by Ca^{2+} (8). The results reported in this paper demonstrate that the oxidation of β-hydroxybutyric acid in heart mitochondria is also influenced by Ca^{2+}, and is strongly inhibited in mitochondria from which Ca^{2+} has been extracted by the combined action of uncouplers and chelating agents. Since the oxidation of other NAD-dependent substrates is unaffected by the treatment, the tentative conclusion is reached that Ca^{2+} specifically controls the interaction of β-hydroxybutyric acid dehydrogenase with NAD^+/NADH (or a fraction of it) within the inner membrane.

Methods and Materials: Rat heart mitochondria were prepared according to the method of Pande and Blanchaer (9) in 210mM Mannitol, 70mM sucrose, 10 mM Tris-Cl pH 7.4 (MST), containing 0.1 mM EDTA. The pellets were washed twice, the second time without EDTA. Occasionally mitochondria were prepared according to the method of Sordahl and Schwartz (10) (the polytron method). The same results were obtained with the two mitochondrial preparations. Submitochondrial particles were prepared in 10 mM Tris pH 8.5 by sonication for 1-3 min. in a Branson sonifier, output 100 Watts. The sonicated suspension was centrifuged for 10 min. at 10.000xg to remove intact mitochondria and large mitochondrial fragments. The particles were collected at 105.000xg for 30 min. Protein determination was carried out with a Biuret procedure. Endogenous Ca^{2+} was determined by Atomic Absorption Spectrophotometry (Perkin-Elmer mod.503). The consumption of oxygen was followed in a Clark electrode at 25° (the details are to be found in the legend for Fig.1). Fluorescence was measured in an Eppendorf Photometer (primary filter Hg 313+366nm, and secondary filter 470-3000nm). The swelling of mitochondria was measured by recording the change in absorbance of the suspension at 540nm in a DB/GT Beckman Spectrophotometer. β-hydroxy-butyrate dehydrogenase activity was measured spectrophotometrically at 340nm by measuring the formation of NADH from exogenously added NAD^+ in 10 mM Tris-Cl pH 8.10, in the presence of 1μM rotenone.

Results and Discussion: Heart mitochondria respiring on β-hydroxybutyric acid fail to respond to the addition of uncouplers with the normal increase in oxygen consumption if they have been preincubated with EGTA (Fig.1 a-b). In fact, some minutes after the addition of the uncoupler, the respiration in general even falls below the original State 4 level. The failure of uncouplers to stimulate respiration is correlated with the original Ca^{2+} content of mitochondria, since it depends critically on the length of time during which mitochondria are preincubated with EGTA (Fig.2). This resulted clearly also from occasional experiments in which uncouplers could induce an evident (albeit not optimal) transition of respiration to the activated state even after 5 min. of preincubation

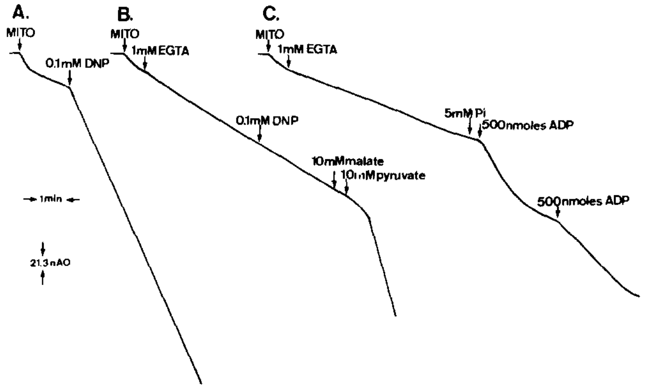

Fig.1: Effect of EGTA on the respiration of heart mitochondria in the presence of β-hydroxyburyrate. The incubation medium contained 210mM Mannitol, 70mM sucrose, 10mM Tris-HCl, pH 7.4, 1.2mg mitochondrial protein, 5mM Na- β-hydroxybutyrate. Total volume 1.8ml, temp.25° C.

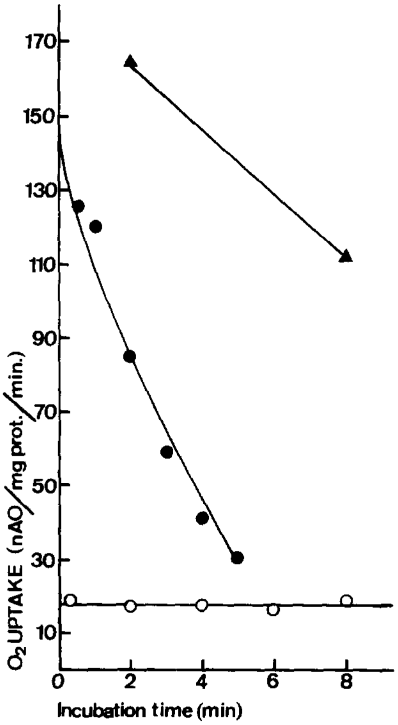

Fig.2: Time-dependence of the EGTA effect. Experimental conditions as in Fig.1. ○-○, rate of State 4 respiration; ▲-▲ , DNP-induced respiration of mitochondria incubated for the time indicated on the abscissa, in the absence of EGTA; ●-● , DNP-induced respiration of mitochondria incubated with 1mM EGTA.

with EGTA. In these cases, determination of the endogenous mitochondrial Ca^{2+} showed levels in excess of 30 nmoles per mg of protein, i.e. 2-3 times the content normally obtained under the present experimental conditions. Fig.1b shows that, after preincubation with EGTA and addition of uncouplers, the respiration could be fully reactivated by the addition of any of a variety of NAD-dependent substrates, thus excluding both the extensive loss of NAD^+/NADH from the intramitochondrial compartment (5-6), and a rotenone-like effect on the respiratory chain, as possible mechanisms for the EGTA-plus-uncouplers-induced effect. The failure of uncouplers to stimulate respiration was not the consequence of some non-specific damage to β-hydroxybutyric acid dehydrogenase induced by the EGTA incubation.Optimal cyclic responses(Fig.1c)of the respiration in the presence of β-hydroxybutyric acid were observed upon addition of ADP and phosphate to EGTA-incubated mitochondria.The apparent requirement for uncouplers to demonstrate the inability of EGTA-treated mitochondria to oxidize β-hydroxybutyric acid optimally was at this point tentatively explained with the complete removal of endogenous Ca^{2+} which is induced only by the combined addition of EGTA and uncouplers. The latter would promote the efflux of Ca^{2+} from mitochondria, the former would complex it outside. This suggestion was tested in experiments with specific Ca^{2+} ionophores, which also promote a complete and rapid efflux of Ca^{2+} from mitochondria. In addition, in the presence of ruthenium red to prevent the re-uptake of the Ca^{2+} lost under the influence of ionophores, mitochondria remain tightly coupled. The effect of ADP and phosphate on the oxidation of β-hydroxybutyric acid under conditions of complete Ca^{2+} deprivation could thus be tested. As shown in Fig.3, A23187, added to mitochondria in the presence of ruthenium red, abolished the stimulation of respiration by DNP when β-hydroxybutyric acid was the substrate (but not when other NAD-dependent substrates were used). Under these conditions, however, A23187 also abolished the normal cyclic response of respiration to ADP plus phosphate (Fig.3b). In fact, not only did the treatment prevent the stimulation of respiration by either uncouplers or ADP and phosphate, but it decreased it in some minutes below the State 4 level. It was thus clear at this point that in the absence of Ca^{2+} β-hydroxybutyric acid could not be oxidized at maximal rates.

To explain the mechanism of the observed effects, several hypotheses were tested. A direct effect of the Ca^{2+} deprivation on the β-hydroxybutyric acid dehydrogenase was ruled out on the basis of experiments carried out

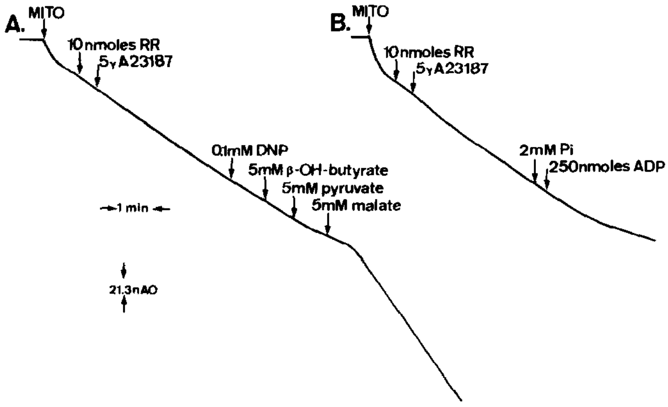

Fig.3: Effect of the Ca^{2+} ionophore A23187 on the respiration rate of heart mitochondria in the presence of β-hydroxybutyrate. Experimental conditions as in Fig.1. RR was added to prevent the re-uptake of the released Ca^{2+}.

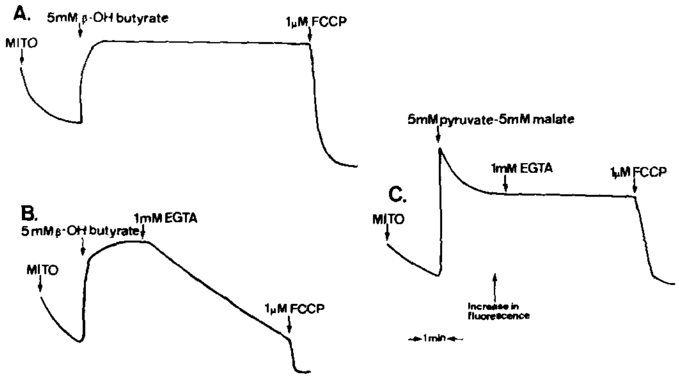

Fig.4: Effect of EGTA on the fluorescence of mitochondrial NADH. Incubation medium 210mM Mannitol, 70mM sucrose, 10mM Tris-HCl, pH 7.4, 0.6mg mitochondrial protein. Total volume 1ml, temp.22°. Additional details are to be found in the "Methods" section.

on inside-out submitochondrial vesicles, which expose the dehydrogenase to the outside medium. When supplemented with NAD^+, these inside-out vesicles oxidize β-hydroxybutyric acid very rapidly and at the same rate in either the presence or the absence of EGTA, plus or minus uncouplers. The possibility was then considered that in the absence of Ca^{2+} the penetration of β-hydroxybutyric acid became inhibited. The possibility was considered unlikely, since it is generally assumed (11) that the penetration of β-hydroxybutyric acid into mitochondria is not a permease-mediated process, and was tested by experiments in which the penetration of β-hydroxybutyric acid was studied using iso-osmotic concentrations of the ammonium salt (12). The rate of penetration, as evaluated by the rate of swelling of mitochondria, was unaffected by EGTA plus or minus uncouplers. It must be pointed out, however, that experiments of this type are carried out in the presence of concentrations of β-hydroxybutyric acid which are considerably higher than those used in the experiments on oxygen consumption. Indeed, if intact mitochondria are presented with 150mM β-hydroxy-butyric acid as a substrate (instead of the normal 5mM) the rate of oxygen consumption becomes extremely high, and comparable to that observed in maximally uncoupled mitochondria.

If the assumption is made that β-hydroxybutyric acid dehydrogenase does not normally react with NAD^+ in the matrix compartment, but with NAD^+ in the inner membrane, the results presented in this paper could be rationalized, since it has already been shown (5) that Ca^{2+} is essential for the transfer of NAD (NADH) into the apolar region of the mitochondrial membrane. The dehydrogenase in this case (i.e. in the absence of Ca^{2+}) would be unable to deliver the reducing equivalents to the intramembrane NAD^+, thus explaining the inhibition of respiration. That β-hydroxybutyric acid dehydrogenase reacts with a special "pool" of NAD^+ is a suggestion which has already been made in the past (13), and is also supported by the observation that no other NAD-linked dehydrogenase is so firmly associated with the inner membrane. Alternatively, one could consider the less likely possibility that in the absence of Ca^{2+} the dehydrogenase itself undergoes some sort of "dislocation", so that its interaction with its special NAD^+ pool is prevented. The experiment on inside-out submitochondrial vesicles (see above), in which the dehydrogenase apparently reacts optimally with exogenous NAD^+ even in the presence of EGTA and uncouplers, is, however, an obstacle to that interpretation. It could perhaps be rationalized by suggesting a functional modification (or a dislocation) of the dehydrogenase due to the sonication procedure and to the turning of the membrane inside-out.

The effect of Ca^{2+} deprivation on the interaction of β-hydroxybutyric acid dehydrogenase with NAD^+ was also studied by following the fluorescence of intramitochondrial NADH. Fig.4 shows that the addition of β-

hydroxybutyrate to heart mitochondria is followed by the expected increased fluorescence at 340 nm, due to the reduction of NAD^+. The addition of EGTA at this point induces a gradual decrease of the fluorescence, similar to what would be observed under conditions of active respiration, i.e. upon addition of ADP and phosphate. In this case, however, the respiration is not increased and remains in State 4. That the phenomenon is not associated with a leakage of pyridine nucleotides from the mitochondria is shown by the fact that other NAD-dependent substrates, added after the EGTA-induced decrease in fluorescence, promote an evident and rapid increase of the fluorescence. These observations are, therefore, in agreement with the tentative conclusion that the decrease of respiration consequent upon the extensive loss of Ca^{2+} from mitochondria is not due to leakage of NAD^+ from the matrix compartment. They support the suggestion that the effects observed are due to modifications of a special intramembrane pool of NAD^+/NADH.

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