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POSSIBLE PARTICIPATION OF MEMBRANE THIOL GROUPS ON THE MECHANISM OF NAD(P)$^+$-STIMULATED Ca^{2+} EFFLUX FROM MITOCHONDRIA

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SUMMARY: NAD(P)$^+$-stimulated Ca^{2+} efflux from mitochondria is inhibited by bongkrekate and slightly stimulated by carboxyatractylate. Addition of oxaloacetate, an NAD(P) oxidant,or diamide, a thiol oxidant, to de-energized mitochondria incubated in Ca^{2+}-free medium induced a small decrease in turbidity of the mitochondrial suspension compatible with small structural changes of mitochondria. Similar to NADP$^+$-stimulated Ca^{2+} efflux these changes were also inhibited by bongkrekate and slightly stimulated by carboxyatractylate. The similarity between the effects of oxaloacetate and diamide, on both Ca^{2+} efflux and mitochondrial structure, indicates the existence of a common denominator, possibly the oxidation of specific thiol groups, regarding the mechanism by which these agents stimulate Ca^{2+} efflux from mitochondria.

We have demonstrated that ruthenium red-insensitive Ca^{2+} efflux from isolated liver (1) and heart (2) mitochondria is stimulated by the oxidized steady-state of matrix NAD(P)H. Although these results were confirmed by other laboratories with isolated mitochondria (3-10), intact cells (11,12) and isolated liver (13), there is no agreement regarding the biological relevance (5,6,10) and the molecular mechanism of such a process (7,14,15).

Regarding the factors controlling the rate of Ca^{2+} efflux from isolated mitochondria some reports have indicated that small structural changes of mitochondria (from aggregated to

Abreviations: EGTA, ethylene glycol bis (β-aminoethyl ether) N,N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane-sulfonic acid; TMPD, N,N,N',N'-tetramethyl-p-phenylene diamine; OAA, oxaloacetate; BOH, β-hydroxybutyrate; BA, bongkrekate; CAT, carboxyatractyloside; DIAMIDE, diazenedicarboxylic acid bis-dimethylamide.

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orthodox configuration) induced by Ca^{2+} and other agents increase the membrane permeability to the cation (16-18). Bongkrekate and atractylate, both inhibitors of the adenine nucleotide translocase, induce opposite effects on mitochondrial configuration (19). Bongkrekate causes a change of the mitochondria to a more condensed configuration and enhances their ability to retain Ca^{2+} under certain conditions (20-23). Atractylate, on the other hand, stimulated Ca^{2+} release from mitochondria (16,21,23). Other data indicate that the redox state of membrane thiol groups also affects the membrane permeability to Ca^{2+} (9,22,24). In fact, diamide, an oxidant of membrane-bound thiol groups, stimulates Ca^{2+} efflux and dithioerythritol a thiol reductant decreases the rate of Ca^{2+} efflux from mitochondria (24). The results shown in this communication indicate that $NADP^{+}$-stimulated Ca^{2+} efflux from mitochondria is correlated to both a Ca^{2+}-independent change in mitochondrial configuration as evidenced by a decrease in turbidity of the mitochondrial suspension, and an oxidation of membrane thiol groups.

MATERIALS AND METHODS

Rat liver mitochondria were prepared by the method of Schneider (25). Changes in Ca^{2+} concentration in the suspending medium were followed using a Ca^{2+}-selective electrode (Radiometer, F 2112 Calcium Selectrode) calibrated with internal standards added to the reaction medium. Pyridine nucleotide fluorescence (excitation at 366 nm, emission at 450 nm) was monitored in an Aminco-Bowman Spectrophotofluorometer. Small structural changes of mitochondria, as measured by absorbance changes (19), were followed at 700 nm in the split-beam mode in an Aminco DW-2 dual wavelength spectrophotometer.

RESULTS AND DISCUSSION

Fig.1 shows the effect of bongkrekate and carboxyatractylate on the rate of mitochondrial Ca^{2+} efflux induced by the oxidized state of NAD(P). The upper traces show the NAD(P)H fluorescence in the presence of exogenous β-hydroxybutyrate or oxaloacetate included in the medium after Ca^{2+} uptake was

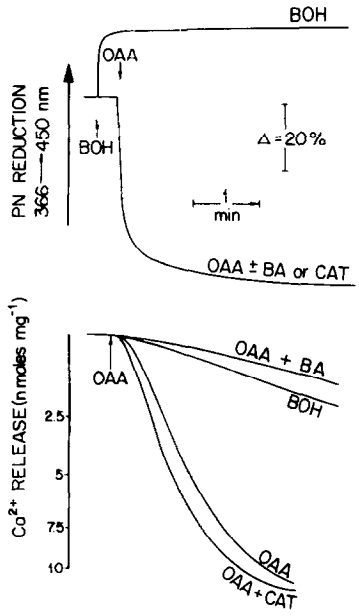

Figure 1. Effect of bongkrekate and carboxyatractylate on Ca^{2+} efflux induced by oxaloacetate. The system (2.0 ml, 35°C), contained: 125 mM sucrose, 60 mM KCl, 20 mM K^{+}-acetate, 3.0 mM Hepes buffer, pH 7.1, 1.0 mM phosphate, 4.0 µM rotenone, 0.1 nmol antimycin A mg^{-1}, 1.0 µg oligomycin mg^{-1}, 0.5 mM Mg^{2+} and 4.0 mg mitochondrial protein. TMPD (12 µM) and ascorbate (1.0 mM) were used as respiratory substrates. Ruthenium red (0.7 µM) was used to interrupt Ca^{2+}uptake. Bongkrekate (7.0 µM) and carboxyatractylate (20 µM) when present were added prior to the addition of mitochondria. β-hydroxybutyrate (3.0 mM) and oxaloacetate (0.5 mM) were added at the points indicated by the arrows.

interrupted by ruthenium red (not shown). The addition of β-hydroxybutyrate increased the reduction of pyridine nucleotides while oxaloacetate caused a large oxidation of these nucleotides notwithstanding the presence of bongkrekate or carboxyatractylate. The lower traces show that in the presence of β-hydroxybutyrate the ruthenium red-insensitive Ca^{2+} efflux occurred at a rate of 1 nmol $min^{-1}mg^{-1}$. In the presence of oxaloacetate, however, the rate of Ca^{2+} efflux was increased to about 7 nmol Ca^{2+} $min^{-1}mg^{-1}$. In spite of the lack of effect of bongkrekate and carboxyatractylate on the oxidation of NAD(P)H by oxaloacetate, it was observed that the former completely prevents, and the latter slightly stimulates, the ruthenium-red insensitive Ca^{2+} efflux. It has been proposed that the effect of these agents

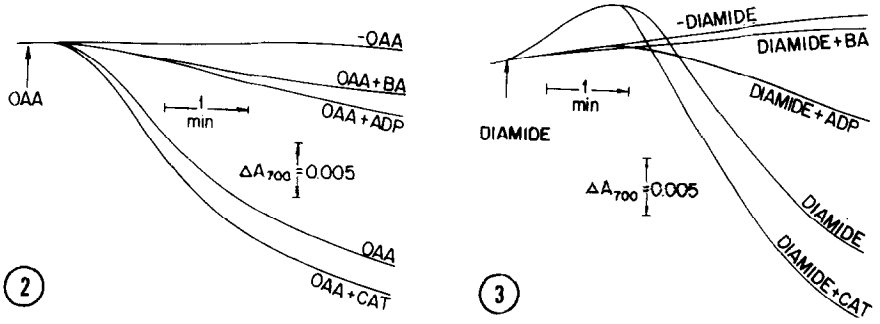

Figure 2. Changes in turbidity of the mitochondrial suspension induced by oxaloacetate. Mitochondria (5.0 mg protein) were preincubated in a system (2.5 ml, 30°C) containing: 250 mM sucrose, 3.0 mM Hepes buffer pH 7.1, 1.0 mM phosphate, 0.4 mM EGTA, 4.0 µM rotenone, 0.1 nmol antimycin A mg^{-1}, 1.0 µg oligomycin mg^{-1}. Oxaloacetate (1.0 mM) was added 5 minutes after the mitochondrial preincubation. Bongkrekate (10 µM), carboxyatractylate (20 µM) and ADP (200 µM) when present were added prior to the addition of mitochondria.

Figure 3. Changes in turbidity of the mitochondrial suspension induced by diamide. Experimental conditions were exactly as in figure 2. Diamide (0.3 mM) was added at the point indicated by the arrow.

on Ca^{2+} efflux from mitochondria is related to the inhibition by bongkrekate and stimulation by carboxyatractylate of the Ca^{2+}-induced configurational transition of mitochondria (16).Related to this possibility, fig.2 shows that addition of oxaloacetate induced a decrease in absorbance ($\Delta A < 0.05$) of the mitochondrial suspension compatible with a small change in mitochondrial configuration (19). It can be seen that bongkrekate, which inhibits Ca^{2+} efflux, also inhibited the decrease in absorbance induced by oxaloacetate. ADP, another inhibitor of Ca^{2+} efflux (23) showed a similar effect. On the other hand, carboxyatractylate, which stimulates Ca^{2+} efflux, increased the change in absorbance induced by oxaloacetate. To avoid the interference of the configurational changes of mitochondria induced by Ca^{2+} accumulation (16-18) or energization (26), in these experiments the mitochondria were preincubated under de-energized conditions in the presence of EGTA.

The experiments in fig.3 show the effect of diamide, an -SH oxidant, on the turbidity of the mitochondrial suspension.

The comparison between the effects of oxaloacetate and diamide was aimed at examining the possible participation of thiol groups on the mechanism of $NADP^+$-induced Ca^{2+} efflux from mitochondria since the oxidation of pyridine nucleotides may lead to an oxidation of critical membrane -SH groups by decreasing the availability of reduced glutathione in the matrix as shown by Bellomo et al (27). Like oxaloacetate, diamide induced a decrease in absorbance of the mitochondrial suspension at 700 nm. Most striking was the similarity between the effects of bongkrekate, ADP and carboxyatractylate on the decrease of absorbance induced by both diamide (fig.3) and oxaloacetate (fig.2). This similarity strongly suggests that the changes in mitochondrial configuration and the Ca^{2+} release induced by $NAD(P)^+$ and diamide have a common denominator. The fact that diamide is an oxidizing agent of membrane-bound thiol groups (24) indicates that the oxidation of NADPH may also cause an oxidation of critical membrane -SH groups that is linked to the oxidation of pyridine nucleotides as proposed by Harris et al (22) and Jung and Brierley (15). These results are in line with cumulative evidence that agents which react with thiol groups induce Ca^{2+} efflux from mitochondria, such as diamide (24), mercurials (22), quinones (27), and t-butyl hydroperoxides (4), and provides an indication for the participation of a membrane component in the mechanism of $NAD(P)^+$-induced Ca^{2+} efflux from mitochondria. The alterations in mitochondrial structure observed under these experimental conditions are of the same order of magnitude of those induced by the binding of bongkrekate or atractylate to the AdN-translocase in heart mitochondria (19) and certainly do not reflect gross membrane damage since our recent work (29) shows that at low Ca^{2+} concentrations the $NADP^+$-induced Ca^{2+} efflux from mitochondria is completely reversible.

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