THE MECHANISM FOR Ca^{2+} RELEASE INDUCED BY *N*-ETHYLMALEIMIDE IN RAT LIVER MITOCHONDRIA

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1. Introduction

Mitochondrial uptake of Ca^{2+}, in the absence of phosphate or permeant weak acids, leads to the formation of a ΔpH, which is proportional to the amount of Ca^{2+} taken up, and to a proportional decrease of the transmembrane electrical potential [1]. The rise of ΔpH and decrease of $\Delta\psi$ following Ca^{2+} uptake are more pronounced when the uptake of endogenous phosphate is blocked by *N*-ethylmaleimide [1,2], *p*-chloromercuribenzenesulfonate [2] or mersalyl [3].

The SH-group reagent *N*-ethylmaleimide has become widely used as inhibitor of P_i transport in mitochondria. MalNEt decreases the ability of mitochondria to retain Ca^{2+} and evidence is provided that the Ca^{2+} efflux observed in MalNEt-treated mitochondria is not due to membrane damage [2]. In contrast, *N*-ethylmaleimide-induced Ca^{2+} loss from mitochondria was accompanied by drastic alterations of e^- flow and membrane permeability [4]. We aim to clarify the mechanism of *N*-ethylmaleimide-induced Ca^{2+} release. It will be shown that *N*-ethylmaleimide does not alter per se the inner mitochondrial membrane but its effects are rather mediated by the rise of ΔpH following divalent cation uptake. Thus, it appears that the rise of ΔpH, and not *N*-ethylmaleimide per se, is responsible for the decreased e^- transfer at site II and for the increased permeability to K^+ and H^+.

Abbreviations: EGTA, ethylene bis (oxoethylenenitrilo) tetraacetic acid; BSA, bovine serum albumin; MalNEt, *N*-ethylmaleimide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Mops, 4-morpholinepropanesulfonic acid; FCCP, carbonylcyanide-*p*-trifluoro-methoxypheylhydrazone; TMPD, tetramethyl-*p*-phenylenediamine

2. Materials and methods

Rat liver mitochondria were prepared as in [5] in 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, and 0.1 mM EGTA. The last washing was carried out in an EGTA-free medium and mitochondrial protein was assayed with the biuret method, using BSA as a standard.

Oxygen consumption was nomitored with a Clark oxygen electrode (Yellow Springs Instruments, OH) in a magnetically stirred, thermoequilibrated vessel. Ca^{2+} and K^+ movements were monitored simultaneously with a Ca^{2+}-selective electrode (W. Möller, Zürich) and a K^+-selective electrode (Schott, Mainz), respectively, in a water jacket-thermostatted vessel equipped with magnetic stirring. The Ca^{2+}- and K^+-selective electrodes were calibrated with internal standards. The contaminating Ca^{2+} (mostly due to BSA) was 35–40 μM, as determined by atomic absorption spectroscopy. This amount of Ca^{2+} has been taken into account in calculating the final Ca^{2+} concentrations. Lower Ca^{2+} concentrations were obtained by titrating the excess Ca^{2+} with EGTA.

Cytochrome *b* redox changes were monitored with an Aminco DW 2a dual wavelength spectrophotometer, in a thermoequilibrated cuvette equipped with magnetic stirring, using the wavelength pair 563 minus 575 nm. All chemicals were of analytical grade.

3. Results

Fig.1A,B shows the effects of MalNEt and acetate on the Ca^{2+} and K^+ fluxes. In this experiment, as well as in all others reported below, the medium was supplemented with BSA and Mg^{2+}, in order to minimize the effect of phospholipases and the liberation of lyso-

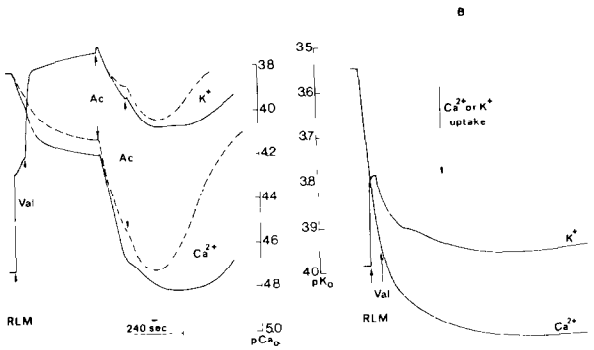

Fig.1. Effect of MalNEt and acetate on Ca^{2+} and K^+ fluxes. The incubation medium contained 0.12 M sucrose, 40 mM choline, 10 mM Tris/Mops, pH 7.4, 2 mM succinate, 2 mM $MgCl_2$, 1 mg/ml BSA, 1 μg/ml oligomycin, 5 μM cytochrome *c*, 104 μM KCl and 149 μM $CaCl_2$. When indicated, 7 mg of mitochondria (RLM), 0.5 μg valinomycin (Val) and 10 mM acetate (Ac). Final vol. 7 ml, 30°C. ——, 50 μM MalNEt (in fig.2B 20 mM acetate (± 50 μM MalNEt)) were present in the incubation medium.

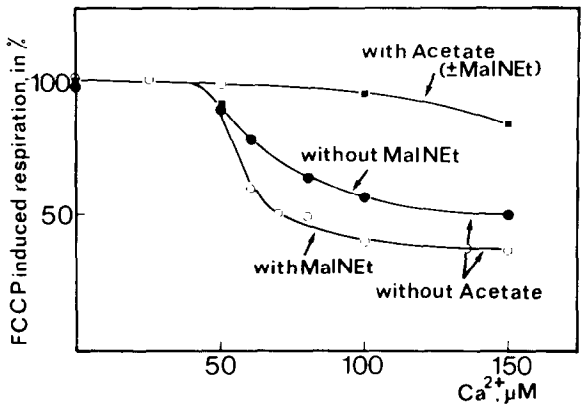

Fig.2. Relationship between Ca^{2+} uptake and e^- transfer from succinate to oxygen: effect of MalNEt and acetate. The incubation medium was the same as fig.1, except that KCl was omitted and $CaCl_2$ was added as indicated in the abscissa. 1 mg/ml mitochondria, final vol. 2 ml, 30°C. After 5 min of incubation 400 pmol FCCP/mg protein were added. ●——●, no further additions; ○——○, 50 μM MalNEt; and ■——■, 40 mM acetate (± 50 μM MalNEt).

phosphatides [4], and with 5 μM cytochrome *c* to compensate for the recently described decreased binding of endogenous cytochrome *c* during Ca^{2+} uptake in salt-containing media [6]. In the presence of valinomycin K^+ is at electrochemical equilibrium and therefore is a probe of the transmembrane $\Delta\psi$. Addition of mitochondria to the medium initiated a phase of Ca^{2+} uptake, only slightly enhanced by valinomycin. A more marked Ca^{2+} uptake followed the addition of acetate. After 4–5 min the phase of Ca^{2+} uptake was followed by a phase of Ca^{2+} release, which was accelerated by the presence of MalNEt (dashed trace). The K^+ trace indicates that Ca^{2+} efflux was paralleled by K^+ efflux and, again, that K^+ efflux was accelerated by MalNEt. Thus Ca^{2+} efflux may be attributed in both cases to a collapse of $\Delta\psi$. In the experiment of fig.1A acetate was added about 4 min after initiation of Ca^{2+} uptake. In fig.1B acetate was present from the beginning. In this case Ca^{2+} uptake was not followed by Ca^{2+} efflux and no collapse of $\Delta\psi$ ensued whether MalNEt was present or absent. Thus when Ca^{2+} uptake occurs in the presence of weak acids Ca^{2+} release is abolished even in the presence of MalNEt.

Ca^{2+} uptake in the presence of MalNEt results in inhibition of the FCCP-induced respiration and that nupercaine protects against this inhibition [4]. However, even in the presence of nupercaine a 50% inhibition of e^- flow from succinate to oxygen was observed (cf. fig.3 in [4]). The experiment of fig.2, carried out in the presence of BSA and Mg^{2+}, essentially confirms this observation. However, fig.2 also shows that the inhibition of the FCCP-induced respiration was present also in the absence of MalNEt, although to a lesser extent and at slightly higher Ca^{2+} concentrations. On the other hand in the presence of acetate the inhibitory effect of Ca^{2+} uptake on the FCCP-induced respiration was abolished both in the presence or absence of MalNEt. Thus the 50% inhibition of respiration observed even in the presence of nupercaine [4] or of BSA plus Mg^{2+} (fig.2) is presumably due to the rise of ΔpH following Ca^{2+} uptake, since it is completely prevented by acetate.

The results of fig.2 are further strengthened by the experiment of fig.3. Mitochondria were tested for their ability to retain K^+. It is seen that after Ca^{2+} uptake mitochondria started to loose K^+ and that the extent of K^+ release was proportional to the amount of Ca^{2+} added. Again, as in fig.2, addition of MalNEt potentiated the Ca^{2+}-induced K^+ release. On the other hand when mitochondria were supplemented with acetate, whether or not MalNEt was present, the extent of K^+ release was virtually abolished.

The experiments of table 1 and fig.4 are an attempt to localize the site of respiratory inhibition occurring during Ca^{2+} uptake, both in the presence or absence of MalNEt. Table 1 shows the respiratory rates of mitochondria treated with FCCP with succinate,

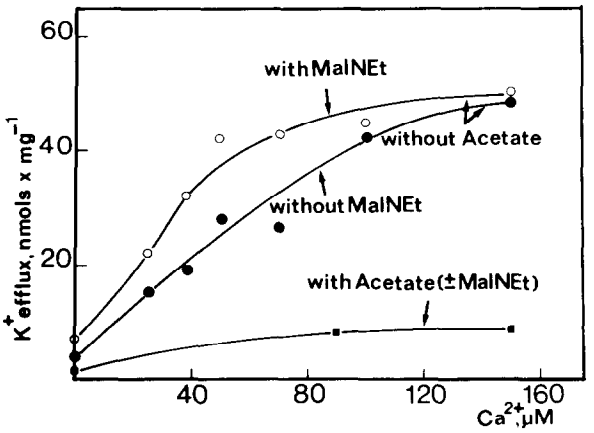

Fig.3. Relationship between Ca^{2+} uptake and spontaneous K^+ efflux: effect of MalNEt and acetate. The incubation medium was the same as fig.1, with $CaCl_2$ as indicated in the abscissa. 1 mg/ml mitochondria, final vol. 4 ml, 30°C. Values on the ordinate refer to spontaneous K^+ efflux after 5 min of incubation. ●——●, no further additons; ○——○, 50 μM MalNEt, and ■——■, 40 mM acetate (± 50 μM MalNEt) were present.

duroquinol, and ascorbate plus TMPD, which provide e^- to complex II, III and IV, respectively. It is seen that a marked respiratory inhibition was observed with succinate and duroquinol, providing e^- to complexes II and III, but not with TMPD, providing e^- to complex IV. It must be noted that the respiratory inhibition was not released when acetate was added 4 min after Ca^{2+} uptake (not shown).

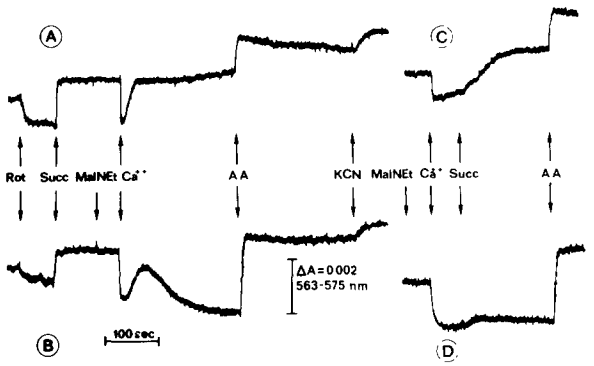

Fig.4. Relationship between Ca^{2+} uptake and redox state of cytochrome *b*: effect of MalNEt and acetate. The incubation medium contained 0.12 M sucrose, 40 mM choline, 10 mM Tris/Mops, pH 7.4, 2 mM $MgCl_2$, 1 mg/ml BSA and 1 μg/ml oligomycin. (A,C) 10 mM acetate, 4 mg/ml mitochondria, final vol. 2 ml, 30°C. When indicated 2 μM rotenone (Rot), 5 mM succinate (Succ), 50 nmol MalNEt/mg protein, 80 nmol $CaCl_2$/mg protein, 50 pg antimycin A/mg protein (AA) and 2 mM KCN. The incubation medium was saturated with oxygen before each experiment.

Table 1

Expt.	Addition(s)	FCCP-induced respiration (natoms oxygen/mg per min)	
		+ Acetate + EGTA	+ MalNEt + Ca^{2+}
1	–	218	101
2	2 mM ascorbate 200 μM TMPD, 0.1 μg antimycin A	210	210
3	200 μM DQH_2	359	149

The incubation medium was the same as in fig.2, plus 2 μM rotenone. When indicated, 20 mM acetate and 100 μM EGTA or 50 μM MalNEt and 100 μM $CaCl_2$. The experiments were started with 1 mg/ml of mitochondria. After 3 min of incubation 400 pmol FCCP/mg protein were added. In Expts 2 and 3 the additions listed in the first column were made 1 min before FCCP. Final vol. 2 ml, 30°C

Fig.4 shows the redox behaviour of cytochrome *b* after a Ca^{2+} pulse in the presence of acetate (A,C) or of MalNEt (B,D). In the presence of acetate (fig.4A) Ca^{2+} addition caused a rapid shift to a more oxidized state followed by recovery of the steady-state redox level preceeding Ca^{2+} addition in about 30 s. On the other hand, when MalNEt was added prior of Ca^{2+} (fig.4B) the shift to a more oxidized state was followed by a slow, incomplete reduction and then by a levelling off at a more oxidized steady state, as compared to that preceeding Ca^{2+} addition. When Ca^{2+} was added before succinate, the subsequent addition of succinate was unable to reduce significantly cytochrome *b* in the presence of MalNEt (fig.4D), while in the presence of acetate the same steady-state reduction level of fig.4A was reached (fig.4C).

4. Discussion

The present results confirm and extend previous observations on the ability of MalNEt to induce Ca^{2+} release from mitochondria [2,4]. The Ca^{2+} release is accompanied by inhibition of the FCCP-induced respiration and has been shown to be associated with the liberation of free fatty acids and lysophosphatides, consequent to the activation of phospholipases [4].

However, in [4] the inhibition of the FCCP-induced respiration occurs in a time considerably shorter than initiation of mitochondrial swelling and liberation of free fatty acids. Furthermore, the respiratory inhibition is almost completely prevented by ruthenium red, the specific inhibitor of mitochondrial Ca^{2+} uptake, and only 50% prevented by nupercaine, whereas both compounds share the same ability in preventing phospholipase activation [4]. This discrepancy suggests that some other factor, in addition to the liberation of lysophosphatides and free fatty acids, is involved in the early Ca^{2+} efflux induced by MalNEt. Two observations suggest that this factor is the rise of matrix pH accompanying Ca^{2+} uptake:

(i) Respiratory inhibition and Ca^{2+} release are not prevented by BSA and Mg^{2+}, although these compounds strongly protect against the effect of free fatty acids;

(ii) Respiratory inhibition, Ca^{2+} release and K^+ loss are completely prevented by acetate, both in the presence or absence of MalNEt.

This interpretation is also supported by the observation that the processes of Ca^{2+} release, K^+ loss and respiratory inhibition are not strictly dependent, although enhanced by, MalNEt. These results support the concept that MalNEt does not alter per se the properties of the inner mitochondrial membrane and that its effects are rather mediated by the rise of matrix pH. Our data with MalNEt confirm the previous results with *p*-chloromercuribenzenesulfonate [2].

The alkalinization of the matrix seems to modify the properties of the inner membrane through two mechanisms: on one hand it causes increased permeability to H^+ and K^+, on the other hand it inhibits e^- transfer at the level of complex III. The first effect may be related to the increased permeability for anions at alkaline pH as in [7], although the mechanism for this effect is still not understood. As to the inhibition of e^- transfer our data show that, under conditions of intramitochondrial alkaline pH, the steady-state redox level of cytochrome *b* is more oxidized and that addition of succinate is unable to reverse this oxidized state. Furthermore, the data of table 1 demonstrate that oxidation of succinate and duroquinol, but not oxidation of TMPD, are inhibited at about the same extent. This pattern suggests that the inhibitory effect is located mainly in the span of the respiratory chain between endogenous CoQ and cytochrome c_1, and that cytochrome *b* reduction by a pH-sensitive component of the respiratory chain is slower at high intramitochondrial pH.

In view of the extensive use of *N*-ethylmaleimide as inhibitor of P_i uptake during determination of the stoichiometries of the H^+ pump [8–10] it may be recalled that, since H^+ fluxes have to be measured under conditions of minimized uptake of weak acids, the rise of matrix pH is maximized. Under these conditions inhibition of e^- transfer and increased permeability may be the cause of the fast levelling off of H^+ extrusion and the subsequent H^+ reuptake.

Acknowledgements

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