CALCIUM RELEASE INDUCED BY *N*-ETHYLMALEIMIDE IN RAT LIVER MITOCHONDRIA

N. E. LOFRUMENTO and F. ZANOTTI

Istituto di Chimica Biologica, Università di Bari e Centro di Studio sui Mitocondri e Metabolismo Energeticò del CNR, 70126 Bari, Italy

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1. Introduction

Recently we have found that in rat liver mitochondria the energy-dependent respiration induced by the addition of Ca^{2+} ions or K^+ plus valinomycin is inhibited by NEM [1]. The inhibition could not be ascribed to the extensively studied effect of NEM on the translocase system which mediate P_i transport and citric cycle intermediates [2,3], in that the respiration was supported by endogenous substrates. The finding that the inhibition was also observed when TMPD + ascorbate was used as source of reducing equivalents, excluded the possibility of a direct effect of NEM on both NAD- and FAD-dependent dehydrogenases. Preliminary experiments on calcium transport have revealed that NEM promotes a back-flow rather than inhibit the efflux of H^+ induced by Ca^{2+} uptake [4,5]. In this paper the effect of NEM on calcium transport following the distribution of $^{45}Ca^{2+}$ across the inner mitochondrial membrane as well as the pH changes in the external medium, have been studied. The data obtained are inconsistent with an unspecific effect of NEM on the permeability of the membrane. As a working hypothesis it is proposed that NEM may influence the energetic state of inner membrane responsible of calcium gradient between cytosol and matrix compartments.

Abbreviations: NEM, *N*-ethylmaleimide; TMPD, *N,N,N',N'* tetramethylphenylenediamine; Hepes, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid; P_i, inorganic phosphate; TCA, trichloroacetic acid; DNP, 2,4-dinitrophenol

2. Materials and methods

Rat-liver mitochondria isolated in 250 mM sucrose were incubated at 25°C in 1.5 ml following medium: 200 mM sucrose, 30 mM KCl and 2 mM Hepes. The pH was adjusted to 7.8 to avoid external pH dropping below 7.0 on addition of Ca^{2+}, with the consequent decrease of NEM effectiveness. A combined glass electrode connected to a Radiometer pH meter (PHM-64) and to a Honeywell recorder for continuous determination of external pH, was used. At the time indicated by arrows, 1 ml mitochondrial suspension was centrifuged for 4 min at 20 000 × *g* in a refrigerated centrifuge. P_i [6] and $^{45}Ca^{2+}$ determinations were carried out on aliquots of 10% TCA extracts of both pellet and supernatant. Endogenous content of calcium (10 nmol/mg protein) was taken into account in determining the specific activity of $^{45}Ca^{2+}$. Spectrophotometric determination of mitochondrial swelling was carried out in parallel samples at 625 nm.

3. Results and discussion

It has been well established that the energy-dependent Ca^{2+} accumulation in mitochondria occurs with a concomitant release of protons in the external medium [7]. Therefore, in appropriate conditions, the transport of calcium can be followed determining the changes in external pH, a method which is only qualitative since the mechanism and the stoicheiometry of H^+ released per Ca^{2+} taken up, is still a matter of debate [8,9]. In fig.1 it is shown

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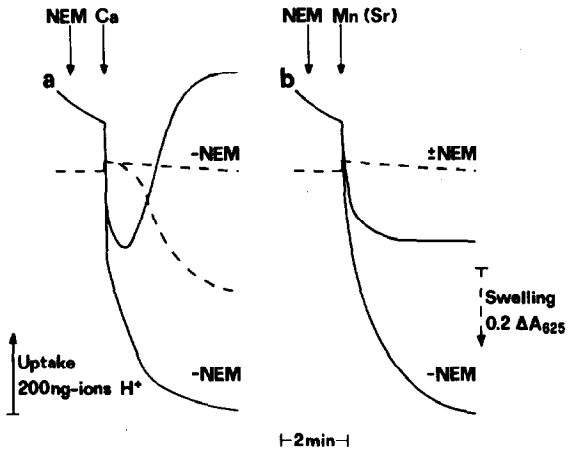

Fig.1. Effect of NEM on mitochondrial swelling and H^+ efflux induced by Ca^{2+}, Mn^{2+} and Sr^{2+}. Mitochondria (4.8 mg protein) were incubated as in section 2. Additions: 0.33 mM *N*-ethylmaleimide (NEM) and 0.5 mM, respectively, of $CaCl_2$ (Ca), $MnCl_2$ (Mn), $SrCl_2$ (Sr). Full line (pH recordings); dashed line (swelling).

that an extensive release of protons occurs when 0.5 mM $CaCl_2$ is added to freshly isolated rat liver mitochondria. The absorbance of mitochondrial suspension followed in parallel experiments at 625 nm, is not influenced during this process. A decrease–increase cycle of external pH and a mitochondrial swelling occur when NEM is added before Ca^{2+}. It can be noted that NEM per se has no effect on both swelling and pH. In expt. (b) the H^+ release induced by Mn^{2+} or Sr^{2+} uptake is strongly inhibited by NEM but in this case no back-flow of protons and no swelling occur. These results together with the findings that when Ca^{2+} uptake is inhibited by the prior addition of DNP or rotenone, NEM had no effect at all on H^+ distribution and swelling [10], indicate that the swelling observed in expt. (a) cannot be ascribed to an unspecific labilization of the inner membrane induced by NEM. This is also consistent with the experiments on transport of P_i and anionic substrates where the intactness of the membrane is a prerequisite to follow the kinetics of exchange diffusion reactions [2,3]. Once accepted that the transport of divalent cations occurs with an identical mechanism [11], the results of fig.1 may suggest that the factors involved to maintain a Ca^{2+} gradient across the inner membrane are different from the ones required for Mn^{2+} or Sr^{2+} gradient. In the experiments of fig.2 quantitative analyses of calcium and P_i, present in the matrix at different stages of pH changes, were done. It has been found that H^+ efflux is directly linked to a net uptake of Ca^{2+} and an increase of endogenous P_i in the matrix. NEM added when Ca^{2+} uptake and H^+ efflux have reached a steady state, promotes a back-flow of both ions and a complete release of endogenous P_i. Mitochondrial swelling induced by NEM in these conditions, becomes evident at least 30 s after the re-uptake of H^+. Although the sensitivity as well as the response time of pH recordings and spectrophotometric determinations of absorbance are not comparable, the high value of delayed time would indicate that swelling is secondary to H^+ movement. No back-flow of protons or swelling could be observed when NEM was added to mitochondria loaded with Mn^{2+} or Sr^{2+} [10]. The addition of rotenone (expt. b) promotes a slight release of Ca^{2+} and H^+ re-uptake and causes a marked decrease in the rate of both swelling and H^+ influx induced by NEM, but has no effect on the total amount of Ca^{2+} and P_i released. The increase of lag-time in swelling onset observed in presence of rotenone is inversely correlated to NEM concentration so that the swelling

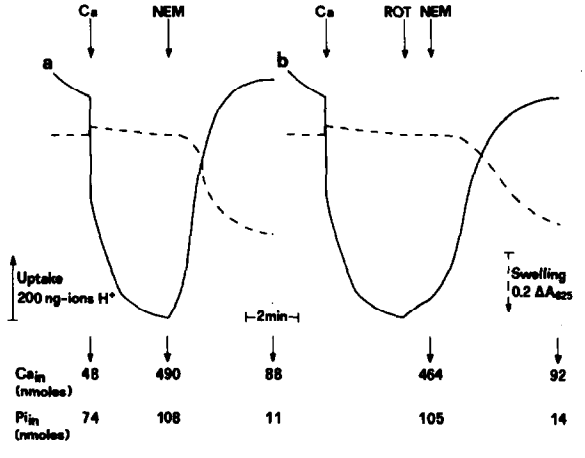

Fig.2. Effect of NEM and rotenone on mitochondrial swelling and on distribution of Ca^{2+}, P_i and protons. Mitochondria (4.8 mg protein) were incubated as in section 2. Additions: 0.5 mM $CaCl_2$ (Ca); 0.33 mM *N*-ethylmaleimide (NEM); 1 μg rotenone (ROT). The nmol calcium and nmol P_i, present in the matrix at the time indicated, were determined as in section 2. Full line (pH recordings); dashed line (swelling).

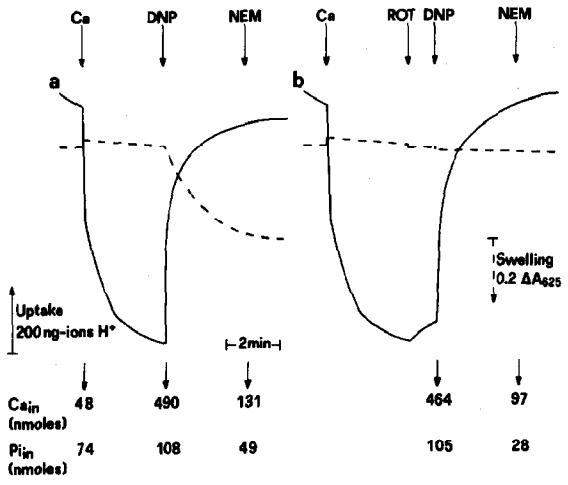

Fig.3. Effect of rotenone on mitochondrial swelling and on re-distribution of Ca^{2+}, P_i and protons induced by DNP. Mitochondria (4.8 mg protein) were incubated as in section 2. Additions: 0.5 mM $CaCl_2$ (Ca); 70 μM 2,4-dinitrophenol (DNP); 1 μg rotenone (ROT); 0.33 mM *N*-ethylmaleimide (NEM). Full line (pH recordings); dashed line (swelling).

induced by NEM at 50 nmol/mg protein, is completely prevented by rotenone. In other experiments it has been found that NEM effect is inhibited by rotenone plus antimycin. These results are inconsistent with the possibility that NEM may promote a further increase of the permeability of the membrane already labilized by Ca^{2+} uptake. NEM inhibits the Ca^{2+}-induced configurational transition of beef heart mitochondria which appears to go parallel with an increased permeability of the inner membrane to sucrose [12]. As illustrated in fig.3 uncouplers, added when Ca^{2+} has been taken up, mimic the effect of NEM promoting together with the expected back-flow of H^+, Ca^{2+} and P_i release, also an extensive mitochondrial swelling. Further addition of NEM has no effect on both swelling and H^+ distribution. Rotenone (expt. b) does not influence the net Ca^{2+} and P_i release even if a decrease in the rate of H^+ re-uptake can be observed, but it completely abolishes the swelling induced by DNP. The generally accepted inverse correlation between the intactness of mitochondrial membrane and swelling is not consistent with the findings until now described which show also that both the uptake and the efflux of Ca^{2+} are not necessarily accompanied or preceded by swelling. From the comparison of our data with [7,13,14] it comes out that swelling phenomenon could be linked to a not well-defined metabolic and/or functional state of mitochondria in relation to the composition of incubation medium. Further support to this is given in fig.4 where is shown that in presence

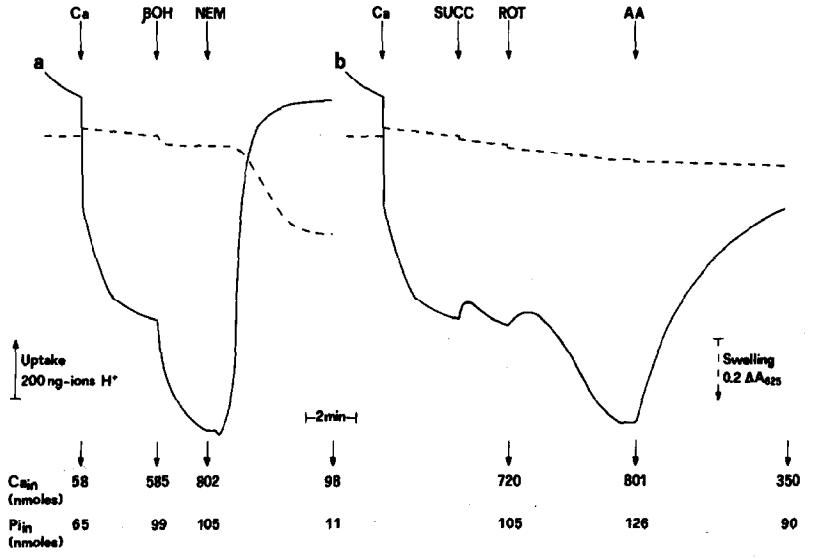

Fig.4. Effect of NEM and respiratory inhibitors on the distribution of Ca^{2+}, P_i and protons in presence of β-hydroxybutyrate and succinate. Mitochondria (5.8 mg protein) were incubated as in section 2. Additions: 0.5 mM $CaCl_2$; 2 mM β-hydroxybutyrate (βOH); 2 mM succinate (SUCC); 0.33 mM *N*-ethylmaleimide (NEM); 1 μg rotenone (ROT); 0.5 μg antimycin A (AA). Full line (pH recordings); dashed line (swelling).

of respiratory substrates, when mitochondria have accumulated 138 ng-ions Ca^{2+}/mg protein, no appreciable swelling can be observed. β-Hydroxybutyrate but not malate or succinate promotes a slight decrease of absorbance which is not comparable to the one induced by NEM. With succinate as substrate (expt. b) all the calcium added is taken up provided that rotenone is also present. In this case the further addition of antimycin promotes the release of Ca^{2+} and P_i but no changes in the absorbance. Identical results were obtained if in expt. (a) rotenone instead of NEM, was added. It is clearly shown that Ca^{2+} and P_i uptake supported by respiratory substrates are both completely reversed by NEM. Swelling also in this case appears to be secondary to the H^+ efflux. Concomitant determinations of oxygen uptake have revealed that even in presence of uncoupler or succinate, anaerobiosis was far from being reached in all the experiments described.

4. Conclusions

Several lines of evidence indicate that in rat liver mitochondria the effect of NEM on the transport of calcium can hardly be ascribed to an unspecific labilization of the inner membrane. They may be summarized as follows:

(1) The H^+ distribution, the absorbance of mitochondrial suspension and the matrix content of P_i are not influenced by NEM per se (fig.1) or when Ca^{2+} transport is inhibited [10].

(2) NEM inhibits the uptake of Mn^{2+} and Sr^{2+} but does not promote swelling or back-flow of protons (fig.1) also when added to mitochondria loaded with one of the two cations [10].

(3) Swelling induced by NEM appears to be secondary to the re-uptake of H^+ (fig.2,4).

(4) Swelling induced by NEM is inhibited by respiratory inhibitors (fig.2).

(5) NEM added after DNP does not promote swelling (fig.3).

In oxygen-pulse experiments [8] NEM raised the H^+/site ratio in presence of Ca^{2+} or K^+ plus valinomycin. Although the experimental conditions are completely different, these results are consistent with our findings. NEM promoting the back-flow of protons may increase the rate of H^+ decay following an oxygen pulse with the consequence of an apparent increase in the H^+ ejection, determined with the extrapolation method [8]. In theory a back-flow of protons would give an inhibition of respiration and indeed it has been found that NEM inhibits the oxygen uptake [1,8]. Therefore the ratio H^+/O and thus the ratio H^+/site calculated from the previous one, may appear to be increased by NEM. However, further experiments are required to test if in oxygen-pulse experiments NEM has an effect identical to the one described here. The findings that uncouplers, respiratory inhibitors and NEM, influence the transport of calcium in a quite similar way may suggest that the energetic state of the inner membrane could be involved in all the three cases. NEM with a mechanism different from that of uncouplers and respiratory inhibitors may interfere with the process which drives the energy of the respiratory chain towards the uptake of Ca^{2+}. The data here reported are consistent with the possibility that the H^+ ejected during the uptake of Ca^{2+} may be the expression of both the activity of respiratory chain [8,9] and the binding of Ca^{2+} to the translocator [15]. Further experiments are in progress to clarify the role of P_i in the overall calcium transport in mitochondria.

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References

[1] Lofrumento, N. E. and Zanotti, F. (1976) Boll. Soc. It. Biol. Sper. 52, 1337–1342.
[2] Lofrumento, N. E. and Zanotti, F. (1976) FEBS Lett. 63, 129–133.
[3] Zanotti, F. and Lofrumento, N. E. (1977) 11th FEBS Meet., Copenhagen, Abstr. Late B-402-4/5.
[4] Lofrumento, N. E. and Zanotti, F. (1977) Boll. Soc. It. Biol. Sper. 53, 164–170.
[5] Lofrumento, N. E. and Zanotti, F. (1977) 11th FEBS Meet., Copenhagen, Abstr. Late B-401-4/5.

[6] Wahler, B. E. and Wollenberger, A. (1958) Biochem. Z. 329, 508.
[7] Chappell, J. B. and Crofts, A. R. (1965) Biochem. J. 95, 378–386.
[8] Brand, M. D., Reynafarje, B. and Lehninger, A. L. (1976) J. Biol. Chem. 251, 5670–5679.
[9] Moyle, J. and Mitchell, P. (1977) FEBS Lett. 73, 131–136.
[10] Lofrumento, N. E. and Zanotti, F. (1978) Boll. Soc. It. Biol. Sper. in press.
[11] Puskin, J. S., Gunter, T. E., Gunter, K. K. and Russell, P. R. (1976) Biochemistry 15, 3834–3842.
[12] Hunter, D. R., Haworth, R. A. and Southard, J. H. (1976) J. Biol. Chem. 251, 5069–5077.
[13] Lehninger, A. L. (1974) Proc. Natl. Acad. Sci. USA 71, 1520–1524.
[14] Brand, M. D., Chen, C. and Lehninger, A. L. (1976) J. Biol. Chem. 251, 968–974.
[15] Reed, K. C. and Bygrave, F. L. (1975) Eur. J. Biochem. 55, 497–504.