H+ GRADIENT IN SUBMITOCHONDRIAL PARTICLES GENERATED BY AN ELECTRONEUTRAL H+/Mn2+ EXCHANGE

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1. Introduction

It has been found that submitochondrial particles, obtained by sonication of mitochondria in the presence of Mg^{2+} and ATP [1] or EDTA [2], can promote several respiration linked ion movements, in addition to proton uptake [3–9].

Certain anions, such as NO_3^- or PCB^-, can be taken up by respiring submitochondrial particles [8,9]. Due to the electroneutral K^+/H^+ or Na^+/H^+ exchange system of the inner mitochondrial membranes [10], K^+ or Na^+ uptake occurs [6–8,11–12] at the expense of H^+ uptake. However, at variance with mitochondria, ion fluxes have seldom been explored in respiration inhibited submitochondrial particles. In the present work, we give evidence of a Mn^{2+}/H^+ exchange, in a respiration inhibited Mn^{2+}–SMP preparation, mediated by the ionophore A23187 [13], in the presence of EDTA.

To monitor H^+ translocation we have followed acridine absorbance [14] or fluorescence changes [14,15]. The paramagnetic Mn^{2+} ion was followed by EPR technique, which has recently been shown to be useful in the study of divalent cation translocation in mitochondria [16–19].

Abbreviations: EPR, electron paramagnetic resonance; PCB⁻, phenyldicarbaundecaborane anion; SMP, submitochondrial particles; FCCP, *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; 9 AA, 9-aminoacridine

2. Materials and methods

Submitochondrial particles with a high Mn^{2+} content (Mn^{2+}–SMP) were prepared according to Hansen and Smith [20] and suspended in a medium containing 0.2 M sucrose, 20 mM Hepes, pH 6.5. The final pH was 6.8–6.9. Protein was determinated by means of biuret procedure [21]. EPR spectra at X band (9100 Mc/s) were registered by means of a Varian V-4502 spectrometer at room temperature. Samples of 75 μl were placed in a flat quartz cell and closed with a teflon plug. The microwave frequency was approx. 9.5 GHz. The modulation amplitude (M), recorder time constant (tc) and scanning rate (SR) were as follows: M 5 G, tc 0.1 s, SR 500 G/min. 9-Aminoacridine fluorescence was measured with a Hitachi MP-2A spectrofluorimeter. The following wavelengths were employed: 400 nm for excitation and 440 nm for emission. The changes in acridine orange absorbance were recorded with a dual wavelength spectrophotometer made in the workshop of the Institute of Physics at the University of Padova. pH measurements were made by a Schott and Gen 9259/8 combination electrode (Jena Glasswerk, Mainz, FRG).

Acridine orange and 9-aminoacridine were obtained from E. Merck. All other chemicals used were of the highest purity available commercially. When not otherwise specified, experiments were performed at room temperature.

3. Results

Figure 1 shows the response of the dye acridine orange to the addition of EDTA and A23187 in the presence of Mn^{2+}–SMP (pH 6.9). EDTA induced a quenching of the dye absorbance at 492 nm and a greater change was induced by the antibiotic A23187. However, after incubation of the SMP for twenty or more minutes at room temperature, A23187 was ineffective. Uptake was followed by a slow release of the dye, which could be stimulated by KCl (see also fig.1c,d). A faster reversal was induced by nigericin, in the presence of K^+ (fig.1a) while preincubation with nigericin abolished the effect (fig.1b). The reversal of the absorbance change was never complete since the change due to EDTA was not reversed. Contrary to nigericin, FCCP was ineffective in preventing the effect (not shown) or in stimulating its reversal (fig.1c). Figure 1d shows that the acridine

orange response is dependent upon the $Mn^{2+}_{in}/Mn^{2+}_{out}$ gradient. The ionophore A23187, without EDTA in the medium, did not trigger the acridine orange response, which became evident only after the external divalent cations were chelated by EDTA. It must be observed that the change of the dye absorbance is in the same direction as that induced by ATP or respiratory substrates [14], which monitor dye uptake by submitochondrial particles. However the acridine orange energy-linked uptake required the presence of anions [14,22], while the experiments shown in fig.1 (except the experiment 1b) were performed in a medium containing sucrose 0.2 M, without added anions.

Similar results could be obtained with 9-aminoacridine (fig.2). The fluorescence quenching of this acridine derivative has been extensively used to measure ΔpH across membranes in liposomes [23], chloroplasts [24], chromatophores [25] and submito-
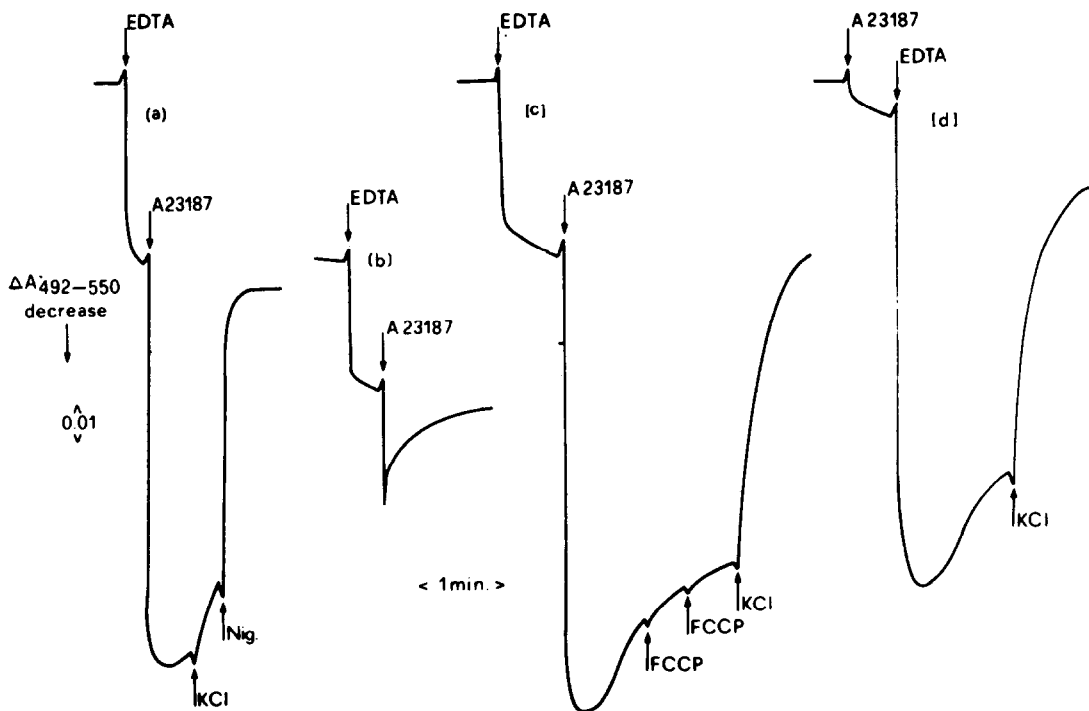

Fig.1. Acridine orange response to A23187 and EDTA additions. The medium (2.5 ml) contained: sucrose 0.2 M, Hepes 10 mM, pH 6.9, Mn^{2+}–SMP 0.56 mg/ml, rotenone 2 μM, acridine orange 8 μM. In the experiment (b) KCl 10 mM and nigericin 1 μg were also present. Additions were: A23187 2 μg, nigericin 1 μg, KCl 20 mM, EDTA (a–c) 1 mM, (d) 250 μM, FCCP 1 μM each addition.
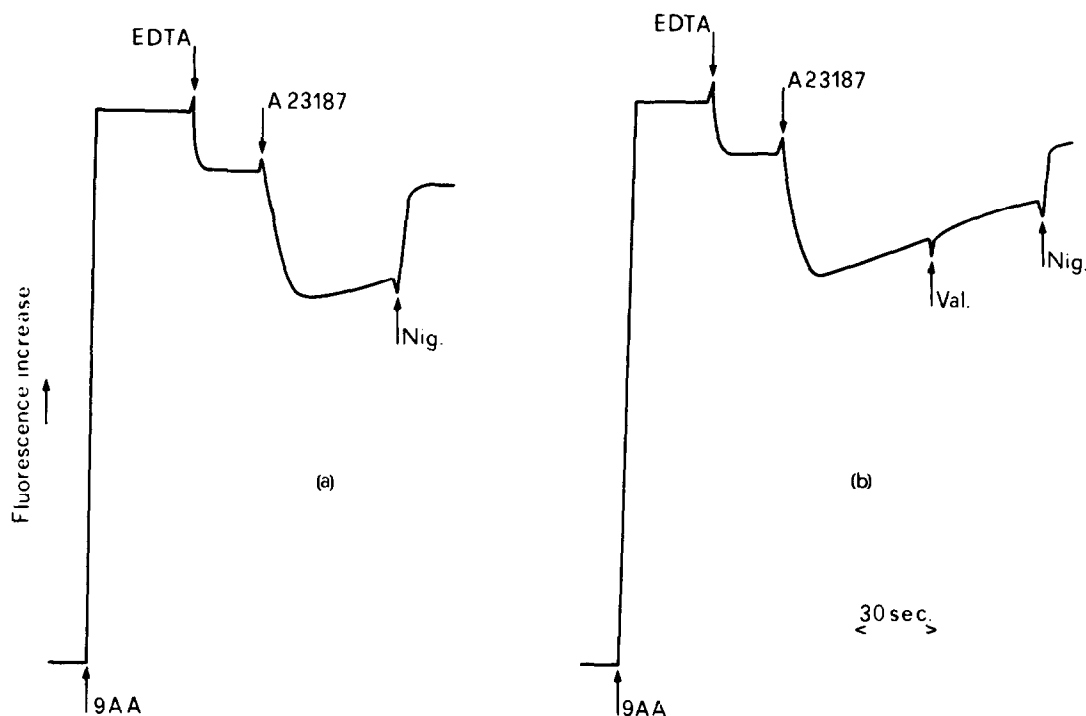
Fig.2. 9-Aminoacridine fluorescence response to EDTA and A23187 additions. The medium (2.5 ml) contained: sucrose 0.2 M, Hepes 10 mM, pH 6.9, Mn^{2+}–SMP 0.56 mg/ml, rotenone 2 μM. In (b) KCl 4 mM was also present. Additions were: 9-aminoacridine 5 μM, EDTA 1 mM, A23187 2 μg, valinomycin 1 μg, nigericin 1 μg.

chondrial particles [15]. The fluorescence quenching was about 20–30% of the initial fluorescence. For the transition de-energized–energized state in EDTA–SMP, a quenching has been observed [15] ranging from 20–50%, depending upon the experimental medium. Figure 2b also shows that, valinomycin, in the presence of K^+, did not stimulate the reversal of the fluorescence quenching.

The quenching of the dye absorbance, induced by EDTA, prior to addition of A23187, was further investigated. Figure 3a shows that Mn^{2+} in excess with regard to EDTA abolished the A23187 as well as the EDTA effect on the acridine orange absorbance. Figure 3b shows that NH_4^+ abolished the A23187, but not the EDTA effect. Finally by supplementing the medium by choline chloride, it was possible to reduce the EDTA effect, without affecting the A23187 effect (fig.3c).

The inhibition of the A23187 effect by Mn^{2+} indicates again that the acridine response monitors

the Mn^{2+}/H^+ exchange, which, in the presence of Mn^{2+} in the external medium, drives H^+ release versus cations uptake.

The effect of Mn^{2+} on the EDTA-induced acridines response will be discussed below.

Figure 4 shows the EPR spectrum of a Mn^{2+}–SMP sample in the presence of 10 mM EDTA. Our EPR experiments, as compared to those of acridine, required a high protein content. This in turn compelled us to use higher EDTA concentrations (see legends of figures). The six line spectrum, shown in fig.4, with a line-width about 25 G, corresponds to free hexahydrated Mn^{2+} ions [17]. This spectrum is practically identical to the one observed with different EDTA concentrations, unless not less than 2.5 mM. At lower EDTA concentrations the signal amplitude was larger, probably due to free external Mn^{2+} ions. Prolonged incubation for twenty or more minutes, with EDTA at room temperature induced disappearance of the six line structure of the EPR spectrum
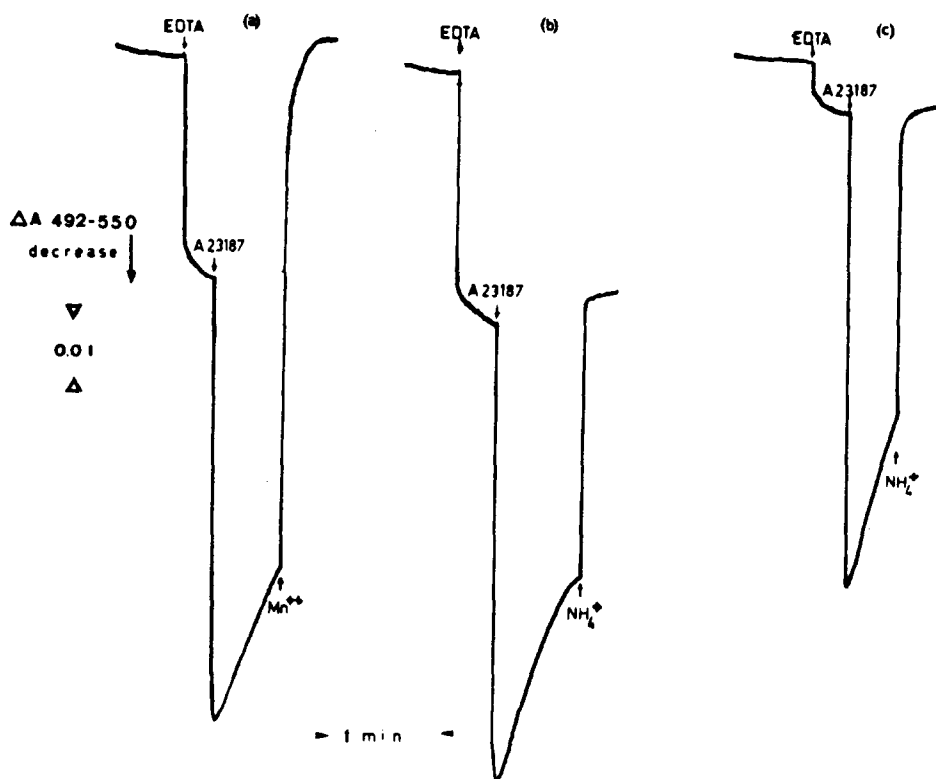
Fig.3. Inhibitory effect of cations on the acridine orange response to EDTA and/or A23187 additions. The medium (2.5 ml) contained: sucrose 0.2 M, Hepes 10 mM, pH 6.9, Mn^{2+}–SMP 0.44 mg/ml rotenone 2 μM, acridine orange 10 μM. In (c) coline chloride 40 mM was present. Additions were: A23187 2 μg, EDTA 250 μM, $(NH_4)_2SO_4$ 2 mM, $MnCl_2$ 1 mM.
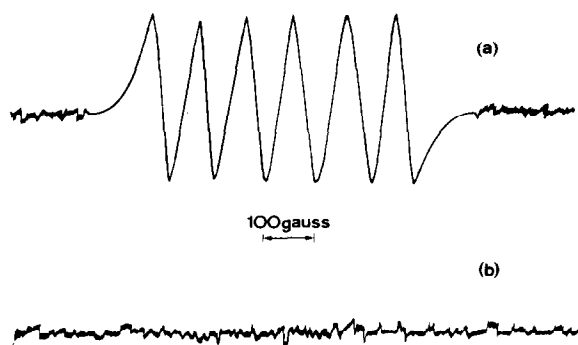

Fig.4. Mn^{2+}–SMP EPR spectra. Effect of A23187. Mn^{2+}–SMP were prepared as described in Materials and methods. EPR measurements were made without further dilution of the preparation (70 mg/ml protein), a few minutes after 10 mM EDTA was added. During the time preceding the measurements, the sample was maintained at 0°C. In (b) A23187 2 μg was present.

(not shown). It is therefore very likely that this signal corresponds to free Mn^{2+} ions inside the vesicles. Further support of this interpretation was given by the effect of the ionophore A23187 on the Mn^{2+} EPR spectrum, which in the above experiments was shown to catalyze the H^+ uptake. In agreement with the acridine response, fig.4 shows that the ionophore A23187 induces the complete disappearance of the six line structure. Control experiments showed no effect of A23187 on the EPR Mn^{2+} spectrum in solution, at the ionophore concentration employed. This result suggests that the free internal Mn^{2+} is translocated to the external medium, where it is chelated by EDTA. A proton ionophore, such as FCCP, was not able to mimic the A23187 effect, when tested either with the acridine or the EPR technique (not shown).

Moreover, in the experimental conditions of the

acridine response, attempts to observe pH changes of the external medium with a glass electrode (see Materials and methods) were unsuccessful.

4. Discussion

The EPR technique gives evidence that Mn–SMP (prepared according to [20]) retains a pool of free Mn^{2+}, which cannot be chelated by externally added EDTA, unless A23187 is added to the medium. It seems therefore very likely to localize this pool in the interior of the SMP.

The usefulness of acridines to measure ΔpH in different vesicular systems (liposomes, chloroplasts, chromatophores, SMP) has been extensively explored [22–24,15], though criticism as to quantitative estimation has been raised [27–29] due to binding properties of some acridines to membranes [14,30].

Acridine responses, shown in the upper experiments, give evidence of an electroneutral Mn^{2+}–H^+ exchange, mediated by the antibiotic A23187. This exchange trasmutes a Mn^{2+} gradient into a H^+ gradient. Since A23187 is also a ionorphore for Mg^{2+}, though at a lower grade [13], Mg^{2+} could participate in the process.

Indeed acridine orange monitors that a H^+ gradient is created, only after the external free Mn^{2+} is chelated by EDTA. Moreover, the response is abolished by K^+ plus nigericin or NH_4^+. Other observations give support to the above suggestion:

(i) The acridine orange (or 9-aminoacridine) response does not require anions. Strong anion requirement has always been observed, when H^+ uptake was driven by ATP or respiratory substrates [14,21] by an electrogenic process [3].

(ii) FCCP does not prevent or abolish the acridine response. This suggests that the H^+ gradient cannot be discharged unless cation counterflow occurs.

(iii) Discharge of the H^+ gradient is stimulated, also in the absence of a K^+ ionophore, by K^+ addition probably due to the natural K^+/H^+ antiporter of the inner mitochondrial membrane [10,11]. The slower rate of discharge observed in the absence

of added K^+, could be due to proton leakage. However, one must also suppose that some other ions present in the medium, or delivered by the SMP, provide charge equilibration by permeating the membranes. Another possibility, suggested by the different rate of spontaneous release observed when the responses of the two probes, acridine orange and 9-aminoacridine are compared (fig.1 and 2), is that the probe itself contributes to ΔpH discharge. It is obvious that this point deserves further investigation.

As mentioned above, FCCP was ineffective to induce the Mn^{2+}/H^+ exchange, whether tested by the acridine or the EPR technique. This could indicate that the submitochondrial preparation used in the present study does not possess the mitochondrial Mn^{2+} or Ca^{2+} carrier which, in the presence of a H^+ ionophore should cause discharge of the Mn^{2+} gradient.

Moreover the mitochondrial membrane seems unable to operate the Mn^{2+}/H^+ exchange without A23187, even if a Mn^{2+} gradient exists. Indeed the EDTA effect, in the absence of A23187, does not monitor a ΔpH across the membranes, the interior more acidic, because the portion of the acridine response due to EDTA is not inhibited by K^+ plus nigericin or NH_4^+ (fig.1a and 3b).

Our interpretation is that as EDTA complexes divalent cations, some negatively charged sites presumably of phospholipids could bind some dye molecules. This binding implies spectral effects as observed for acridine binding to negatively charged liposomes [31]. In agreement with the upper interpretation, addition of Mn^{2+} by inhibiting the dye binding also abolished the absorbance response. A similar interpretation could be given for the choline effect.

Finally, in agreement with previous findings [32], our results indicate that EDTA and/or Mn^{2+} equilibration does occur, across the Mn^{2+}–SMP membranes, after prolonged incubation at room temperature.

References

[1] Low, H. and Vallin, I. (1963) Biochim. Biophys. Acta 69, 361–374.

[2] Lee, C. P. and Ernster, L. (1966) in: Regulation of Metabolic Processes in Mitochondria p. 218, Elsevier, Amsterdam.

[3] Mitchell, P. and Moyle, J. (1965) Nature 208, 1205–1206.

[4] Chance, B. C. and Mela, L. (1967) J. Biol. Chem. 242, 830–844.

[5] Papa, S., Guerrieri, F., Rossi Bernardi, L. and Tager, J. M. (1970) Biochim. Biophys. Acta 197, 100–103.

[6] Montal, M., Chance, B. and Lee, C. P. (1970) J. Memb. Biol. 2, 201–234.

[7] Papa, S. (1966) in: Energy Transduction in Respiration and Photosynthesis, p. 173, Adriatica Editrice, Bari.

[8] Papa, S., Guerrieri, F., Simone, S., Lorusso, M. and Larosa, D. (1973) Biochim. Biophys. Acta 292, 20–38.

[9] Skulachev, V. P. (1971) in: Current Topics in Bioenergetics, p. 127, Academic Press, New York and London.

[10] Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147–1162.

[11] Douglas, D. G. and Cockrell, R. S. (1974) J. Biol. Chem. 249, 5464–5471.

[12] Cockrell, R. S. and Racker, E. (1969) Biochem. Biophys. Res. Commun. 35, 414–419.

[13] Reed, P. and Lardy, H. A. (1972) J. Biol. Chem. 247, 6970–6977.

[14] Dell'Antone, P., Colonna, R. and Azzone, G. F. (1972) Eur. J. Biochem. 24, 553–565.

[15] Rottenberg, H. and Lee, C. P. (1975) Biochemistry 14, 2675–2680.

[16] Gunther, T. E. and Puskin, J. S. (1972) Biophys. J. 12, 625–635.

[17] Puskin, J. S. and Gunther, T. E. (1973) Biochem. Biophys. Res. Commun. 51, 797–803.

[18] Gunther, T. E., Puskin, J. S. and Russell, P. R. (1975) Biophys. J. 15, 319–332.

[19] Bragadin, M., Dell'Antone, P., Pozzan, T., Volpato, O. and Azzone, G. F. (1975) FEBS Lett. 60, 354–358.

[20] Hansen, M. and Smith, A. L. (1964) Biochim. Biophys. Acta 81, 214–222.

[21] Layne, E. (1957) in: Methods in Enzymology, Vol. 3, p. 437, Academic Press, N.Y.

[22] Dell'Antone, P., Frigeri, L. and Azzone, G. F. (1973) Eur. J. Biochem. 34, 448–454.

[23] Deamer, D. W., Price, R. G. and Crofts, A. R. (1972) Biochim. Biophys. Acta 274, 323–335.

[24] Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.

[25] Casadio, R., Baccarini Melandri, A. and Melandri, B. A. (1974) Eur. J. Biochem. 47, 121–128.

[26] Crofts, A. R. (1967) J. Biol. Chem. 242, 3352–3359.

[27] Fiolet, J. W. T., Bakker, E. P. and Van Dam, K. (1974) Biochem. Biophys. Acta 368, 432–445.

[28] Fiolet, J. W. T., Haar, L. V. D. E., Kraayenhof, R. and Van Dam, K. (1975) Biochim. Biophys. Acta 387, 320–334.

[29] Pick, V. and Avron, M. (1976) FEBS Lett. 65, 348–353.

[30] Dell'Antone, P., Colonna, R. and Azzone, G. F. (1972) Eur. J. Biochem. 24, 566–576.

[31] Massari, S. and Pascolini, D. (1977) Biochemistry 16, 1189–1195.

[32] Azzone, G. F. and Massari, S. (1972) FEBS Lett. 28, 61–64.