THE COUPLING OF ELECTRICAL ION FLUXES IN RAT LIVER MITOCHONDRIA

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1. Introduction

Although the molecular mechanism of the mitochondrial proton pump is not understood [1], the ion fluxes driven by the proton pump are phenomenologically distinguished into two groups: (a) the electrical fluxes, whereby strong acids and bases come into equilibrium with $\Delta\psi$ and (b) the electroneutral fluxes whereby weak acids and bases come into equilibrium with ΔpH [2]. Two questions arise. First, this scheme has difficulties to cope with the observation that intact mitochondria catalyze both the active uptake and extrusion of strong electrolytes (i.e. KCl) without inversion of the membrane polarity [3,4]. Second, the notation of the fluxes of strong acids and bases as electrical, leaves still open the dilemma as to whether the coupling of the fluxes involve long range or short range interactions (delocalized or localized potentials). We have examined the effect of divalent cation transport inhibitors on the steady state distribution of divalent cations and on the active divalent cation extrusion [5]. The two processes possess properties which can be explained by assuming a tight coupling of the fluxes. The view is therefore discussed that electrical fluxes may be microscopically coupled through short range interactions (localized potentials).

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2. Experimental

Rat liver mitochondria were prepared according to standard procedures [6]. The last washing was carried in an EDTA free medium. Each preparation was routinely assessed for respiratory control. The protein concentration was determined with the biuret reaction. The medium was routinely bubbled with oxygen except in the oxygen pulse experiments when it was bubbled with N_2. Kinetic measurements were carried out in a dual wavelength spectrophotometer made in the workshop of the Institute of Physics of the University of Padova. The murexide measuring wavelengths were 542–500 nm. Oxygen pulse experiments were made essentially as suggested by Mitchell and Moyle [7]. The mitochondrial suspension was covered with paraffin oil and was allowed to reach anaerobiosis in a cuvette equipped with a stirring device. pH changes were followed by a H^+ sensitive glass electrode. The reaction was started by adding 100 μl of the suspending medium saturated with oxygen.

The active extrusion of $Sr(NO_3)_2$ was followed as described previously [5]. The changes in absorbance were followed either in an Eppendorf Photometer at 546 nm or in a Perkin–Elmer spectrophotometer Mod. 124 at 600 nm. The penetration of $Sr(NO_3)_2$ was initiated by adding the mitochondria to a medium containing $Sr(NO_3)_2$ in the presence of rotenone. At the end of the electrolyte penetration the active extrusion was initiated by the addition of succinate. All experiments were made at room temperature. Ruthenium Red was a commerical product by Sigma and used after purification. All other chemicals were analytical grade.

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3. Results

La^{3+} and Ruthenium Red act as specific inhibitors of divalent cation transport in mitochondria [8–10]. Rossi et al. [11] reported that addition of Ruthenium Red after completion of Ca^{2+} uptake does not affect the steady state accumulation of the cation. In contrast Sordahl [12] reported a slight release of Ca^{2+} induced by the addition of Ruthenium Red to aerobic mitochondria in steady state conditions. Stucky and Ineichen [13] showed that Ruthenium Red induces a loss of endogenous calcium from aerobic mitochondria. The Ruthenium Red or La^{3+} induced Ca^{2+} release is opened to different interpretations. One is that Ruthenium Red or La^{3+} cause, in addition to inhibition of divalent cation transport, also a lowering of the mitochondrial energy level. However this occurs at concentrations 20–30 times higher [9]. The effect of Ruthenium Red (or La^{3+}), at the concentrations used here, was further tested on respiratory rate, respiratory control, ADP/O ratio and K^+ accumulation ratio. None of these parameters was affected.

Another possibility is that intact mitochondria posses a neutral H^+/Ca^{2+} antiporter. The operation of this antiporter, driven by ΔpH, would drive the extrusion of Ca^{2+}. Such a process would be compensated by a constant uptake in the absence, but not in the presence, of Ruthenium Red. The electroneutral divalent cation ionophores, A23187 and X537A [14,15], in concentrations causing only a negligible release of Ca^{2+}, induce a complete release of Ca^{2+} if Ruthenium Red is added [12]. The presence of a natural H^+/Ca^{2+} exchange can be studied by following the penetration of Ca $(acetate)_2$ in a way similar to that used to follow the penetration of the acetate and phosphate salts of NH_4^+ or K^+ (+ nigericin).

Figure 1 shows the rate of absorbance decrease induced by the addition of the electroneutral ionophore X537A (or A23187 not shown) to mitochondria incubated in acetate salts of various divalent cations. The swelling is due to penetration of osmotically active solutes. It is seen that the rate of swelling increased proportionally to the amount of ionophore. The rate of cation transport was roughly proportional to the reported p*K*a of formation of the cation ionophore complex except in the case of

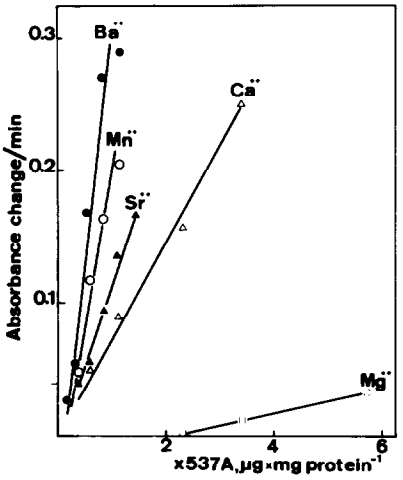

Fig.1. Rate of X537A induced transport of divalent cations. The medium contained acetate salts of the various divalent cations at a concentration of 30 mM, supplemented with 5 mM Tris–Cl, pH 7.0 and 3 µM Rotenone. The reaction was started by the addition of X537A in the amounts indicated on the abscissae. On the ordinate are reported the initial rates of the absorbance changes. 1 mg mitochondrial protein/ml.

Mn^{2+} [16]. Figure 1 shows that at zero ionophore concentration the rate of swelling was practically negligible in the case of all cations. This does not support the existence of a natural antiporter for divalent cations [17].

Another interpretation for the Ruthenium Red or La^{3+} induced Ca^{2+} release is that it is due to slow H^+ ion leak. If this is the case addition of FCCP which also causes a H^+ ion leak should increase the rate of Ca^{2+} release. Figure 2 shows the La^{3+} induced Ca^{2+} release in the absence and presence of FCCP. After addition of FCCP there was a small release of Ca^{2+} and then a new steady state was reached. Addition of La^{3+} caused a release of Ca^{2+} which was considerably faster than in the absence of FCCP. In the insert of fig.2 it is shown that the rate of La^{3+} induced Ca^{2+} release was proportional to the concentration of FCCP. Thus in this experiment the increased rate of Ca^{2+} release is coupled to an increase of H^+ ion permeability induced by the uncoupler.

In another experiment 40 nm FCCP was added from the beginning which resulted in a steady state level of Ca^{2+} uptake slightly lower than in the

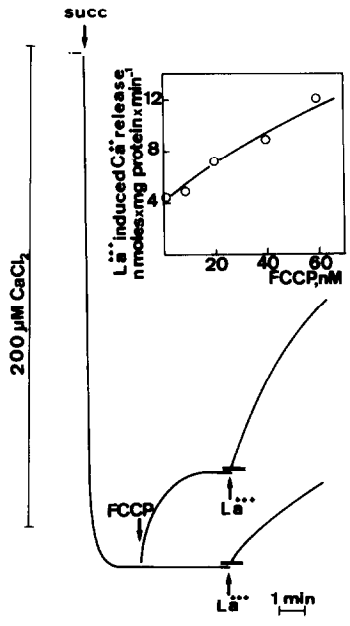

Fig.2. Enhancement by FCCP of the La^{3+} induced Ca^{2+} release. The medium contained 200 mM sucrose, 20 mM Hepes, pH 7.3, 5 mM $MgCl_2$, 3 μM rotenone, 2.4 mM acetate Tris, 200 μM $CaCl_2$, 2 mg/ml mitochondrial protein, 100 μM Murexide. When indicated were added 2.5 mM succinate–Tris, 60 nM FCCP, 8 μM La^{3+}. In the insert the amount of FCCP was variable.

absence of FCCP. Again Ruthenium Red induced a Ca^{2+} release that was faster in respect to the FCCP-free sample. Ageing of mitochondria also resulted in an increase of the Ruthenium Red or La^{3+} induced Ca^{2+} release. The effect was removed by the addition of albumin.

Mitchell and Moyle [7] considered the H^+ ejection following the addition of O_2 to anaerobic mitochondria as expression of the operation of an electrogenic proton pump. Chappell and Haarhoff [18] and Chance and Mela [19] however showed that addition of EDTA abolished the H^+ ejection. Figure 3 shows that the H^+ ejection following the addition of O_2 pulses to anaerobic mitochondria consisted in a rapid and in a slow phase. Pretreatment of the mitochondria with either EDTA or Ruthenium Red abolished completely the rapid phase of H^+ ion ejection while the slow phase was not affected. Presumably the slow phase is related to a Ruthenium Red insensitive cation flux.

In fig.4 it is seen that incubation of mitochondria in $Sr(NO_3)_2$ resulted in an extensive swelling [5,17]. Addition of succinate at the end of the swelling phase induced first a very rapid osmotic shrinkage and then a respiration dependent extrusion of $Sr(NO_3)_2$. Figure 4 shows also that the process of active $Sr(NO_3)_2$ extrusion was inhibited by La^{3+} at about the same concentrations at which it inhibited the active divalent cation uptake. The titration with Ruthenium Red yielded similar results (not shown).

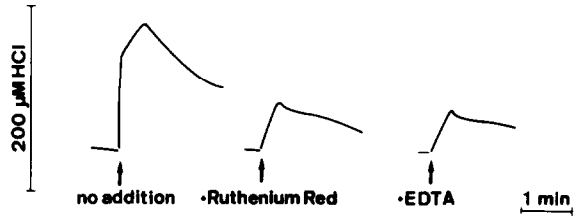

Fig.3. Inhibition of H^+ ion extrusion during oxygen pulses by Ruthenium Red. The medium contained 250 mM sucrose, 1 mM Tris, pH 7.4, 2.5 mM succinate – Tris, 13 mg/ml mitochondrial protein. Ruthenium Red was 3 nmol/mg protein and EDTA 1 mM. When indicated were added 100 μl medium saturated with oxygen.

Figure 5 shows the effect of electroneutral ionophores on the process of active $Sr(NO_3)_2$

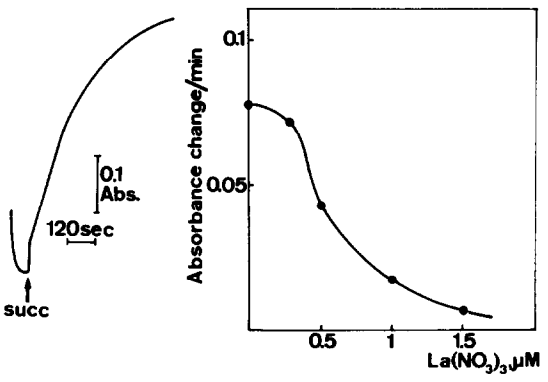

Fig.4. Active extrusion of $Sr(NO_3)_2$ and effect of La^{3+} The medium contained 12.5 mM $Sr(NO_3)_2$, 10 mM Tris–Cl, pH 7.9, 1 μM rotenone and 1 mg/ml mitochondrial protein. The reaction was initiated by the addition of mitochondria. At the end of swelling the active extrusion was initiated by 3 mM succinate. In the left part the kinetics of swelling and shrinkage phases. In the right part the inhibition by La^{3+} of Sr^{2+} extrusion.

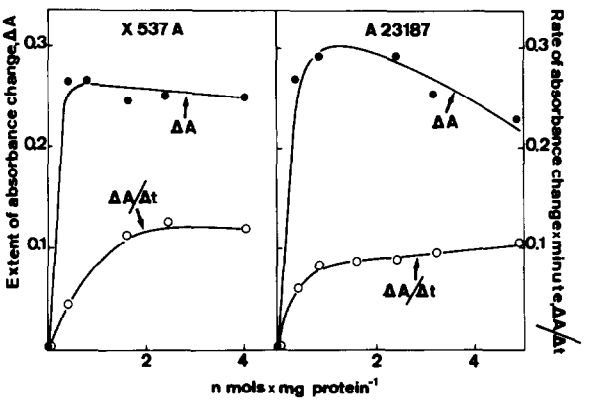

Fig.5. Effect of A23187 and X537A on active extrusion of $Sr(NO_3)_2$ in the presence of Ruthenium Red. The medium contained 20 mM $Sr(NO_3)_2$, 5 mM Tris–Cl, pH 7.6, 3 μM rotenone. Penetration of electrolytes was initiated by adding mitochondria, 1.5 mg protein/ml to the incubation medium. After the penetration of electrolytes was completed, the mitochondria were supplemented with 4 μM Ruthenium Red and ionophores and extrusion started with 3 mM succinate.

extrusion in the presence of La^{3+} or Ruthenium Red. After the process of active extrusion was completely inhibited by La^{3+} or Ruthenium Red addition of A23187 or X537A restored the active extrusion. The rate of the ionophore-induced extrusion was lower than that observed in the La^{3+} or Ruthenium Red uninhibited mitochondria.

4. Discussion

In the electrogenic proton pump $\Delta\psi$ is used to move primarily strong electrolytes and ΔpH to move secondarily weak electrolytes. The coupling of fluxes then involves electrical forces in the primary reaction moving the strong electrolytes and chemical forces in the secondary reaction moving the weak electrolytes. The electrogenic proton pump predicts that the polarity of the fluxes of strong bases and acids depends on the membrane polarity. Therefore the inversion of the fluxes of strong bases and acids requires an inversion of the membrane polarity.

Recently Azzone et al. [3,4] have reported two ion fluxes which are anomalous in respect to this prediction: (1) the active uptake of Cl^- and (2) the active extrusion of strong bases. The observations have been explained by assuming that the primary reaction through the proton pump is tightly coupled either to the uptake of cations or to the extrusion of anions, thus leading to the formation of ΔpH. Then the secondary reaction consists of an electrical H^+ influx down the ΔpH tightly coupled either to the influx of Cl^- or to the efflux of cation. The concept of tight coupling between electrical ion fluxes is supported by the present data.

According to the electrogenic H^+ pump, in steady state permeant cations are distributed at electrochemical equilibrium with a respiratory chain generated $\Delta\psi$. A Ruthenium Red or La^{3+} induced cation release would be explained by the presence of a H^+/divalent cation antiporter. However the data of fig.1 do not support the existence of such an antiporter. Furthermore the rate of Ruthenium Red and La^{3+} induced Ca^{2+} release increases proportionally to the uncoupler concentration. This indicates that the H^+ ion influx induced by uncouplers cannot be compensated by operation of the proton pump although an excess of respiration is available.

Two explanations may be considered. One, the influx of H^+ via FCCP is tightly coupled with the efflux of Ca^{2+}. FCCP is then equivalent to an electroneutral ionophore which causes release of Ca^{2+} when the reuptake of Ca^{2+} via the proton-pump is inhibited. Two, once the influx of H^+ via FCCP has occurred, the extrusion of H^+ ions via the proton pump does not take place unless coupled with the uptake of Ca^{2+}. This is equivalent to saying that the operation of the proton pump is inhibited by divalent cation transport inhibitors when divalent cations are the only permeable ion. Such a conclusion is supported by the oxygen pulse experiments where the rapid phase of H^+ ion ejection is abolished by Ruthenium Red in the same way as it is abolished by EDTA.

The inhibition of $Sr(NO_3)_2$ extrusion by La^{3+} or Ruthenium Red indicates that the divalent cations move via the natural carrier for divalent cations. The movement of divalent cations through the natural carrier is known to be electrical [17]. After the process of $Sr(NO_3)_2$ extrusion has been inhibited by La^{3+} or Ruthenium Red addition of A23187 or X537A restores the extrusion. Thus the

extrusion occurring via the natural carrier coupled to the reuptake of H^+ and that occurring via the electroneutral ionophores are essentially equivalent. The mitochondrial membrane does not distinguish between an electroneutral transport due to the extrinsic ionophore X537A or A23187 and an electroneutral transport due to tight coupling between electrical H^+ influx and electrical cation efflux via the natural carrier.

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