INDUCTION OF ELECTRONEUTRAL EXCHANGES OF H^+ WITH K^+ IN RAT LIVER MITOCHONDRIA

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1. Introduction

The view of ΔpH-driven native H^+–K^+ carriers catalyzing an electroneutral H^+/K^+ exchange in native mitochondria meets a crucial dilemma. On one side, the active and rapid matrix shrinkage of mitochondria swollen in a K^+ medium necessarily involves the occurrence of an electroneutral H^+/K^+ exchange in order to account for a K^+ flux opposite in polarity to that driven by the membrane potential. On the other side if native mitochondria would possess such a fast-operating H^+/K^+ antiporter the K^+ distribution in state 4 mitochondria would never approach quasi-thermodynamic equilibrium. Hence the respiratory rate of valinomycin-treated mitochondria in low K^+ media would not decline to low levels, as usually observed, and it would not be possible to calculate $\Delta\psi$ by applying the Nernst equation to the K^+ distribution [1 9]. The present study suggests one way to overcome this dilemma by showing that a H^+/K^+ exchange activity is induced in rat liver mitochondria following the addition of agents or the establishment of conditions usually thought to result only in an increased K^+ permeability [10–12]. In these cases however it appears that the induction of the H^+/K^+ exchange activity parallels also an increase of H^+ permeability presumably due to some rearrangement of the membrane structure [13]. The view is therefore considered that these induced H^+/K^+ electroneutral fluxes involve a short-range coupling of electrical H^+ and K^+ fluxes and may play a major role in regulating the matrix volume.

2. Experimental

Rat liver mitochondria were prepared according to standard procedures [14]. The kinetics of solute influx and efflux were followed by recording the absorbance changes either with a Hitachi Perkin Elmer Spectrophotometer model 124 at 600 nm or with a dual wavelength spectrophotometer [15]. In the latter case the safranine wavelength was used (520–554 nm) although the dye was omitted. The volume changes calculated from spectrophotometric measurements were always compared with those calculated from gravimetric or isotopic measurements [4,5,15].

$\Delta\psi$ was followed kinetically by measuring the absorbance changes in the presence of 25 μM safranine [16,17]. More precise determinations of $\Delta\psi$ were carried out on the basis of [^{3}H]triphenylmethylphosphonium distribution [14,18]. ΔpH was measured on the basis of [^{3}H]acetate distribution [14,18]. Respiration was measured with a Clark oxygen electrode.

The conductances for H^+, Cm_{H^+}, and for K^+, Cm_{K^+}, are defined as the ratios between the ion currents, J_{H^+} and J_{K^+}, and the H^+ and K^+ electrochemical gradients, $\Delta\tilde{\mu}_H$ and $\Delta\tilde{\mu}_{K^+}$, respectively [19]. The ion currents, J_{H^+} and J_{K^+}, have been calculated by multiplying the respiratory rates for the stoichiometry of the H^+ pump (4 charges/site). Use of a stoichiometry of 2 charges/site does not alter the pattern. $\Delta\tilde{\mu}_H$ has been calculated on the basis of the values of $\Delta\psi$ and ΔpH. $\Delta\tilde{\mu}_{K^+}$ has been calculated on the basis of the values of $\Delta\psi$ and the K^+ gradient. Activity of

matrix K^+ has been assumed as equal to half the osmolarity of the medium.

3. Results

3.1. *Effect of membrane stretching on H^+ and K^+ conductance*

Figure 1 shows that the respiratory rate (and therefore the K^+ current, J_{K^+}) of mitochondria, incubated in the presence of low valinomycin, increased with the decrease of KNO_3 concentration. The K^+ electrochemical gradient $\Delta\tilde{\mu}_{K^+}$ remained roughly constant until 60 mM KNO_3 and then tended to decrease gradually. The K^+ conductance, Cm_{K^+}, increased parallel to the decrease of osmolarity. The mitochondrial surface has been calculated to be 10-times higher at 40 mosM as compared with 200 mosM [20]. In fig.1 the K^+ conductance was 10-times higher at 20 mM KNO_3 as compared with 100 mM KNO_3. Figure 1 then suggests that the rate of valinomycin-catalyzed transport depends on the degree of unfolding of the mitochondrial cristae.

Figure 2 shows that the H^+ current, J_{H^+}, increased with the increase of matrix volume, as obtained by incubating mitochondria in sucrose media of decreasing osmolarity. The $\Delta\tilde{\mu}_H$ decreased only slightly until the matrix volume reached 3 μl × mg protein^{-1} and then

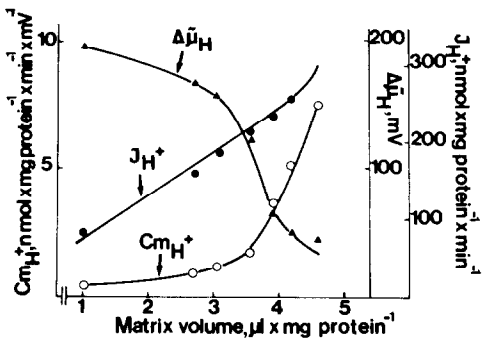

Fig.1. Dependence of K^+ conductance on membrane stretching. The medium contained 5 mM succinate–Tris, 10 mM acetate–Tris (pH 7.4), 1 μM rotenone, 25 μM triphenylmethylphosphonium and various concentrations of KNO_3 to reach the indicated osmolarity. 1.0 pmol valinomycin × mg protein^{-1}. 1 mg protein/ml. The reaction was initiated by the addition of mitochondria to the incubation medium and terminated after 30 s by centrifugation.

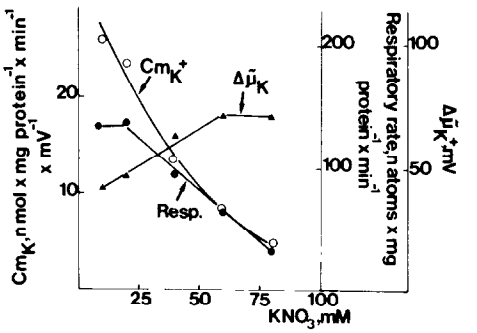

Fig.2. Dependence of H^+ conductance on membrane stretching. The medium contained 2 mM $MgCl_2$, 5 mM acetate–Tris, 5 mM succinate–Tris (pH 7.4), 1 μM rotenone, 25 μM triphenylmethylphosphonium and various concentrations of sucrose to osmotically change the matrix volume 1 mg protein/ml. The reaction was initiated by the addition of mitochondria to the incubation medium and terminated after 30 s by centrifugation.

sharply above this value. The H^+ conductance, Cm_{H^+}, followed a similar pattern, undergoing a slight increase up to a volume of 3 μl × mg protein^{-1} and then an exponential increase above this volume. Figure 2 suggests that the stretching of the membrane results in increase of the H^+ conductance. There is however a critical degree of stretching correlated with a dramatic increase of H^+ conductance.

3.2. *H^+/K^+ exchange in phosphate salts*

The low rate of K_2HPO_4 influx in fig.3, indicates a negligible rate of H^+/K^+ exchange [1,2]. This rate calculated on the increase of matrix volume was < 7 nmol × mg protein^{-1} × min^{-1}. Addition of 1 nmol A23187 × mg protein^{-1} resulted, after a lag phase of ~4 min in an increase of the rate of exchange. EDTA, together with A23187, resulted in a shorter lag phase and in a more rapid exchange. Valinomycin at 1 nmol × mg protein^{-1} had a double effect. (1) It increased the rate of exchange. (2) It caused a further increase of the rate of exchange after a lag phase. When mitochondria were incubated in phosphate media of lower osmolarity, say 40 mM, the effects of the addition of valinomycin and of A23187 + EDTA were more marked, in that the time for onset of exchange was shortened and the rate of exchange accelerated.

Figure 4 shows the effect of valinomycin on two

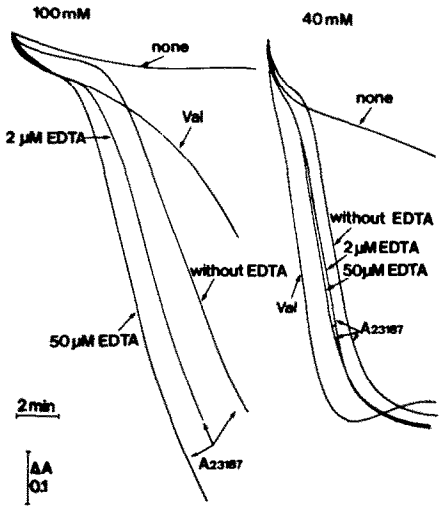

Fig.3. H^+/K^+ exchange induced by valinomycin and A23187 + EDTA. The medium contained either 100 mM or 40 mM K_2HPO_4 (pH 7.4), 2 μM rotenone, 1 mg protein/ml. Other additions were as follows: 1 nmol valinomycin × mg protein^{-1}, 1 nmol A23187 × mg protein^{-1}.

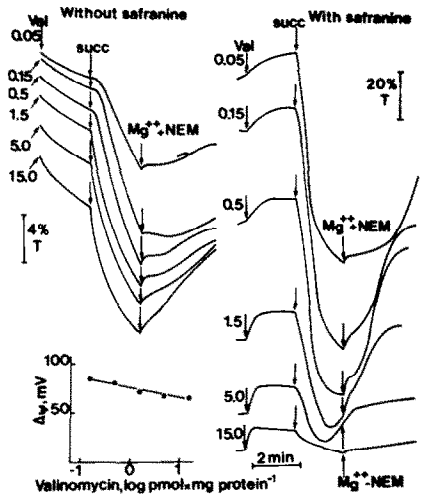

Fig.4. Effect of various valinomycin concentrations on H^+/K^+ exchange and $\Delta\psi$ in phosphate salts. The medium contained 40 mM K_2HPO_4 (pH 7.4), 1 μM rotenone, 1 mg protein/ml. When indicated, 25 μM safranine.

processes of H^+/K^+ exchange. There was first a H^+/K^+ exchange following addition of valinomycin to rotenone-treated mitochondria. Then followed a phase of active K^+ uptake due to succinate oxidation. Finally there was a H^+/K^+ exchange during succinate oxidation in the presence of Mg^{2+} + NEM. The rate of exchange was always correlated with the amount of valinomycin. At 0.05 pmol valinomycin × mg protein^{-1} the rate of H^+/K^+ exchange was low both during passive influx and during active efflux. At 15 pmol valinomycin × mg protein^{-1} the rate of H^+/K^+ exchange was high both during passive influx and during active efflux. Figure 4 shows also the dimension of $\Delta\psi$. The slight increase of safranine response following addition of valinomycin to anaerobic mitochondria is probably related to a collapse of Donnan potential. Addition of succinate caused a rapid rise of $\Delta\psi$ at low but not at high valinomycin concentrations. Furthermore while at low valinomycin $\Delta\psi$ remained high, at high valinomycin tended to collapse after ~2 min. Addition of Mg^{2+} + NEM caused a collapse of $\Delta\psi$, after a long lag phase of low valinomycin and a short lag phase at high valinomycin. The maximal rate of H^+/K^+ exchange coincided with the phase of collapse of $\Delta\psi$. After 3 min addition of Mg^{2+} + NEM the mitochondrial suspension was centrifuged and assayed for the ion distribution. Values of $\Delta\psi$ were < 50% those found with native mitochondria and tended to be lower at higher valinomycin.

Figure 5 shows the effect of increasing EDTA concentrations, in presence of 1 nmol A23187 × mg protein^{-1} on the H^+/K^+ exchange during both passive influx and active efflux. As already seen in fig.3,

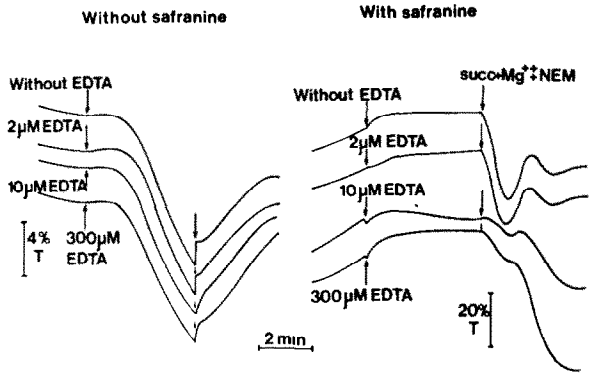

Fig.5. Effect of various A23187 + EDTA concentrations on H^+/K^+ exchange and $\Delta\psi$ in phosphate salts. The medium was as in fig.4. 1 nmol A23187 × mg protein^{-1} was always present.

after 2 min incubation in the presence of A23187 + EDTA there was a marked enhancement of the rate of exchange. Addition of succinate + Mg^{2+} + NEM initiated active efflux. The rate of H^+/K^+ exchange during active efflux increased proportionally to the concentration of EDTA. The safranine response indicates a slight collapse of $\Delta\psi$ after addition of A23187 + EDTA. After 4 min addition of succinate + Mg^{2+} + NEM caused a rise of $\Delta\psi$ the dimension of which decreased with the amount of EDTA. The safranine trace showed large oscillations. In the absence of EDTA after the rapid rise, $\Delta\psi$ tended to collapse and then to rise again. There was a gradual damping of the oscillations. At higher EDTA the initial rise of $\Delta\psi$ was more limited. However after ~2 min there was a more extensive rise. The more extensive rise at higher EDTA occurred in coincidence with the more extensive shrinkage of the mitochondria.

3.3. *H^+/K^+ exchange in strong acid salts*

Figure 6 shows the effect of increasing valinomycin concentrations first on passive electrical K^+ influx and then on H^+/K^+ exchange during active efflux [5]. The increase of valinomycin affected both the rate and extent of the initial passive electrical K^+ influx and the rate of the subsequent H^+/K^+ exchange during succinate oxidation. In Cl^- media, at 0.05 pmol valinomycin × mg protein^{-1}, initiation of respiration resulted in a phase of further electrolyte influx, accompanied by a slight oscillation. Further electrolyte influx and oscillation occurred also at 0.5 pmol valinomycin. At 5.0 pmol valinomycin the initial influx was more extensive and initiation of respiration was accompanied by rapid electrolyte efflux. Addition of succinate was accompanied by a rapid rise of $\Delta\psi$ at 0.05 pmol and 0.5 pmol but not at 5.0 pmol valinomycin. Replacement of Cl^- with I^- resulted in a larger electrolyte influx also at low valinomycin. Initiation of respiration resulted always, in phase of active efflux, although rate and dimension of efflux were dependent on the amount of valinomycin. There was still a rise of $\Delta\psi$ at low valinomycin and a complete collapse of $\Delta\psi$ at high valinomycin, in correspondence with the more extensive shrinkage. $\Delta\psi$ decreased in the presence of Cl^- from >130 mV at low valinomycin to ~100 mV at high valinomycin. In the presence of I^-, $\Delta\psi$ decreased from 80 mV at low valinomycin to 57 mV at high valinomycin. Similar experiments were also carried out with other lipophilic anions such as NO_3^- or SCN^-. Dimension of passive electrolyte influx or active efflux increased with the anion lipophilicity while dimension of $\Delta\psi$ decreased with the anion lipophilicity.

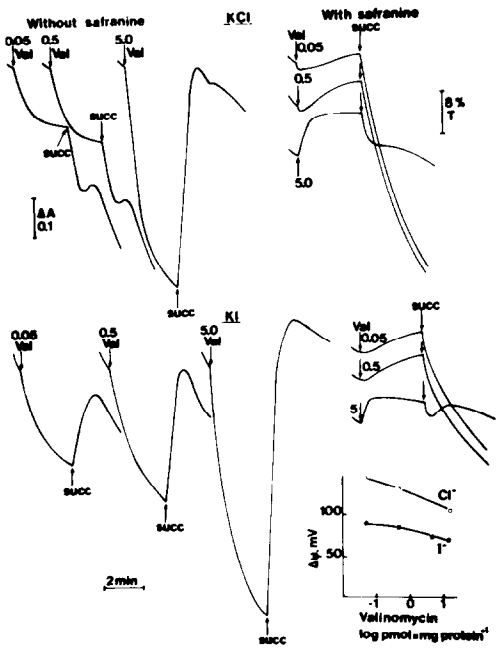

Fig 6. Effect of anion lipophilicity on H^+/K^+ exchange and $\Delta\psi$ in strong acid salts. The medium contained either 40 mM KCl, 10 mM Tris–Cl or 40 mM KI, 10 mM Tris–NO_3 (pH 7.4), 1 μM rotenone. Amounts of valinomycin were 0.05, 0.5 and 5.0 pmol × mg protein^{-1}. Succinate was 2.5 mM.

4. Discussion

This study substantiates the conclusion [25,26] that the rate of H^+/K^+ exchange in native mitochondria is negligible. An exchange rate of ~7 nmol × mg protein^{-1} × min^{-1}, is lower than the electrical H^+ and K^+ leaks in intact mitochondria.

A marked increase of the rate of exchange occurs after addition of valinomycin and of A23187 + EDTA. Valinomycin is known to induce an electrical permeability for K^+ [10] while A23187 + EDTA has been suggested to increase the electrical univalent cation permeability by removing membrane-bound Mg^{2+} [12]. Induction of H^+/K^+ exchange, where the K^+

permeability is electrical in nature, implies an increase also of the electrical H^+ permeability. Such an increase is supported by the following observations:

(i) While induction of K^+ transport by valinomycin shows no lag phase, a lag phase occurs during induction of H^+/K^+ exchange;

(ii) There is a marked correlation between rate of exchange and dimension of $\Delta\psi$; high rates of exchange are usually accompanied by collapse of $\Delta\psi$ and viceversa;

(iii) Although the membrane of native mitochondria has a negligible electrical permeability for the charged phosphate anion, an active phosphate extrusion takes place after passive influx of K_2HPO_4: the extrusion implies an electrophoretic movement of P_i. The sensitivity to mersalyl of P_i extrusion suggests an involvement of the P_i carrier operating electrogenically under these conditions [4].

Membrane-bound Mg^{2+} may play a role in controlling the permeability for H^+:

(i) A23187 + EDTA is a most powerful depleting agent for membrane Mg^{2+};

(ii) There is a correlation between enhancement of exchange and depletion of membrane-bound Mg^{2+} (unpublished).

The lag phase for the induction of the exchange may be due to the time required to remove Mg^{2+} from the binding sites controlling H^+ permeability. A lag phase during permeability changes of the mitochondrial membrane has been observed and attributed to release of Ca^{2+}, activation of phospholipase and release of endogenous fatty acids. Different factors may converge in causing a release of membrane-bound Mg^{2+}.

Although membrane stretching seems to have a profound effect on the membrane conductances for H^+ and K^+, the molecular mechanism for the increase of conductance differs. The membrane conductance for K^+ in the presence of valinomycin increases continuously with the increase of membrane surface. Apparently the ionophoretic activity of valinomycin is restricted when the cristae are folded. On the other hand the H^+ conductance declines slightly until the osmolarity of the medium falls below 40 mosM. This indicates that the increase of H^+ conductance is not related to the unfolding of the cristae but rather to the ultrastructural rearrangement during membrane stretching.

A deviation from linearity around 100 mosM was found [21] in plots of light scattering versus reciprocal of osmolarity and attributed to membrane damage. The break was observed [15,22] only when the light scattering values but not the matrix volumes are plotted against osmolarities. Another break was found [15] below 30 mosM in plots of matrix volumes versus osmolarity and attributed to ultrastructural rearrangement following mechanical stretching. This latter break may be related with the increase in equivalent pore radius [23] in swollen mitochondria and may explain the depression of $\Delta\psi$ and the enhancement of electrical anion efflux.

The present study suggests that alteration of the membrane, primarily release of Mg^{2+} and stretching, leads to increase of H^+ permeability and then to short range coupling of electrical fluxes of H^+ and K^+. The mitochondrial membrane may be seen as a mosaic of domains with low or high H^+ conductance. In the domain of low H^+ conductance there is a high electrical field and the proton pump drives active cation uptake. In the domain of high H^+ conductance there is a low electrical field and the H^+ fluxes are coupled through short range interactions with the cation fluxes. This results in the exchange reactions.

The induction of H^+/K^+ exchange may represent a physiological means to regulate the matrix volume. Electrical cation uptake leads to osmotic swelling and membrane stretching with induction of H^+/K^+ exchange. This in turn leads to cation efflux and matrix shrinkage. Restoration of the membrane architecture leads to lowering of the ion leak and of the exchange. The tendence of the matrix to swell is balanced by an increase of exchange with efflux and shrinkage. Conversely, the tendence of the matrix to shrink is balanced by a decrease of exchange: the influx predominates and swelling is restored. Alternation of the two phases leads to oscillations.

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