INDUCTION OF ELECTRONEUTRAL EXCHANGES OF H^+ WITH Na^+, DIVALENT AND ORGANIC CATIONS IN RAT LIVER MITOCHONDRIA

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1. Introduction

In the preceding paper we have shown that the apparent dilemma between occurrence of a fast H^+/K^+ exchange during active shrinkage and a quasi-equilibrium distribution of K^+ in valinomycin-treated rat liver mitochondria may be explained by the concept that the electroneutral exchanges are induced membrane reactions presumably following rearrangements of the membrane structure. A similar dilemma concerns the electroneutral exchanges involving Ca^{2+}, organic cations and Na^+ [1–7]. Divalent cations have been shown to undergo a process of rapid active extrusion which presumably involves the operation of a H^+/Cat^{2+} exchange carrier [6,7]. However the existence of such a rapid operating exchange carrier contrasts with the quasi-equilibrium distribution of divalent cations and the low respiratory rate in steady state mitochondria after divalent cation uptake. Organic cations may also be actively extruded from the mitochondrial matrix [5]; this implies the existence of exchange carriers for organic cations. However the transport of organic cations is generally assumed to be electrophoretic and furthermore the distribution of organic cations a reliable indicator of the steady state $\Delta\psi$. Finally among various univalent cations Na^+ is actively extruded from the mitochondrial matrix at the fastest rate [1,2]. However the occurrence of such a rapid H^+/Na^+ exchange carrier in native rat liver mitochondria is still open [11,12].

The present study shows the following features of the exchanges concerning Na^+, divalent and organic cations:

(i) The rate of H^+/Na^+ exchange is low in native mitochondria and markedly increased by A23187 + EDTA,
(ii) The rates of H^+/organic cation exchange correlate with the cation lipophilicity;
(iii) The H^+/Cat^{2+} exchange is inhibited by Ruthenium Red.

These observations together with the correlation between increase of exchange and decrease of $\Delta\psi$ suggest that the exchange reactions are induced rather than native membrane reactions following some rearrangement of the membrane structure. It is likely that the exchange reactions for Na^+, organic and divalent cations depend, as in the case of K^+, on short-range coupling of electrical ion fluxes and are involved in regulation of matrix volumes.

2. Experimental

Rat liver mitochondria were prepared according to standard procedures [8]. Solute influxes and effluxes were measured, as in [8]. Membrane potential was followed by measuring the safranine response in a dual wavelength spectrophotometer [8].

Nitrate and phosphate salts of quaternary ammonium cations were obtained chromatographically after elution of an anion exchange resin. The resin

Abbreviations TMA, tetramethylammonium; TPA, tetrapropylammonium, TBA, tetrabutylammonium, TMPA, trimethylphenylammonium; TEBA, triethylbenzylammonium; DMBPA, dimethylbenzylphenylammonium

(Dowex 1, Cl^- form, 200–400 mesh) was converted into nitrate and phosphate form by treatment with a 1 M solution of Na-nitrate and Na-phosphate as in [9]. From a 0.1 M solution of the organic cation in Cl^- form, 5 ml was added to a column (1 × 30 cm) containing the resin in nitrate or phosphate form. The efflux velocity was 0.5 ml/min and 5 ml fractions were collected. Absence of Cl^- was tested with a strongly acidic, 0.1 M solution of $AgNO_3$. The amount of electrolyte in the eluate in the nitrate form was determined either spectrophotometrically after extraction with picrate in dichloromethane [10] or gravimetrically after liophilization. The P_i content was determined colorimetrically.

3. Results

3.1. *The H^+/Na^+ exchange*

Figure 1 shows that the absorbance change following incubation of rotenone-treated mitochondria in sodium salt of phosphoric acid was very slow. A23187-induced influx of Na phosphate after a lag phase. The rate of H^+/Na^+ exchange increased in the presence of EDTA and with the EDTA concentration. In the presence of 300 μM EDTA the rate of H^+/Na^+ exchange was ~2 orders of magnitude faster than that observed in native mitochondria.

Table 1 reports the rates of H^+/Na^+ exchange calculated from the volume changes. The initial rate of H^+/Na^+ exchange, in Na-acetate, was 37.5 nmol × mg protein^{-1} × min^{-1} with phosphate and 16.9 with Tris–Cl buffer, while the steady rate of exchange were 7.5 and 3.4 nmol × mg protein^{-1} × min^{-1}, respectively. 0.25 and 1 nmol nigericin × mg protein^{-1} increased the initial rate of exchange to 127 and 203 nmol × mg protein^{-1} × min^{-1}, respectively. The rates of influx of NH_4 acetate were 500 and 135 nmol × mg protein^{-1} × min^{-1} with phosphate

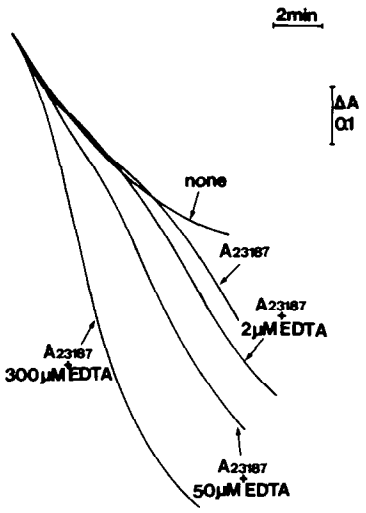

Fig.1. Absorbance changes due to passive influx of sodium salt of phosphoric acid. Effect of A23187 plus EDTA. The medium contained 0.14 M Na_2HPO_4 (pH 7.4), 2 μM rotenone. Additions were 1 μg A23187 ± 2, 50 or 300 μM EDTA. 1 mg/ml mitochondrial protein.

Table 1
Rate of H^+/Na^+ exchange in liver mitochondria

Cation	Anion	Buffer	Nigericin	Rate of exchange (nmol × mg protein^{-1} × min^{-1})	
				Initial	Steady
Na^+	Acetate	15 mM Na-phosphate	None	37.5	7.5
Na^+	Acetate	15 mM Na-phosphate	0.25 nmol/mg	127.0	–
Na^+	Acetate	15 mM Na-phosphate	1 nmol/mg	203.0	–
Na^+	Acetate	15 mM Tris–Cl	None	16.9	3.4
NH_4^+	Acetate	15 mM NH_4-phosphate	None	500.0	–
NH_4^+	Acetate	15 mM Tris–Cl	None	135.0	–
Na^+	Phosphate	None	None	12.0	2.0

The rates of exchange were calculated on the increase of matrix volume [8] under the conditions in fig.1. Acetate was 135 mM and phosphate 120 mM. Both the initial and the steady state rate of exchange are reported

and Tris–Cl as buffers, respectively. Finally the rates of H^+/Na^+ exchange, in Na-phosphate, were 12 in the initial phase and 2.0 in the steady phase, respectively.

Figure 2 shows the H^+/Na^+ exchange during both passive influx or active efflux of Na_2HPO_4. The rate of exchange was low at 120 mM Na_2HPO_4, while at 90 mM Na_2HPO_4 was higher and furthermore showed an enhancement after a lag phase. This suggests induction of exchange after membrane stretching. At 60 and 30 mM Na_2HPO_4 the rate of exchange was even more rapid. Addition of succinate + 5 mM Mg^{2+} + 100 μM *N*-ethylmaleimide resulted in an efflux which was slight at 120 mM and more marked at lower osmolarities. This indicates a correspondence between H^+/Na^+ exchange during passive influx and active efflux. Addition of succinate + Mg^{2+} + NEM at 120 mM Na_2HPO_4 resulted first in a rapid rise of $\Delta\psi$, then a short phase of collapse, and then a more extensive rise. The short collapse was concomitant to the initiation of the slight electrolyte efflux. At 90 and 60 mM Na_2HPO_4 addition of succinate + NEM + Mg^{2+} caused a rise of $\Delta\psi$ only after a lag phase concomitant with the electrolyte efflux. At 30 mM Na_2HPO_4 the rise in $\Delta\psi$ occurred after a shorter lag and was more extensive. This correlated with a more rapid and complete shrinkage.

3.2. *The H^+/organic cation exchange*

Figure 3 shows the influx of three organic aliphatic cations of increasing lipophilicity: TMA, TPA and

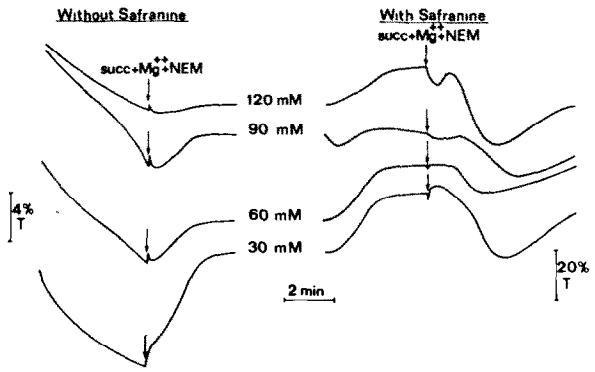

Fig.2. H^+/Na^+ exchange and $\Delta\psi$ during active efflux in Na_2HPO_4. The medium contained Na_2HPO_4 (pH 7.4), at various osmolarities. Rotenone, 1 μM, 1 mg protein/ml. Where indicated, 5 mM $MgCl_2$, 100 μM *N*-ethylmaleimide and 5 mM succinate. Safranine, 25 μM.

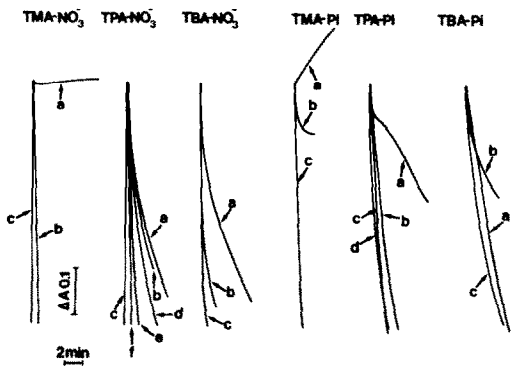

Fig.3. Passive influx of TMA, TPA and TBA. The medium contained nitrate salts of organic cation at 0.1 M and phosphate salts at 70 mM, 10 mM Tris–nitrate (pH 7.4). Rotenone, 1 μM, 0.2% BSA and 1.5 mg mitochondrial protein. Volume 2 ml. The reaction was initiated by the addition of mitochondria. Other additions were as follows: TMA–NO_3 – (a) none, (b,c) 20 and 40 μM tetraphenylboron; TPA–NO_3 – (a) none, (b,c) 1 and 2 μM tetraphenylboron, (d,e,f) 1,3,6 μM FCCP, TBA–NO_3 – (a) none, (b,c) 3 and 6 μM tetraphenylboron, TMA–P_i – (a) none, (b,c) 50 and 100 μM tetraphenylboron; TPA–P_i – (a) none, (b,c) 5 and 20 μM tetraphenylboron, (d) 20 μM tetraphenylboron + 2 μM FCCP; TBA–P_i – (a) none, (b) 20 μM mersalyl, (c) 2 μM FCCP.

TBA. Consider first the fluxes of the nitrate salts:

(i) The rate of spontaneous influx was correlated with the lipophilicity of the cations TMA < TPA < TBA;

(ii) The rate of influx was increased by, and the effect was proportional to, the concentration of tetraphenylboron;

(iii) The rate of influx of TBA and TPA was enhanced by FCCP, and the effect in the case of TPA, was proportional to the concentration of FCCP.

Both the sequence TMA < TPA < TBA and the enhancing effect of tetraphenylboron are in accord with the view that the NO_3^--driven cation influx depends on the lipophilicity of the organic cation.

Consider now the influxes of the phosphate salts:

(i) The rate of exchange increased in the order TMA < TPA < TBA;

(ii) As in the case of the NO_3^- salts the rate of exchange was increased by, and the effect was proportional to, the concentration of tetraphenylboron;

(iii) The rate of exchange either of TPA in the pres-

ence of 20 μM tetraphenylboron or of TBA in the absence of tetraphenylboron, was only slightly enhanced by FCCP.

(iv) The rate of exchange with TBA was markedly inhibited by the inhibitor of the P_i carrier, mersalyl.

The sequence TMA < TPA < TBA and the enhancing effect of tetraphenylboron are in accord with the view that also the H^+/organic cation exchange depends on the cation lipophilicity. The mersalyl sensitivity indicates an electroneutral transport of P_i via the carrier and not an electrical diffusion of the phosphate anion.

Figure 4 shows the influx of three organic aromatic cations of increasing lipophilicity, TMPA, TEBA and DMBPA. In the case of the NO_3^- salts:

(i) The rate of spontaneous influx was correlated with the lipophilicity of the cations TMPA < TEBA < DMBPA;

(ii) The rates of influx were increased by tetraphenylboron, the concentrations of tetraphenylboron were in the range of those for the aliphatic cations;

(iii) The rates of influx were stimulated by FCCP.

In the case of the P_i salts:

(i) The rates of H^+/cation exchanges were correlated with the cation lipophilicity;

(ii) The effect of tetraphenylboron was close to that observed with the NO_3^-salts, similar concentrations of tetraphenylboron were required;

(iii) The FCCP increased only slightly the rates of exchange;

(iv) The absorbance change was inhibited by mersalyl.

Table 2 shows a correlation between the partition coefficient of the organic cations (between water and organic solvent) and the rate of absorbance decrease in absence and in presence of 25 μM tetraphenylboron and 50 μM picrate. The partition coefficient α increased in the order TMA < TMPA < TEBA < TPA < TBA. A similar sequence was observed for the absorbance decrease with the exception of TBA. This may be due to the high lipophilicity of TBA. It is known that hydrophobic complexes tend to dissociate in hydrophobic environments.

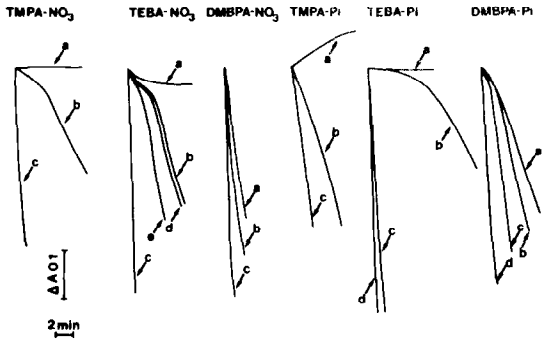

Fig.4. Passive influx of TMPA, TEBA and DMBPA. Experimental conditions as in fig.5. Other additions were as follows: TMPA–NO_3 – (a) none, (b,c) 2 and 5 μM tetraphenylboron, TEBA–NO_3 – (a) none, (b,c) 2 and 5 μM tetraphenylboron, (d,e) 2 and 5 μM FCCP; DMBPA–NO_3 – (a) none, (b,c) 2 and 5 μM tetraphenylboron, TMPA–P_i – (a) none, (b,c) 5 and 10 μM tetraphenylboron; TEBA–P_i – (a) none, (b,c) 2 and 5 μM tetraphenylboron, (d) 5 μM tetraphenylboron + 1 M FCCP, DMBPA–P_i – (a) none, (b,c) 5 and 10 μM tetraphenylboron

Table 2
Correlation between extraction or organic cations in organic solvents and rate of organic cation transport

Cation	Extraction in organic solvents (α)	Rate of electrolyte influx (ΔA/min × 10^3)		
		No addition	+25 μM TPB^-	+0.5 mM picrate
TMA	0.04	2	20	4
TMPA	0.11	7	37	80
TEBA	0.64	4.6	54	105
TPA	0.91	12.8	375	540
TBA	0.97	118	246	211

The medium for electrolyte influx contained: 60 mM cation Cl, 10 mM Tris–Cl (pH 8), 2 μM rotenone, 0.2% BSA. Protein, 1.45 mg. Volume 2 ml. α indicates the partition of organic cations between H_2O and dichloromethane. The values of α were determined as in [15]

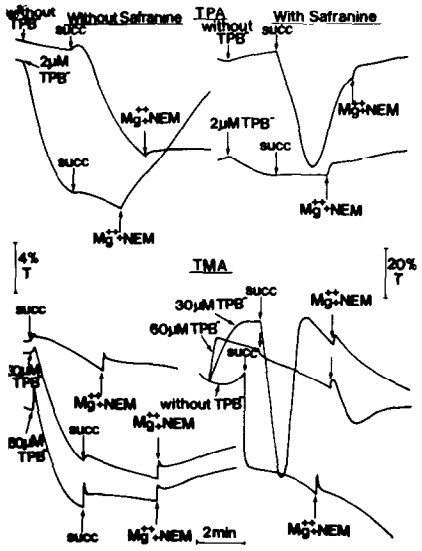

Fig.5. Effect of tetraphenylboron on H^+/organic cation exchange and $\Delta\psi$. The medium contained either 40 mM tetramethylammonium phosphate (TMA) or 40 mM tetraphenylammonium phosphate (TPA), pH 7.4, 1 μM rotenone, 1 mg protein/ml. Where indicated, 5 mM $MgCl_2$, 100 μM *N*-ethylmaleimide and 5 mM succinate. Safranine, 25 μM.

Figure 5 shows a correlation between exchange and $\Delta\psi$. With TPA the exchange was very slow in the absence of tetraphenylboron. Succinate caused active TPA uptake, while Mg^{2+} + NEM blocked the uptake but did not initiate efflux. At 2 μM tetraphenylboron induced a rapid exchange during both passive influx and active efflux. In the absence of tetraphenylboron, succinate caused a large rise of $\Delta\psi$ which then collapsed. This suggests that the active TPA uptake is accompanied by membrane damage. In the presence of 2 μM tetraphenylboron, succinate did not cause a rise of $\Delta\psi$. There was therefore a correlation between diminution of $\Delta\psi$ and increase of H^+/cation exchange. In the case of TMA the rate of exchange was negligible in the absence of tetraphenylboron and rapid at 30 and 60 μM tetraphenylboron. Active efflux did not occur in the absence of tetraphenylboron while it did so at 30 and 60 μM tetraphenylboron. A large rise of $\Delta\psi$ occurred in the absence of tetraphenylboron. At 30 μM tetraphenylboron there was a rise and then a collapse of $\Delta\psi$, while at 60 μM tetraphenylboron there was no rise of $\Delta\psi$ after succinate and a slight increase after Mg^{2+} + NEM.

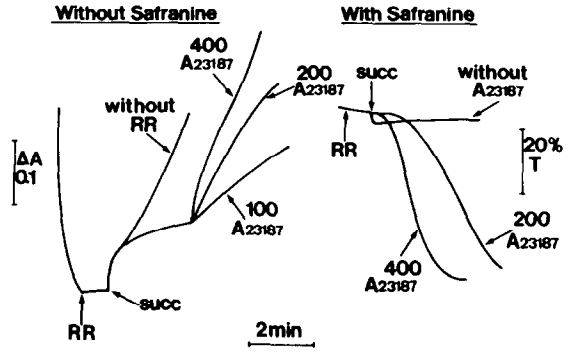

Fig.6. Effect of A23187 on active divalent cation efflux and $\Delta\psi$. The medium contained 20 mM $Sr(NO_3)_2$, 10 mM Tris–NO_3 (pH 7.4); 1 μM rotenone, 1 mg protein/ml. Where indicated, 0.4 μM Ruthenium Red, 5 mM succinate. A23187 is indicated in pmol × mg protein^{-1}. Safranine, 25 μM.

3.3. *The H^+/Cat^{2+} exchange*

Figure 6 shows the effect of A23187 on the efflux of Sr^{2+} [7]. Rotenone-treated mitochondria were swollen in $Sr(NO_3)_2$ and addition of succinate started an active efflux implying a H^+/Sr^{2+} exchange. The efflux was almost completely abolished by Ruthenium Red and restored by A23187 at a rate increasing with the A23187 concentration.

Addition of succinate, in the presence of Ruthenium Red, was not followed by a rise in $\Delta\psi$. However a rise of $\Delta\psi$ followed after addition of A23187 and proportionally to the amount of ionophore. Presumably the lack of rise of $\Delta\psi$ was due to being $\Delta\tilde{\mu}_H$ partly in form of ΔpH. By catalyzing a H^+/Cat^{2+} exchange A23187 causes transformation of ΔpH into $\Delta\psi$, and then increase of safranine response.

4. Discussion

Unlike the ammonium salts, the sodium and potassium salts of phosphoric and acetic acid were reported [11] not to undergo net translocation through the cristae membrane. At variance with [11] sodium phosphate was found [12] to undergo a rapid equilibration across the cristae membrane. The calculated rate of translocation of sodium phosphate was at least half as fast as that of ammonium phosphate. The rate of H^+/Na^+ exchange has been

calculated [13] to be as high as 88 nmol × mg protein^{-1} × min^{-1}. It was proposed [2–4] that the natural H^+/Na^+ exchange is responsable for the ΔpH driven efflux of Na^+ during active shrinkage.

According to the present data:

(i) The rate of H^+/Na^+ exchange is very low in native mitochondria, in accord with [11];

(ii) A rapid H^+/Na^+ exchange is induced after removal of membrane-bound Mg^{2+} by means of A23187 + EDTA: the rate of A23187 + EDTA-induced H^+/Na^+ exchange is almost 2 orders of magnitude higher than the rate occurring in native mitochondria;

(iii) After incubation of mitochondria in isotonic Na_2HPO_4 addition of succinate is not followed by H^+/Na^+ exchange during active efflux; however incubation of mitochondria in hypotonic Na_2HPO_4 is followed by H^+/Na^+ exchange; induction of H^+/Na^+ exchange is accompanied by collapse of $\Delta\psi$.

Lipophilic organic cations were first used in mitochondrial ion transport in [14] the purpose being that of showing that any ion, natural or unnatural, penetrating through a phospholipid membrane could be transported by means of an energy-linked process. The conclusion was drawn that processes capable of driving active uptake or extrusion of organic ions are electrical in nature. However the observation of an active efflux with organic cations [5] suggests also the occurrence of an electroneutral H^+/organic cation exchange.

In the present study the exchanges of H^+ with organic cations increase in rate:

(i) With the cation lipophilicity;

(ii) After addition of tetraphenylboron.

Both evidences suggests that the translocation of the organic cation during H^+/cation exchange takes place in the lipid bilayer [14].

The exchange of H^+ with divalent cations is inhibited by Ruthenium Red at the same concentration at which is inhibited the electrical Ca^{2+} transport [7,15]. This suggests either that the exchange goes via the native carrier or that the membrane has an exchange carrier with identical sensitivity to Ruthenium Red. However the process of divalent cation efflux is rapid. If the native membrane would possess a H^+/Cat^{2+} exchange carrier operating at such fast rate, mitochondrial respiration would be uncoupled through divalent cation cycling in the same manner as it is uncoupled by A23187 [16].

A major argument has been considered [4] in favour of the existence of antiporters in the native membrane of rat liver mitochondria that the rates of cation efflux during active shrinkage and the rates of passive influx during swelling in weak acids show a similar selectivity sequence. In order to explain the marked increase in rate of the exchange during shrinkage a subtle regulation of the antiporters was proposed. Evidence is now growing in favour of a number of such antiporters also in the membrane of a variety of bacterial systems. However, we have now shown that electroneutral exchanges occur with all sorts of ions and that the rate of exchange undergoes a major increase after modifications of the mitochondrial membrane. The present study then suggests that the antiporter concept should be widened to incorporate two new features:

(i) Low specificity, which reminds the 'everything translocase' argument [14] supporting the view that the cation fluxes driven by the H^+ pump are electrophoretic and do not involve interactions with intrinsic membrane carriers,

(ii) inducibility, which recalls the concept of membrane sites possessing a variable accessibility to ionic species in the water phase.

A major argument in favour of the inducibility is that an enhancement of the exchange reactions analyzed in this study require a substantial increase of the membrane electrical permeability for H^+. It is possible that the exchange reactions reflect a compulsory electrostatic interaction of various ionic species with a fixed charge system. However, the low specificity and the inducibility give rise, through short range interactions, to an infinite variety of electroneutral ion fluxes, which provide a major feature to these reactions.

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