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Electrophoretic polyamine transport in rat liver mitochondria

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Summary. Naturally occurring polyamines (spermidine, putrescine, cadaverine), as the well studied spermine, are transported into rat liver mitochondrial matrix provided that mitochondria are energized and the electrical membrane potential has a value of about 180 mV. This condition is achieved by the presence of inorganic phosphate, or acetate, or nigericin in the incubation medium. Valinomycin plus K^+ almost completely blocks polyamine transport.

The obtained results clearly show that all naturally occurring polyamines are transported by an electrophoretic mechanism in responce to a high negative inner electrical potential.

The distribution ratio of polyamines across the mitochondrial membrane is far from the thermodynamic equilibrium by many orders of magnitude. This result might suggest the existence of a different pathway for polyamine efflux.

Keywords: Amino acids – Mitochondria – Polyamines – Transport – Membrane potential

Introduction

In a previous paper, Toninello et al. (1985) demonstrated that spermine is transported across the inner membrane of rat liver mitochondria by a mechanism requiring membrane energization and the parallel transport of phosphate (Pi). In addition, it was demonstrated that also phosphate transport is strongly enhanced by the presence of spermine.

In a recent paper we demonstrated that phosphate requirement is not a specific condition for spermine transport because other non-physiological agents, such as acetate or nigericin by inducing a shift of the protonmotive force to a higher membrane potential ($\Delta\Psi$), promote spermine uptake (Toninello et al., 1988a).

Indeed, the observation that valinomycin plus K^+, by collapsing $\Delta\Psi$, induces a block of spermine transport, led us to conclude that this transport is driven by an electrophoretic mechanism.

Spermine, as previously proposed, could be transported in liver mitochondria, as a divalent cation, by a leak pathway or, as a tetravalent cation, by a specific channel. The polyamine must overcome a single sharp energy barrier if the transport is due to a leak pathway. On the contrary, if the transport system is a channel, spermine must overcome two lower symmetrical energy barriers (Toninello et al., 1988a)

The existence of one or more sharp energy barriers is due to the effect of the membrane field on the energy barrier by adding the electrostatic potential of an ion in the field to the field-independent energy barriers (Garlid et al. 1989).

Spermidine, putrescine and cadaverine have different molecular size, different net electric charges and therefore different "Born charging energy" (Parsegian, 1969) from spermine.

This difference in "Born charging energy" could be relevant for the height of energy barrier, thus influencing the rate of transport.

In this view it seems of particular interest to determine if an electrophoretic mechanism exists also for spermidine, putrescine and cadaverine transport and if they are transported at the same rate as spermine.

Diwan et al. (1988) demonstrated that spermidine transport occurs in outer-membrane-depleted mitochondria by suggesting the hypothesis that the outer membrane has a barrier function for polyamines transport. However, in that paper no indication was given concerning the transport mechanism.

Another important point is to calculate the distribution ratio for all these polyamines. This is of particular interest, especially in the case of an electrophoretic transport, to determine the existence of an efflux pathway as already proposed for spermine (Toninello et al., 1988a; Toninello et al., 1988b).

Material and methods

Rat liver mitochondria were isolated in 0.25 M sucrose and 5 mM Hepes (pH 7.4) by conventional differential centrifugation. Mitochondrial protein concentration was assayed by a biuret method with bovine serum albumin as standard.

Mitochondrial incubations were carried out at 20°C with 1 mg mitochondrial protein/ml in the following standard medium: 200 mM sucrose, 10 mM Hepes (pH 7.4), 1.25 μM rotenone. 1 mM [^{14}C] polyamines (spermidine, putrescine, cadaverine) (50 μCi/mmol) were presents as indicated in the figures.

Other additions or variations in the composition of the medium are indicated in the descriptions of specific experiments.

Membrane potential was measured in a open thermostatically controlled and stirred vessel, by monitoring the distribution of lipophilic cation tetraphenylphosphonium (TPP^+) across the mitochondrial membrane with a selective electrode prepared in our laboratory according to published procedures (Kamo et al., 1979; Affolter and Siegel 1979). TPP^+ concentration was 2 μM.

The membrane potential measured with the TPP^+ electrode was corrected as proposed by Jensen et al. (1986).

Mitochondrial matrix volume was calculated according to Palmieri and Klingenberg (1979).

Uptake of [^{14}C] polyamines was determinated by a centrifugal filtration method as previously described (Toninello et al., 1985; Toninello et al., 1988a).

Results

Fig. 1 shows that 1 mM [^{14}C] spermidine, putrescine and cadaverine, when added to rat liver mitochondria suspended in a sucrose medium in the presence of rotenone, are rapidly taken up as already demonstrated for spermine (Toninello et al., 1985).

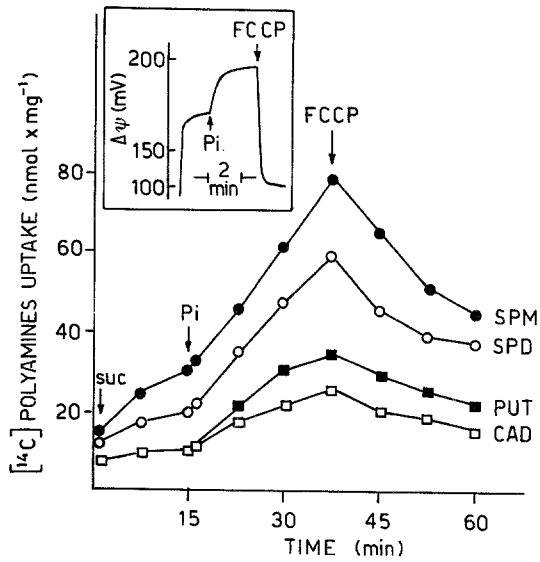

Fig. 1. Polyamine transport: dependence on energized state and Pi transport. Rat liver mitochondria were incubated in the standard medium in the presence of labelled polyamines as indicated. At the arrows 5 mM succinate (*suc*), 1 mM Pi and 0.1 μg/mg prot FCCP were added. The results reported in the inset have been obtained in the absence of polyamines *SPM* spermine, *SPD* spermidine, *PUT* putrescine, *CAD* cadaverine

The initial rapid uptake (8–18 nmol/mg prot), due to the binding of polyamines with the mitochondrial membranes, insensitive to uncouplers and inhibitors of the respiratory chain (results not shown), is followed by a very slow uptake after the addition of succinate.

Addition of 1 mM phosphate, that shifts the $\Delta\Psi$ value to about 190 mV (see inset in Fig. 1), strongly enhances the transport while, a subsequent addition of FCCP (see inset), that collapses $\Delta\Psi$, induces a release of a large aliquot of accumulated polyamines.

By replacing FCCP with antimycin A the same results are obtained. The curve of spermine transport is reported in this experiment and in the following for comparison with the other polyamines.

The transport of spermidine, putrescine and cadaverine is concentration dependent (results not reported) as already observed for spermine (Toninello et al., 1985).

It was reported that the action of phosphate on spermine transport is due to its effect on membrane potential (Toninello et al., 1988a). To evaluate if spermidine, putrescine and cadaverine are also subject to this mechanism, incubations

in the presence of other agents such as acetate or nigericin, that shift $\Delta\Psi$ to higher values, have been performed.

The results reported in Fig. 2A show the effect of acetate on the transport of polyamines. [^{14}C] polyamines are present in the medium at concentrations of 1 mM. After an initial period of rapid binding, the addition of succinate induces a slow uptake of polyamines (as observed in the previous experiment). This uptake is accelerated by a subsequent addition of 20 mM acetate that enhanced $\Delta\Psi$ up to 160 mV (see inset in Fig. 2). Both the rate of transport and the total amount of accumulated polyamines are significantly lower than those obtained in the presence of phosphate (compare with Fig. 1).

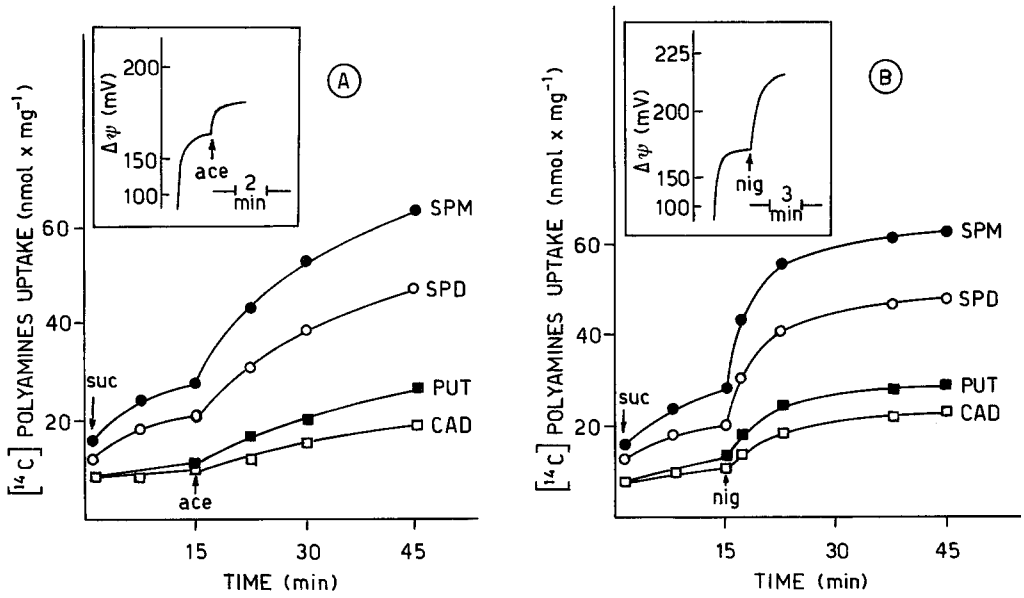

Fig. 2. Effect of acetate (**A**) and nigericin (**B**) on polyamine transport. Incubation conditions as in Fig. 1. At the arrows 5 mM succinate (*suc*), 20 mM acetate (*ace*) (**A**), 0.33 μg/mg prot nigericin (*nig*) (**B**) were added. The results reported in the insets have been obtained in the absence of polyamines

Under the same conditions by deenergizing mitochondria with FCCP or antimycin A, polyamines are not accumulated (results not reported).

Fig. 2B shows polyamine transport in the presence of nigericin, an antibiotic that induces an enhancement of $\Delta\Psi$ higher than that obtainable in the presence of either phosphate or acetate (see inset in Fig. 2). After the addition of nigericin there is a rapid polyamines uptake, faster than in the other conditions. However this accumulation does not continue further on and so the total amount of accumulated polyamines is lower than in the presence of phosphate.

Opposite results are obtained if another antibiotic, valinomycin, is used.

Fig. 3 reports the effect of valinomycin plus K^+ on polyamine transport in the presence of phosphate. An almost complete inhibition is induced in this condition for all the polyamines. Indeed, if valinomycin plus K^+ are added after accumulation of the polyamines, these are released in the incubation medium (results not shown). In the presence of K^+ in the medium, valinomycin induces

a collapse of $\Delta\Psi$ (see inset in Fig. 3) by raising ΔpH, thus still maintaining an energization state of the mitochondrial membrane.

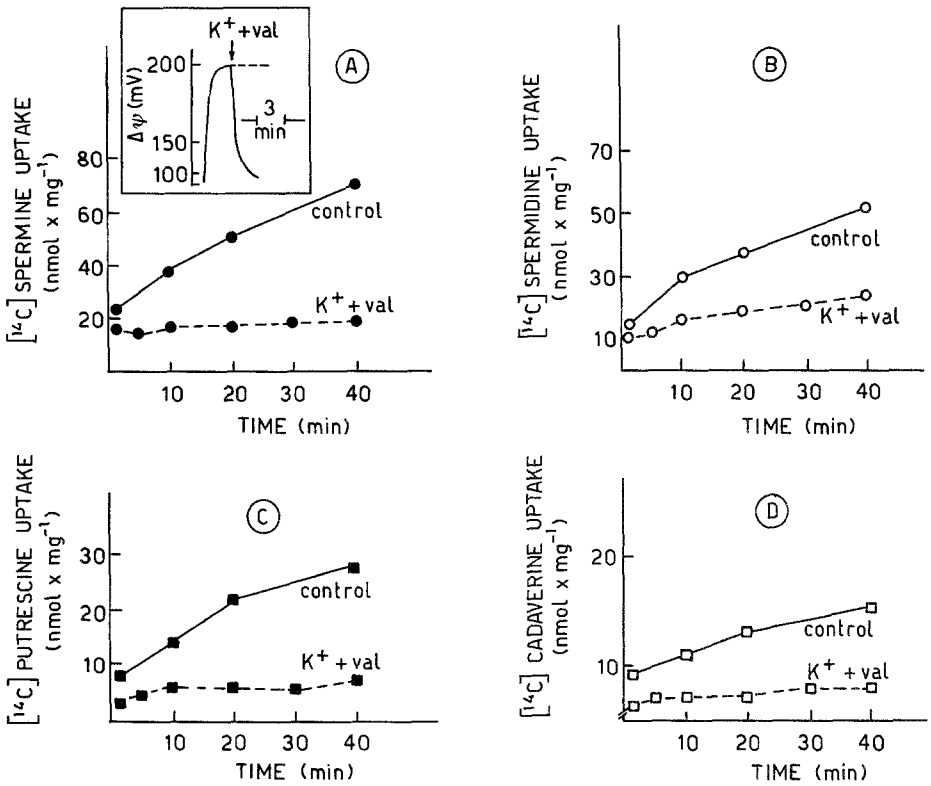

Fig. 3. Effect of valinomycin plus K^+ on polyamine transport. Rat liver mitochondria were incubated in the standard medium in the presence of 5 mM succinate and 1 mM Pi. Labelled polyamines were present are indicated. When present 2 mM KCl and 0.33 μg/mg prot valinomycin (*val*) were present. The results reported in the inset have been obtained in the absence of polyamines

Spermine and the other polyamines are transported into the mitochondrial matrix also at very low external concentrations (results not shown). Hence it is possible to measure the distribution ratios of transported polyamines between the matrix space and the external incubation medium.

Table 1 reports the different distribution ratios for the various polyamines, after 30 minutes of incubation, in mitochondria having a $\Delta\Psi$ value of about 180 mV in the presence of 1 mM phosphate.

The polyamines were initially present in the medium at concentrations of 50 μM. The presented results are corrected for the aliquot of polyamines bound to the membrane.

Discussion

These experimental data clearly demonstrate that spermidine, putrescine and cadaverine are transported into the matrix space by a mechanism requiring the energization of the inner mitochondrial membrane.

Table 1. Distribution ratio of polyamines accumulation

	Matrix concentration (mM)	Medium concentration (μM)	Distribution ratio [polyamines]in/[polyamines]out
Spermine	7.60	36.8	$2.07 \cdot 10^2$
Spermidine	6.00	40.0	$1.50 \cdot 10^2$
Putrescine	1.20	47.8	$0.25 \cdot 10^2$
Cadaverine	0.70	48.5	$0.14 \cdot 10^2$

Rat liver mitochondria were incubated for 30 minutes in the standard medium containing 5 mM succinate and 1 mM Pi, in the presence of 50 μM [^{14}C] spermine, [^{14}C] spermidine, [^{14}C] putrescine, [^{14}C] cadaverine (50 μCi/mmol) as indicated

All conditions that lead to a deenergized state, (e.g. presence of FCCP or antimycin A), completely block the transport or induce a release of the accumulated polyamines (see Fig. 1). The release of accumulated polyamines after addition of FCCP demonstrates that a free and mobile aliquot of polyamines that moves in response to membrane potential variations exists in the matrix space.

The requirement of membrane energization that implies the formation of an electrochemical transmembrane potential, raises the question which gradient, electrical or chemical (or both), is the driving force for the polyamine transport.

The results reported in Fig. 1 demonstrate that phosphate is able to induce an enhancement of polyamine transport. Phosphate is transported into the mitochondrial matrix by a specific and well known mechanism in symport with protons (Fonjo and Bessman 1966). The effect of this transport is a collapse of ΔpH and a rise of $\Delta\Psi$ to about 180–190 mV (see inset in Fig. 1).

The action of phosphate on polyamine transport might be explained by the rise of $\Delta\Psi$ induced by this anion. The transport of polyamines would thus be due to an electrophoretic mechanism requiring a $\Delta\Psi$ 20–30 mV higher than that obtainable in static head conditions.

The results reported in Fig. 2 confirm this assumption. These data demonstrate that by inducing a rise of $\Delta\Psi$ with other agents, i.e., nigericin or acetate, the transport of polyamines is again enhanced, although with different rates and different amounts of accumulation. Compare the results reported in Figs. 1 and 2. These differences are due to the different mechanism by which phosphate, acetate and nigericin induce a rise in $\Delta\Psi$ and to the different final values of $\Delta\Psi$ attained.

Acetate is transported into the mitochondrial matrix, probably as dimer of undissociated acid, by an unknown mechanism that induces a proton uptake and a collapse of ΔpH. This, consequently, induces a rise of $\Delta\Psi$ up to 165–170 mV, lower than that induced by phosphate. To obtain this result a very high concentration of acetate (20–30 mM) is required. The lower $\Delta\Psi$ imposed by acetate accounts for the lower amount of polyamines transported.

Nigericin, in the absence of external K^+, induces an exchange of endogenous K^+ with external H^+. This K^+/H^+ exchange causes a complete collapse of ΔpH and an increase of $\Delta\Psi$ to its maximum value of about 220 mV. This very high

$\Delta\Psi$ is probably responsible for the initial rate of polyamine uptake which is faster than that obtained in the presence of both phosphate and acetate.

The high $\Delta\Psi$ induced by these agents is continuely lowered during polyamine transport. However, in the presence of either phosphate or acetate this lowering is immediately counterbalanced by the uptake of the anions. This uptake can maintain $\Delta\Psi$ at a critical value able to continue polyamine transport.

On the contrary, in the presence of nigericin, the lowering of $\Delta\Psi$ induced by the polyamine uptake is not counterbalanced; this explains why the faster initial transport of polyamines stops after a few minutes.

In the reported results, the sequence of quantitative polyamines transport is the following: spermine > spermidine > putrescine > cadaverine, demonstrating the importance of the electric charge for this electrophoretic accumulation.

The results reported in Fig. 3 demonstrate that $\Delta\Psi$ is the only factor responsible for polyamines transport. In fact, in membrane energization conditions but with ΔpH at the maximal value and $\Delta\Psi$ practically collapsed, the transport of polyamines is completely blocked. This condition is obtainable in the presence of valinomycin plus K^+ in the incubation medium. Valinomycin induces an electrophoretic transport of K^+ inside the mitochondrial matrix that collapses $\Delta\Psi$. The corresponding H^+ ejection, due to an accelerated mitochondrial respiration, maintains ΔpH at the maximal level.

All together these results widely confirm that spermidine, putrescine and cadaverine, as spermine, are exclusively transported by an electrophoretic mechanism in response to a high negative inner electrical potential.

With a membrane potential of 180 mV an electrophoretic transport of tetra, tri and divalent polycations as the above considered polyamines, should reach a Nernstian equilibrium of 10^{12}, 10^9, 10^6 respectively.

If polyamines cross the membrane by a leak pathway, as previously proposed for spermine (Toninello et al., 1988a), it is possible that their charge is halved; then the equilibrium would be 10^6, $10^{4,5}$, 10^3.

Considering both the net charges and the halved charges, the results presented in Table 1 demonstrate that the distribution ratio of polyamines across the mitochondrial membrane is far from the Nernstian equilibrium by many orders of magnitude. These results strongly support the possibility that a pathway for polyamines efflux, with a different mechanism from that of influx, can also exists.

This bidirectionality for polyamines transport could be taken into account for a possible cycling of polyamines across the mitochondrial membrane in view of their important role in the regulation of membrane bound enzymes.

The difference in Born charging energy existing among the polyamines does not account for the differences in the rate of transport and quantitative accumulation.

The more electrically charged polyamines (spermine and spermidine) should be transported to a lesser extent than putrescine and cadaverine because of their higher Born energy and therefore high energy barriers to overcome.

The experimental data demonstrate the existence of a specific system for polyamine transport able to strongly lower the energy barrier, thus allowing their transport with the mentioned sequence: spermine > spermidine > putres-

cine > cadaverine. This transport system is able to eliminate the difference in polyamines charges and in the "Born charging energy" and, on the contrary, it favours the transport of the more charged polyamines.

As in the case of spermine (Toninello et al., 1988a), experiments that take into account the "Eyring rate theory" (Garlid et al., 1989) are in progression to better characterize the transport of spermidine, putrescine and cadaverine.

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