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Uptake of spermine by rat liver mitochondria and its influence on the transport of phosphate

A. Toninello, F. Di Lisa, D. Siliprandi and N. Siliprandi *

Centro Studio Fisiologia Mitocondri, CNR and Istituto Chimica Biologica, Università di Padova, 35131 Padova (Italy)

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Spermine, a polyamine present in the mammalian cells at rather high concentration, has, among other actions, a remarkable stabilizing effect on mitochondria, functions which have generally been attributed to the capability of this and other polyamines to bind to membrane anionic sites. In the present paper evidence is provided that at physiological concentrations spermine may also be transported into rat liver mitochondrial matrix space, provided that mitochondria are energized and inorganic phosphate is simultaneously transported. The close dependence of spermine transport is also demonstrated by the concurrent efflux of spermine and inorganic phosphate when mitochondria preloaded with the two ionic species are deenergized either with uncouplers or respiratory chain inhibitors. Furthermore, Mersalyl, the known inhibitor of phosphate transport, prevents both spermine uptake and release. Mg^{2+} inhibits the transport of spermine conceivably by competing for the some binding sites on the mitochondrial membrane. The physiological significance of these results is discussed.

Introduction

The oligoamines spermine, spermidine and putrescine exert a broad influence on cellular metabolism [1]. In 1960 Tabor [2] reported that these oligoamines have a remarkable stabilizing action on mitochondria, and later Phillips and Chaffee [3] found that spermine not only prevents the loss of respiratory control in heat-aged liver mitochondria, but also has a restorative effect if their phosphorylative capacity has been previously impaired. Indeed, spermine modifies the permeability properties of mitochondrial membranes such that the movements of Ca^{2+} and inorganic phosphate (P_i) are significant slowed down. Since these movements are linked to the efflux of Mg^{2+} and adenine nucleotides from mitochondria, spermine prevents the fall of transmembrane potential ($\Delta\psi$) induced by Ca^{2+} and P_i cycling [4]. It also fully restores collapsed $\Delta\psi$ if adenine nucleotides are subsequently added to the medium [4].

Recently, Solaini and Tadolini [5] observed that spermine binds to submitochondrial particles and activates the adenosine triphosphatase. These findings would assume physiological significance if it were found that in intact mitochondria spermine was transported into the inner membrane-matrix space. However, there is no published evidence that this is so, and it is currently assumed that the oligoamines only bind to the exterior of mitochondrial membranes [6].

In the present paper, evidence is provided that spermine does in fact cross into the inner mitochondrial space, provided that the mi-

* To whom correspondence should be addressed.

Abbreviations: EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*'-tetracetic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; P_i, inorganic phosphate.

tochondria are energized and inorganic phosphate (P_i) is being transported simultaneously. This process is inhibited by Mg^{2+}. It is also shown that spermine transport enhances P_i transport.

Methods

Rat-liver mitochondria were isolated in 0.25 M sucrose/5 mM Hepes (pH 7.4) by the conventional centrifugation method. 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 or 5 mg mitochondrial protein/ml in the following standard medium: 200 mM sucrose, 10 mM Hepes (pH 7.4), 1.25 μM rotenone. Other additions are indicated in the descriptions of various experiments.

[^{14}C]spermine and [^{32}P]phosphate uptake were determined by centrifugal filtration method. Tubes for the 3200 Eppendorf microfuge were prepared as follows: 0.15 ml 12.5% (v/v) sucrose was placed at the bottom of the tube and 0.4 ml of a mixture of silicone oil (AR 100/AR 150 (2:1) Wacker-Chemie GmbH, München, F.R.G.) was carefully layered on top of this. At the times indicated, 1.0 ml portions of the incubation mixture were withdrawn and layered on the top of the silicone oil. The tubes were then centrifuged at 12000 rpm for 2 min. The top layer was removed and the space was washed thrice with bidistilled water. Most of the silicone layer was then removed as well. 0.85 ml of 1 mM sodium EDTA, 0.1% sodium chloride, 0.9% sodium deoxycholate were added and the dissolved pellet was assayed for radioactivity by employing a Beckman LS-100C spectrophotometer.

When needed, that is in the experiments relative to the kinetics of spermine transport, correction of spermine present in the non-matrix space of the pellets was done by subtraction of controls incubated in the presence of 0.1 μg/mg protein FCCP.

Results

When added to rat liver mitochondria suspended in sucrose medium and treated with rotenone to prevent the respiration of endogenous substrates, [^{14}C]spermine was rapidly taken up from the medium, the maximum uptake being achieved in a few seconds (approx. 4 nmol/mg protein) (Fig. 1). This amount, which probably represents the extent of the binding of spermine to mitochondrial membranes [6], was insensitive to uncouplers or respiratory chain inhibitors added either before or after exposure to spermine. It cannot be excluded that part of the polyamine taken up by our mitochondrial preparation in the absence of energy is actually taken up by liposomes or other cellular component contaminating our preparation. A further but very slow uptake of [^{14}C]spermine occurred in the presence of succinate, but when P_i (0.5 mM) was also added, there was an immediate and consistent increase of spermine uptake (in the absence of succinate P_i had no effect). This effect is specific for P_i, since other anions that enter the mitochondria in an H^+-coupled fashion (e.g., acetate, bicarbonate) were practically uneffective. The uptake of spermine by mitochondria in the presence of succinate and P_i was linear with time up to 15 min with a rate of about 0.5 nmoles/min per mg protein. If either FCCP of antimycin A was added to mitochondria loaded in this way with spermine, they initiated a sustained loss of the accumulated oligoamine. However, the amount released was lower than that taken up, perhaps due to mem-

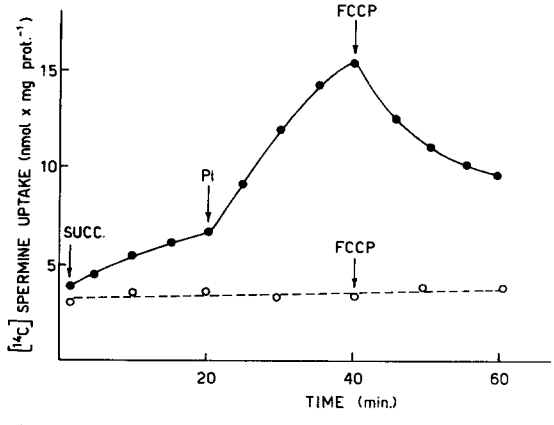

Fig. 1. Spermine uptake by rat liver mitochondria (RLM): dependence on the energized state and P_i transport. 1 mg RLM/ml was suspended at 20°C in the following medium: 200 mM sucrose, 10 mM Hepes (pH 7.4), 1.25 μM rotenone, 0.1 mM [^{14}C] spermine (100 μCi/mmol). Additions: 0.5 mM P_i, 5 mM succinate, 0.1 μg/mg protein FCCP.

brane binding. Another possibility is that part of the spermine was coprecipitated inside the mitochondria together with some of the absorbed phosphate [1].

Apparently Ca^{2+} movements are not involved in spermine uptake by respiring mitochondria. Neither Ca^{2+}, nor EGTA addition affected the rate and the extent of spermine uptake. Moreover, ruthenium red, the known inhibitor of Ca^{2+} electrophoretic uniport, was without any effect on spermine transport (results not reported).

The kinetics of spermine transport into liver mitochondria energized with succinate and P_i (reported in Fig. 2) show that the initial rate of spermine uptake approaches a saturation level as the spermine concentration is increased. The apparent K_m is 0.5 mM, a value in the range of the cytosolic concentration of spermine (0.57 mM, see also Ref. 7). However, the rate also depends on the medium. Thus is an equiosmotic KCl medium the rate of spermine uptake by energized mitochondria was 50% lower than that obtained in sucrose medium. This difference can be accounted for by the interference of the ionic strength on the electrostatic binding of spermine to the membrane sites preliminary to its transport. In other words neutralization of the negative surface charges may interfere with spermine association to the membranes.

Spermine uptake also depends on P_i transport (Fig. 3). Thus (i) spermine taken up by respiring mitochondria is a linear function of the external P_i concentration up to 1.5 mM; (ii) Mersalyl, a known inhibitor of the P_i transport, almost completely abolished spermine uptake. (In this experiment Mersalyl was used in the concentration of 10 μM, which did not affect the respiration of succinate, the inhibition of which might be responsible for the suppression of spermine uptake, see Fig. 1).

The loss of accumulated spermine from mitochondria induced by antimycin A was also inhibited by Mersalyl (Fig. 4a). As expected, this inhibitor also prevented the parallel loss of accumulated P_i (Fig. 4B).

These findings clearly demonstrate that not only the uptake, but also the loss of spermine from liver mitochondria is dependent on the concurrent

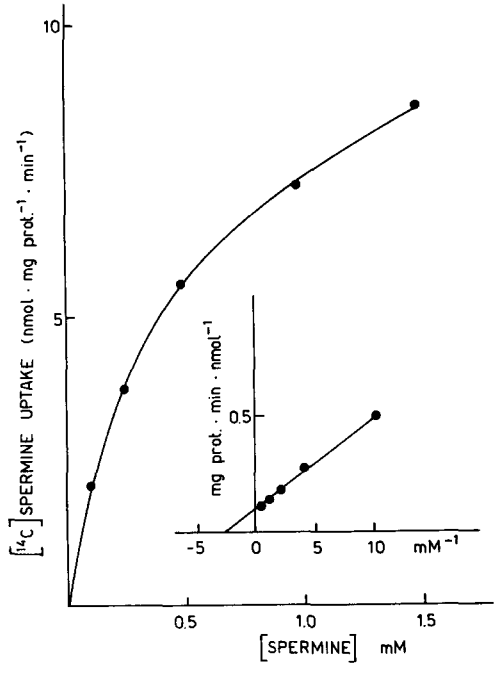

Fig. 2. Kinetics of spermine transport. Conditions as in Fig. 1 in the presence of 5 mM succinate and 2 mM P_i. [^{14}C] spermine (1 mCi/mmol) at various concentrations as indicated. Incubation time: 5 min. The uptake data were corrected for spermine present in the non-matrix space of the pellets by subtraction of controls incubated in the presence of FCCP.

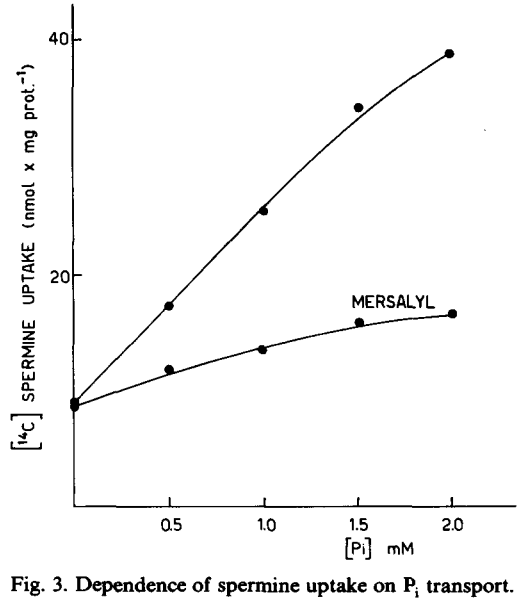

Fig. 3. Dependence of spermine uptake on P_i transport. Conditions as in Fig. 1 in the presence of 5 mM succinate and 1 mM [^{14}C] spermine (1 mCi/mmol). P_i at various concentrations as indicated. When present, 10 μM Mersalyl. Incubation time: 20 min.

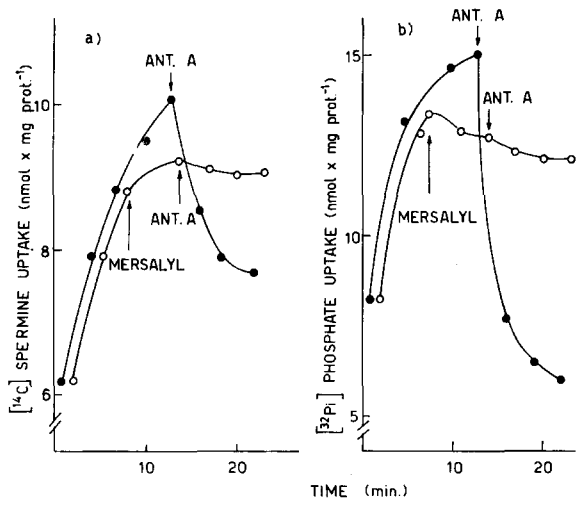

Fig. 4. Parallelism between spermine and P_i movement in rat liver mitochondria (RLM). 5 mg RLM/ml in 200 mM sucrose, 10 mM Hepes (pH 7.4) 1.25 μM rotenone, 5 mM succinate, 0.1 mM [^{14}C] spermine (100 μCi/mmol), 0.5 mM [^{32}P] P_i. Additions: 1 μg/mg protein antimycin A (Ant. A), 50 μM Mersalyl (10 nmoles/mg protein).

transport of P_i. Conversely, the uptake of P_i by energized liver mitochondria is enhanced by spermine to an extent dependent on its concentration in the medium (Fig. 5). Therefore, it would appear that the transport of P_i and spermine in mitochondria are mutually related processes.

Spermine resembles Mg^{2+} in its effects on energy-linked processes [8]. The possible interaction

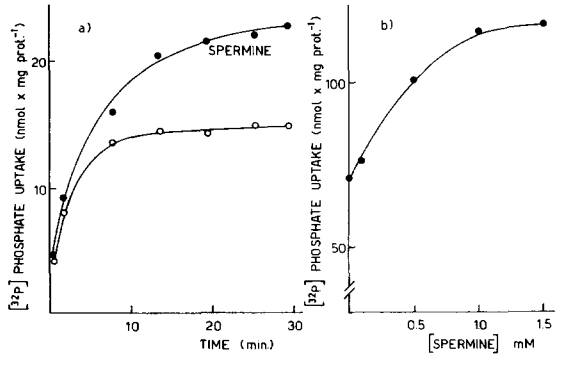

Fig. 5. Enhancement of P_i uptake by rat liver mitochondria induced by spermine transport. Conditions as in Fig. 1 in the presence of 5 mM succinate, 0.5 mM [^{32}P] P_i and 0.1 mM spermine (a); 5 mM succinate, 2 mM [^{32}P] P_i and spermine at various concentrations as indicated (b), incubation time; 20 min.

of these two cations in the transport process was therefore examined. Addition of Mg^{2+} to the suspending medium in fact inhibited spermine uptake (Fig. 6). Ca^{2+} did not have this effect (results not reported).

Mg^{2+} and spermine may compete for the same binding sites on the mitochondrial membrane. Thus addition of Mg^{2+} to mitochondria preloaded with spermine induced a partial release of this amine, which, however, was completed by the further addition of FCCP (Fig. 7). Conceivably, the portion of spermine sensitive to Mg^{2+} is displaced from membrane binding sites, whereas the portion sensitive to FCCP is released from the matrix space as a consequence of deenergizing the mitochondria. This explanation is consistent with the observation that Mg^{2+} ions, unlike FCCP, are capable of displacing spermine from non-respiring mitochondria (Fig. 7), though the amounts were much lower than those taken up by non-respiring mitochondria. However, if Mg^{2+} was added prior to spermine a minimum spermine uptake was observed. This discrepancy may be explained by assuming an immediate electrostatic binding of

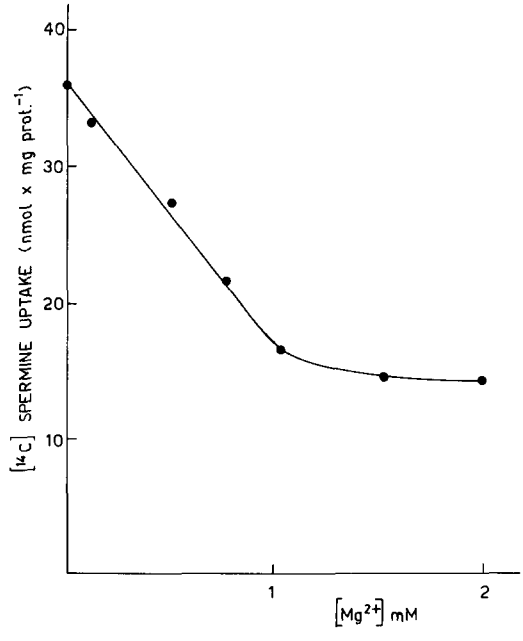

Fig. 6. Inhibition of spermine uptake by Mg^{2+}. Conditions as in Fig. 1 in the presence of 5 mM succinate, 1 mM P_i, 1 mM [^{14}C] spermine (1 mCi/mmol). Mg^{2+} at various concentrations as indicated. Incubation time: 20 min.

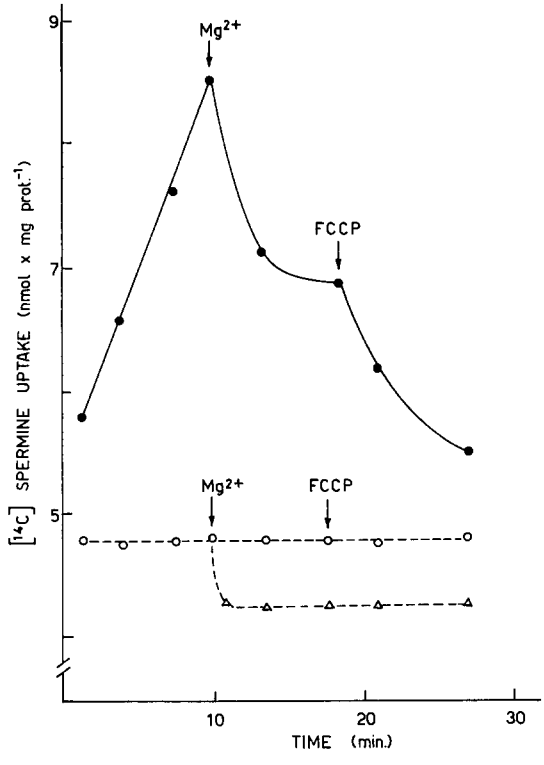

Fig. 7. Release of spermine from respiring and non-respiring mitochondria induced by Mg^{2+} and FCCP. 1 mg rat-liver mitochondria per ml was suspended at 20°C in the following medium: 200 mM sucrose/10 mM Hepes (pH 7.4)/1.25 μM rotenone/0.1 mM [^{14}C] spermine (100 μCi/mmol) (dashed line). With 5 mM succinate and 0.5 mM P_i (continuous line). Additions: 1 mM Mg^{2+}, 0.1 μg/mg protein FCCP.

spermine to anionic sites in the membranes [1] followed by a transaminidases catalyzed incorporation into mitochondrial proteins [9,10].

Discussion

The results here reported provide good evidence that spermine, in addition to its binding to the membranes, may be transported inside the mitochondria in a process driven by respiration and dependent on P_i transport. That a consistent portion of the spermine taken up by respiring mitochondria is transported into the inner space is based on the following evidence: (i) uncouplers or respiratory chain inhibitors completely prevent this uptake and induce the release of much of the accumulated spermine (Fig. 1); (ii) the spermine taken up by respiring mitochondria is entirely discharged upon addition of both Mg^{2+} and FCCP (Fig. 7); (iii) the amount of spermine removed by Mg^{2+} from respiring mitochondria is considerably higher that remove from non-respiring mitochondria, suggesting that the respiration markedly enhances the amount of spermine taken up (Fig. 7). Furthermore, the observation that spermine and P_i are taken up in a parallel way and simultaneously released from loaded mitochondria upon addition of antimycin A (Fig. 4) shows that spermine is transported into respiring mitochondria concurrently with P_i.

Mersalyl prevents spermine uptake (Fig. 3) and also the release of accumulated spermine induced by antimycin A (Fig. 4). This implies that this polyamine is transported bidirectionally across the inner membrane along with P_i. The dependence of spermine transport on that of P_i most probably reflects the necessity to maintain a proper H^+ balance. It is known that P_i acts as a proton donor to the mitochondrial proton pump, and that deprotonated P_i is an important factor in the generation of intramitochondrial negativity responsible for the electrophoretic influx of permeant cations [11]. It has to be outlined that under physiological conditions, polyamines are largely protonated, and therefore exhibit a net positive charge [12]. In turn, spermine enhances the extent of P_i accumulation in mitochondria, possibly by a coprecipitation of the two ions [1] within the matrix space. However, the simultaneous efflux of spermine and P_i from loaded mitochondria upon deenergization (Fig. 4) implies that the supposed precipitate of spermine-P_i is in a reversible equilibrium with the two ions in solution.

It may be noted that Mg^{2+} too are transported into liver mitonchondria in a P_i-dependent process [13]. It is therefore conceivable that the two cations compete with each other (Fig. 6) both for the binding sites on the membranes, and also for transmembrane transport.

It is likely, if not certain, that spermine taken up by non-respiring mitochondria binds to the membranes in part electrostatically [6] and in part by covalent linkages probably catalyzed by widespread transaminidases [9,10]. Preliminary unpublished results showed that part of [C^{14}]spermine added to isolated mitochondria was associated to some proteins separated on SDS-poly-

acrylamide gel electrophoresis.

As to the mechanism of spermine transport into the mitochondria the present data do not allow any precise inference. In spite of the difference in the transport rate there is some analogy with Ca^{2+} transport. The uptake of both spermine and Ca^{2+}, driven by the electrochemical potential generated by the respiration, is facilitated by P_i and also the efflux of these cations occurs concomitantly with that of P_i. Furthermore, both spermine and Ca^{2+} are capable at least in theory, of precipitating as phosphate salts (or complexes) within the mitochondria. On the other hand the insensitivity of spermine transport to ruthenium red makes very improbable that spermine might be transported on the Ca^{2+} carrier.

The modifications induced by spermine on Ca^{2+} transport [14] may perhaps be ascribed to their competition not only for common anionic binding sites on the membranes [6], but also for the transport of P_i, i.e., part of the available P_i may be diverted from co-transport with Ca^{2+} [15], thus attenuating the consequent deterioration of mitochondrial functions (4). Conceivably, the protective action of spermine on mitochondrial function [3,4] may be due both to modifications of membrane properties induced by spermine binding and also to a control on Ca^{2+} and P_i transport.

The present results acquire some relevance in the light of the recent paper of Nicchitta and Willliamson [16] showing that at physiological concentrations spermine accelerates Ca^{2+} cycling in isolated liver mitochondria. Indeed, this polyamine exerts a combined effect on both Ca^{2+} influx and Ca^{2+} efflux system, thereby making the mitochondria responsive to extramitochondrial Ca^{2+} concentration in the physiological range [16]. If the action of external spermine on the electrophoretic Ca^{2+} uniport is quite understandable, its action on the release of Ca^{2+} through the ruthenium red insensitive efflux system might be better explained by the results here reported showing the possibility of a transport of external spermine into the matrix space.

Since the concentrations of spermine employed in our experiments are very close to those present intracellularly [7], the reported results could be relevant to its physiological function.

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