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Review

Effects of polyamines on mitochondrial Ca^{2+} transport

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Abstract

Mammalian mitochondria are able to enhance Ca^{2+} accumulation in the presence of polyamines by activating the saturable systems of Ca^{2+} inward transport and buffering extramitochondrial Ca^{2+} concentrations to levels similar to those in the cytosol of resting cells. This effect renders them responsive to regulate free Ca^{2+} concentrations in the physioloical range. The mechanism involved is due to a rise in the affinity of the Ca^{2+} transport system, induced by polyamines, most probably exhibiting allosteric behaviour. The regulatory site of this mechanism is the so-called S_1 binding site of polyamines, which operates in physiological conditions and is located in the energy well between the two peaks present in the energy profile of mitochondrial spermine transport. Spermine is bidirectionally transported across teh inner membrane by cycling, in which influx and efflux are driven by electrical and pH gradients, respectively. Most probably, polyamine affects the Ca^{2+} transport system when it acts from the outside–that is, in the direction of its uniporter channel, in order to reach the S_1 site. Important physiological functions are related to activation of Ca^{2+} transport systems by polyamines and their interactions with the S_1 site. These functions include a rise in the metabolic rate for energy supply and modulation of mitochondrial permeability transition induction, with consequent effects on the triggering of the apoptotic pathway.

Keywords: Polyamine; Ca^{2+}; Mitochondria; Transport

1. Introduction

Precise and rapid control of the intracellular calcium concentration is critically important for cell function; prolonged exposure to increased Ca^{2+} concentrations can produce cell damage and ultimately cell death. Several cellular processes are implicated in the regulation of Ca^{2+} fluxes in order to maintain intracellular Ca^{2+} homeostasis, among them the calcium transport systems in mitochondria, whose role, however, is a matter of debate.

Calcium transport across the inner membrane of mammalian mitochondria is catalyzed by a complex system of specific transporters. It includes: (i) the Ca^{2+} electrophoretic uniporter and the rapid mode of Ca^{2+} transport (RaM) for inward transport, and (ii) the Ca^{2+}/Na^{+} and Ca^{2+}/H^{+}, electroneutral exchanger, for outward transport. The Ca^{2+}-induced mitochondrial permeability transition (MPT), which is also involved in Ca^{2+} efflux, is also often considered as a Ca^{2+} transport mechanism.

The most important function attributed to mitochondrial Ca^{2+} transport is the regulation of matrix Ca^{2+} levels and hence modulation of oxidative metabolism, as there are several Ca^{2+}-sensitive matrix dehydrogenases. Another important role ascribable to this transport is the buffering of the extramitochondrial Ca^{2+} concentration. However, this effect has remained controversial for a long time. It is generally accepted that the ability of mitochondria to buffer extramitochondrial Ca^{2+} concentrations is exhibited only when cells are injured and a massive increase in Ca^{2+} levels occurs in the cytoplasm. In other words, mitochondria should be considered as the main Ca^{2+} storage compartment in cells rather than as regulators of cytoplasmic Ca^{2+} concentrations. This function was generally attributed to the endoplasmic reticulum until the discovery of the effects of polyamines on Ca^{2+} transport. The natural polyamines are aliphatic molecules that are fully protonated at physiological pH; although they exhibit a plethora of effects at

Abbreviations: MPT, mitochondrial permeability transition; $\Delta\Psi$, membrane potential; RaM, rapid mode of Ca^{2+} uptake; MGBG, methylglyoxal-bis-(guanlyhydrazone)

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biological level, their specific functions have not yet been elucidated. They weakly and reversibly bind to the mitochondrial membrane at the level of specific binding sites. This binding permits their transport into the matrix compartment and interaction with the Ca^{2+} transporters, with important effects on Ca^{2+} bidirectional fluxes. In fact, in the presence of the polyamine spermine, mitochondria shift their capacity in buffering exogenous (cytoplasmic) Ca^{2+} concentrations towards lower levels, indicating that spermine assumes an important role in maintaining Ca^{2+} homeostasis. The present review focuses on the effects of polyamines on the functional properties of the Ca^{2+} transporters and the physiological consequences of Ca^{2+} transport on both mitochondrial function and the modulation of the cytoplasmic Ca^{2+} signal.

2. Mitochondrial Ca^{2+} transport

Higher animals possess a refined mitochondrial Ca^{2+} transport system, most likely a gated channel operating bidirectionally across the inner membrane with different mechanisms. This system, which has been highly conserved during evolution, contributes to the regulation of intracellular calcium homeostasis together with mechanisms controlling Ca^{2+} fluxes across the plasma membrane and endoplasmic reticulum membranes. This process reflects a dynamic state of all these fluxes which at steady state establishes an equilibrium among the opposite movement of Ca^{2+} across the above mentioned membranes. Apart from the important physiological effects linked to the entry of Ca^{2+} into the mitochondrial matrix (see below), accumulation of Ca^{2+} in mitochondria can trigger a phenomenon termed the MPT, characterized by an increase in permeability of the inner membrane due to the opening of a proteinaceous pore (for reviews on MPT see Refs. [1,2]). This phenomenon produces a series of events leading to a redox catastrophe and bioenergetic collapse, and triggers the proapoptotic pathway.

At the beginning of the 1990s, the role of mitochondrial Ca^{2+} transport began to attract renewed interest of a broadened audience, most likely due to the central role played by Ca^{2+} in the MPT. It has become clear that several cellular processes are directly or indirectly dependent on mitochondrial Ca^{2+} uptake.

2.1. The Ca^{2+} uniporter

Ca^{2+} is accumulated in mitochondria by an electrophoretic transport system that is sensitive to membrane potential ($\Delta\Psi$) [3–7]. This uptake is described by a function (see Eq. (1)) which can be obtained from the same rationale used to propose the Goldman constant field equation [8–11]. The combination of Ca^{2+} concentration dependence and $\Delta\Psi$ dependence fits the formula:

$$v = \{[V^*[Ca^{2+}]^2]/[K_{0.5}^2 + [Ca^{2+}]^2]\} \times \{(\Delta\phi/2) \times (e^{\Delta\phi/2})/[\sinh(\Delta\phi/2)]\} \quad (1)$$

where $[Ca^{2+}]$ is the free calcium concentration, V^* is a velocity parameter, which is independent of membrane potential, $K_{0.5}$ is the value of $[Ca^{2+}]$ at half maximal velocity, and $\Delta\phi$ is a dimensionless parameter proportional to the membrane potential expressed as $\Delta\phi = 2F\Delta\Psi/RT$ [1,11].

The electrophoretic nature of this transport mechanism strongly supports its identification as uniporter system which exhibits a cooperative effect involving two binding sites: an activation site and a transport site. The rate of transport is increased upon binding of Ca^{2+} to the activation site [12]. The polycation ruthenium red strongly inhibits this transport [13].

2.2. Rapid mode of Ca^{2+} uptake (RaM)

In 1995, Sparagna et al. [14] identified a novel mechanism of mitochondrial Ca^{2+} uptake in liver mitochondria that is capable of sequestering significant amounts of Ca^{2+} from cytosolic Ca^{2+} in rapid pulses; the same mechanism, termed RaM, was subsequently described in heart mitochondria [15]. In liver, RaM exhibits a much higher initial conductivity (i.e., about 300-fold higher) than that of the uniporter [14]. Although RaM activity in heart mitochondria exhibits almost the same characteristics as in liver, its activation and inhibition are quite different [15], probably due to different physiological adaptations in these two tissues. In both liver and heart, RaM is highly conductive at the beginning of a Ca^{2+} pulse, but is inhibited by the increase in Ca^{2+} concentration caused by the pulse itself. There is evidence that RaM has an external calcium binding site which is responsible for inactivation of the phenomenon in a very short time [14] and accounts for the observed inhibition. However, the time required to reset all of the RaMs for subsequent calcium uptake is as at least 60-fold longer than the inactivation time [15].

2.3. Ca^{2+}/Na^{+} and Ca^{2+}/H^{+} antiporters

Ca^{2+} efflux from mitochondria is mediated by two mechanisms, both of which are electroneutral in nature, one Na^{+}-dependent [16] and the other Na^{+}-independent [17]. The Na^{+}-dependent mechanism is more prominent in brain and heart mitochondria. It exchanges Ca^{2+} for Na^{+}, with a stoichiometry that is most likely greater than two Na^{+} for one Ca^{2+} [18]. Diltiazem and tetraphenylphosphonium are the most important inhibitors of this mechanism [19,20]. The Na^{+}-independent mechanism is predicted to exchange Ca^{2+} for two H^{+}, is most likely energy-dependent [9] and is prominent in liver. It has been proposed that in liver, kidney and most likely in lung, this mechanism might be linked to the redox state of mitochondrial pyridine nucleotides, stimulated by the prooxidants H_2O_2, menadione, and *t*-butylhydroperoxide, and would require an intramitochondrial Ca^{2+}-dependent hydro-

lysis of NAD^+ (reviewed in Ref. [21]). Hydrolysis of NAD^+ would only be possible when a vicinal thiol is cross-linked, either by oxidation [22] or by reaction with phenylarsine oxide [23], and would be prevented when these thiols are alkylated [24] or oxidized to a sulfonate state [25]. However, the proposal that the Na^+-independent pathway of Ca^{2+} efflux is linked to the redox state of pyridine nucleotides is very controversial. Most authors in the field do not agree with this mechanism and instead support the proposal that Ca^{2+} efflux induced by pro-oxidants takes place by transition pore opening [26,27].

2.4. MPT as a Ca^{2+} efflux mechanism

As described above [1,2], the inner mitochondrial membrane can undergo an increase in permeability due to the opening of the MPT pore. This specific channel permits the bidirectional flux of metabolite molecules up to 1500 kDa in size, and has been reported to exist in different conductivity states [28]. The opening of the pore is reversible and induces a total Ca^{2+} efflux from mitochondria, which contrasts with the gradual and uniform release that would be expected from the antiporters. For this reason, the MPT has been proposed to represent an important mechanism promoting efflux of this cation with a specific biological role [27].

2.5. Physiological effects of Ca^{2+} transport

This sophisticated system of uptake and release of Ca^{2+} suggests that it plays a key role in cell physiology, at least in part due to the fact that two enzymes of the Krebs cycle (isocitrate and oxoglutarate-dehydrogenase) and pyruvate dehydrogenase are activated by matrix Ca^{2+} [29]. Based on the sensitivity of the matrix dehydrogenases, to $[Ca^{2+}]$, a first consequence of transient Ca^{2+} accumulation within the matrix is the activation of mitochondrial metabolism. Numerous experimental observations demonstrate a correlation between increased Ca^{2+} concentration and augmented NADH levels, mitochondrial ATP production and O_2 uptake [30,31]. Indeed, this Ca^{2+}-dependent control mechanism works as an integrator of the cytosolic Ca^{2+} signal [30].

Recent data demonstrate that, besides controlling mitochondrial function, uptake of Ca^{2+} by mitochondria plays a role in the control of other events occurring in the cytosol. In this regard, it has been reported that the speed and extent of Ca^{2+} release from the endoplasmic reticulum is modulated by the local Ca^{2+} buffering of mitochondria [32]. There is also evidence for a role of mitochondria in modulating the activity of plasma membrane channels [33] and in buffering Ca^{2+} concentrations in neurons [34].

Mitochondrial Ca^{2+} influx/efflux systems are also involved in controlling hormone synthesis and/or release by endocrine cells. In particular, it has been reported that mitochondrial Ca^{2+} accumulation modulates the secretion of insulin by pancreatic β cells through the activity of a Ca^{2+}-activated coupling factor [35] identified to be glutamate [36]. A similar intriguing role of Ca^{2+} movements in mitochondria has been suggested for secretion of catecholamine from chromaffin cells [37].

Several qualified reviews have described Ca^{2+} transport in mammalian mitochondria [27,38–41]; although the molecular nature of this phenomenon is still unsolved, recent developments have contributed to a revived interest, as pointed out by a very recent historical review [42].

3. Polyamine functions

The natural polyamines, putrescine, spermidine, and spermine, are ubiquitous metabolites in prokaryotic and eukaryotic cells, and are universally known to be essential in important physiological processes such as cell growth and differentiation [43]. These general aspects as well as the complicated highly regulated pathway of their biosynthesis have attracted increased interest in recent years and are dealt with in several reviews [43–49]. Besides the regulation of mitochondrial Ca^{2+} transport, polyamines are involved in a large number of cellular functions including protein synthesis and phosphorylation [50], inhibition of the MPT [51], modulation of programmed cell death [52], regulation of nitric oxide synthase [53] and free radical scavenging [54,55].

4. Polyamine–mitochondria interactions

Polyamines interact with mitochondrial membranes at the level of two monocoordinated binding sites (S_1 and S_2) for spermine and spermidine and one monocoordinated binding site (S_2) for putrescine [56,57]. These sites exhibit low affinity and high binding capacity, with a calculated binding energy of 16–20 kJ/mol, which is typical for weak interactions. These values were calculated using a thermodynamic treatment of ligand–receptor interactions [58], applied to non-equilibrium binding of polyamines [56,57]. Binding constants and consequent interaction energies were calculated by utilizing Eq. (2) for Scatchard and Eq. (3) for Hill analysis.

$$\frac{[B]}{[F]} = \sum_{i=1}^{s} \{[B_{\max,i}] - [B_i]\} \cdot \left[\frac{1}{K_{i,1(t)}} + \varepsilon_i(F)\right] \tag{2}$$

$$\ln\left\{\frac{[B]}{[B_{\max}] - [B]}\right\} = \ln\left\{\sum_{i=1}^{s} x_i(F)\left[\frac{1}{K_{i,1(t)}} + \varepsilon_i(F)\right]\right\} + \ln[F] \tag{3}$$

where:

$$\varepsilon_i(F) = \sum_{k=2}^{n_i} \frac{[F]^{k-1}}{\prod_{j=1}^{k} K_{i,j}(t)}$$

represents the appropriate measure of the extent of multiple coordination on the i-th sites. [$B_{max,\ i}$] is the maximum concentration of the i-th sites which may be bound by the ligand, [B_i] is the concentration of i-th sites bound by the ligand, [B_{max}] is the maximum receptor-bound ligand concentration, [F] is the free ligand concentration, $K_{i,j}(t)$ is the affinity constant of the ligand for the i-th site, j is the occupancy number, and t is time.

The rationale for these equations, both of which were obtained from the same general formula [58], considers receptors having groups of different binding sites (s) where each may have multiple occupancies (n_i).

The observed variations in the polyamine binding parameters most likely reflect differences in their flexibility and hydrophobicity [57]. Polyamine binding sites are probably located in strict proximity to each other on the inner membrane and present the peculiarity of being filled in different ways depending on the polyamine. Both the S_1 and S_2 sites are involved in the mechanism of polyamine transport into mitochondria. This process is carried out by a common uniporter system, is dependent on the $\Delta\Psi$, and exhibits a non-ohmic flux–voltage relationship rationalized by Eq. (4) [59,60]

$$J = J_0 e^{z\beta F\Delta\Psi/RT} \quad (4)$$

where J is the rate of polyamine influx at high values of $\Delta\Psi$, J_0 is the exchange flux at $\Delta\Psi = 0$, F is the Faraday constant, T is the absolute temperature in K, R is the general gas constant, z is the polyamine valence, and β is a parameter describing the shape and position of the energy barrier(s) for ion transport. For a sharp barrier, β equals the fractional distance from the external side of the membrane to the peak of the first energy barrier.

Polyamine transport is insensitive to the pH of the medium, suggesting a strictly electrophoretic behavior [59–61]. It is also temperature-dependent and increases as the charge number of the transported species increases, with an activation enthalpy of about 12 kJ/mol per charge at $\Delta\Psi \cong 175$ mV [60]. Flux–voltage relationship and free-energy profiles derived from analyses of spermine transport provide evidence for the presence of a channel exhibiting two asymmetrical energy barriers with an energy well, at the level of S_1, located at 1/8 of the length of the channel [62]. Upon transport into mitochondria, spermine continuously flows out by a ΔpH-dependent mechanism. This process establishes a bidirectional movement of the polyamine in and out of mitochondria, driven by electrical and pH gradients, respectively, and suggests that there is a continuous energy-dependent cycling of spermine [63] (for a review on the interactions of polyamines with mitochondria and their transport see Ref. [64]). Taking into account the interactions of spermine and other polyamines at the mitochondrial level and their well-documented effects on Ca^{2+} uptake, the following section presents a summary of accumulated knowledge on how polyamines interact with mitochondrial Ca^{2+} transport systems.

5. Polyamines and mitochondrial Ca^{2+} transport

The study of spermine as a regulator of mitochondrial calcium cycling began before the complete rationalization of polyamine–mitochondria interactions was achieved. It was approached on the basis of previous observations regarding the activation by polyamines of plasma membrane Ca^{2+} fluxes in response to testosterone and isoproterenol administration in kidney cortex [65,66]. The first important paper on this argument was published by Nicchitta and Williamson in 1984 [67]. The most significant conclusion from this study of isolated liver mitochondria and permeabilized hepatocytes was that in both systems, in the presence of spermine and physiological free Mg^{2+} concentrations, mitochondria are able to enhance Ca^{2+} accumulation and buffer the extramitochondrial free Ca^{2+} concentration to values very similar to those present in the cytosol of resting hepatocytes, thereby making the mitochondria responsive to free Ca^{2+} concentrations in the physiological range. Indeed, the authors proposed that the rate of mitochondrial Ca^{2+} cycling is increased by effects of spermine on both the Ca^{2+} uniporter and the electroneutral efflux system.

Thus, the finding that besides acting as stores for massive Ca^{2+} concentrations, mitochondria can also behave as fine regulators of physiological Ca^{2+} concentrations in the cytosol, prompted a reevaluation of the role of these organelles in controlling cellular Ca^{2+} homeostasis. The demonstration by Nicchitta and Williamson that spermine stimulates Ca^{2+} uptake conflicted with previous results obtained by Åkerman [68], who observed an inhibitory effect by the polyamine on this process. This discrepancy was later explained with the demonstration of a dual effect of spermine on Ca^{2+} uptake [69] (see below).

Interestingly it was observed that Mn^{2+}, at extramitochondrial levels occurring "in vivo", is also able to alter the balance between the uptake and release of Ca^{2+} in the presence of Mg^{2+} and phosphate, by accelerating the Ca^{2+} uniporter [70]. Substitution of spermine for Mg^{2+} abolishes the effect of Mn^{2+}.

The spermine-induced increase in Ca^{2+} uptake by isolated liver mitochondria incubated in KCl medium in the presence of phosphate is also responsible for the substantial increase in energy-dependent osmotic swelling associated with the electrogenic flux of K^+ into mitochondria [71]. This K^+-dependent swelling is not associated with either uncoupling of mitochondria or any nonspecific permeability changes and is not prevented by inhibitors of mitochondrial phospholipase A_2.

When incubated under the same conditions, mitochondria derived from rats treated with glucagon exhibit less swelling than control mitochondria [71]. In the presence of ruthenium red and high endogenous Ca^{2+}, mitochondria from hormone-treated animals show larger amplitude swelling than controls. These results indicate that a rise in the matrix Ca^{2+} concentration can enhance the electrophoretic flux of K^+ into mitochondria by an unknown mechanism

and thereby induce swelling. It has been proposed that this mechanism also underlies the ability of alpha-agonists and vasopressin to enhance mitochondrial volume "in situ" [71].

In addition to liver mitochondria, the effect of spermine on the regulation of Ca^{2+} transport was further investigated in heart [48] and brain mitochondria [50,72,73].

A general observation gained from these studies is that the stimulation of the initial transport rate and Ca^{2+} cycling is ascribable to a decrease in the apparent K_m for Ca^{2+} uptake by the Ca^{2+}-uniporter. These studies pointed out that the half-maximally effective concentration of spermine (50 μM) is in the range of its physiological concentration in the cell [67].

Spermidine was found to be five times less effective than spermine, and putrescine is ineffective [50]. It has also been observed that the stimulation of Ca^{2+} uptake by spermine is inhibited by Mg^{2+} in a concentration-dependent manner and that spermine is also able to reverse ruthenium red-induced Ca^{2+} efflux from mitochondria [50]. The ability of Mg^{2+} to counteract the activation of Ca^{2+} uptake by spermine was suggested by the authors as a mechanism to protect mitochondria against excessive Ca^{2+} accumulation. It is important to point out that Mg^{2+} exhibits this protective effect when the extramitochondrial Ca^{2+} concentration is low, that is 0.1 μM. At free Ca^{2+} concentrations of 0.5 μM and higher, spermine activates the Ca^{2+} uniporter in the presence of physiological Mg^{2+} concentrations [48]. Furthermore, studies of brain mitochondria demonstrated that this effect of spermine is also due to an inhibition of Na^+-dependent and Na^+-independent Ca^{2+} efflux [72–74]. Studies of brain mitochondria indicated that Ca^{2+} transport is markedly more stimulated by spermine in hippocampal mitochondria than in brainstem mitochondria [75]. A careful examination of the kinetics of Ca^{2+} transport revealed that the effect of spermine takes place at low Ca^{2+} concentrations. As the Ca^{2+} concentration increases beyond this low level, the stimulatory effect of the polyamine becomes less evident until an inhibitory effect is produced [75].

While the main observations of the effects of polyamines on mitochondrial Ca^{2+} transport have been made with liver, heart and brain mitochondria, this effect has also been recognized in mitochondria from other tissues. For example, mitochondria of pancreatic islets exhibit the same effect of spermine on Ca^{2+} uptake [7] observed in liver, heart and brain mitochondria, while studies of intestinal mitochondria revealed a polyamine-induced alteration of Ca^{2+} homeostasis with the stimulation of phospholiphase D [76].

The ability of spermine to decrease extramitochondrial Ca^{2+} to physiological levels by activating its transport system was also evidenced in yeast mitochondria [77,78], in which a regulatory effect by physiological ADP concentrations and by the intramitochondrial NADH/NAD^+ ratio was identified [79]. Indeed, under certain conditions, ruthenium red was found to stimulate the yeast uniporter [78].

Instead, in intact liver cells, the effect of spermine is abolished by preincubation with alloxan or ninhydrin, which reduce mitochondrial membrane potential, inhibit Ca^{2+} uptake and stimulate Ca^{2+} efflux [80].

5.1. Dual effect of polyamines

Studies of rat liver, brain and heart mitochondria have revealed a dual effect of spermine on Ca^{2+} transport depending on the concentration of the cation [81].

In fact, spermine exhibits an accelerating effect on the initial Ca^{2+} uptake rate at 50 μM Ca^{2+} and an inhibitory effect at 180 μM (thus explaining the previous observation of Åkermann [68] who also observed an inhibitory effect). However, spermine invariably increases mitochondrial Ca^{2+} accumulation under both conditions.

The same group demonstrated that a number of other cationic compounds containing amino groups also exert a dual effect on Ca^{2+} transport in liver mitochondria, consisting of a decrease in the rate of Ca^{2+} uptake at high concentrations of the polyamine compound and an increase in Ca^{2+} accumulation [69].

This effect is similar to that previously observed with spermine in mitochondria isolated from different tissues [81]. However, in contrast to the effect of spermine, spermidine and putrescine, the increasing Ca^{2+} accumulation by aminoglucoside, basic polypeptides and metal amine complexes shifts to an inhibition of Ca^{2+} accumulation at higher compound concentrations.

The authors suggested that the ability of structurally similar compounds to decrease Ca^{2+} uptake rate and increase Ca^{2+} accumulation correlated with the number of positive charges. However, the identification of cationic polyamines and basic oligopeptides unable to enhance mitochondrial Ca^{2+} accumulation also raised the proposal that although the presence of multiple, distributed cationic charges is necessary to affect mitochondrial Ca^{2+} transport, it is not sufficient [69].

A study performed by simultaneous measurements of the free matrix and extra mitochondrial Ca^{2+} concentrations demonstrated that spermine is able to decrease the extramitochondrial steady-state Ca^{2+} concentration but at the same time, due to a concentration-dependent reversal of this effect, also induces a decrease in the free matrix Ca^{2+} concentration [82]. These results were explained by the existence of a putative second low affinity binding site for spermine that mediates an inhibition of Ca^{2+} uptake [82] as subsequently demonstrated by other authors [56,57,81]. In other words, at high spermine per mitochondrion ratios, the polyamine has an additional low affinity binding site of action, which contrasts its enhancing effect on free Ca^{2+} uptake mediated by the site with higher affinity.

The demonstration that aliphatic polyamines are able to inhibit the MPT with the order of efficacy spermine>spermidine>putrescine [84] pointed out a structure–reactivity relationship reminiscent of the enhancement of mitochon-

drial Ca^{2+} accumulation by spermine and polyamine analogues [69,85]. Thus, the possibility that the inhibition of the MPT is directly related to the enhancement of mitochondrial Ca^{2+} accumulation by polyamines was considered a reasonable hypothesis [85]. However, it has also been reported that gentamycin and polylysine which, like spermine, are able to enhance mitochondrial Ca^{2+} accumulation [69], are ineffective in protecting against the MPT, while the aliphatic polyamine bis(hexamethylene)triamine, which is unable to enhance mitochondrial Ca^{2+} accumulation, is able to prevent the MPT [85].

All these results led the authors to propose that the enhancement of Ca^{2+} accumulation by spermine is not strictly related to inhibition of the MPT (see below) and that aminoglucosides exert their effects on Ca^{2+} transport by antagonizing, rather than triggering, the effects of natural polyamines.

5.2. RaM and polyamines

As reported above, liver [14] and heart [15,86,87] mitochondria are proposed to possess a mechanism called RaM which functions at the beginning of short pulses and allows mitochondria to sequester a considerable amount of Ca^{2+} from such pulses. Treatment of liver mitochondria with 1 mM spermine causes a sixfold increase in Ca^{2+} uptake via the RaM, while in the same experimental conditions, it causes a twofold increase in the uniporter rate [8]. RaM is activated much less strongly by spermine in heart mitochondria [14]. The mechanism by which spermine exhibits an increased activation of Ca^{2+} uptake via RaM compared with that of the uniporter remains to be determined. Taking into account the characteristics of liver and heart, the data accumulated for RaM suggest that it plays a role in metabolic signaling that controls activation of the Ca^{2+}-dependent metabolic reactions linked to ATP synthesis taking place in the mitochondrial matrix in these tissues. One proposal is that RaM may be involved in the mechanism modulating the rate of ADP phosphorylation in physiological conditions while minimizing the probability of MPT induction [14]. Sparagna et al. [14] favor the hypothesis that RaM is mediated by a specific transport mechanism that might be responsible for mitochondrial Ca^{2+} uptake from cytosolic Ca^{2+} transients in vivo.

Aside from isolated mitochondria, there is also evidence for the presence of this mechanism in intact cells, even though some authors do not use the term RaM to identify this rapid uptake phenomenon [88–91].

5.3. Mechanism underlying the effect of polyamines

The stimulatory effect of spermine on mitochondrial Ca^{2+} uptake is generally accepted as physiologically relevant [67]. Generally, the activation of Ca^{2+} uptake by energized mitochondria exposed to physiological concentrations of spermine provokes an approximate 70% reduction in the steady-state Ca^{2+} concentration (from 0.8 to 0.25 μM) in the incubation media, a concentration approximating the levels of cytosolic free Ca^{2+} in intact hepatocytes [50,67,92]. In spite of efforts documented in these reports, the precise mechanism by which spermine influences mitochondrial Ca^{2+} transport remained uncertain for many years, and new observations continue to lead to different proposals.

Nicchitta and Williamson [67] proposed that the mechanism responsible for spermine's effect on Ca^{2+} transport is related to a spermine-dependent alteration in the Ca^{2+} transport system and lipid bilayer interactions. The same authors also suggested that spermine causes a decrease in the apparent K_m for Ca^{2+} uptake by the Ca^{2+} uniporter [67]. A number of studies suggested an allosteric activation of the Ca^{2+} uniporter [50,93–95], which is independent of other important effectors such as Mg^{2+} [67,95] when the Ca^{2+} concentration is higher than 0.5 μM [67]. The lack of an effect of Mg^{2+} on the ability of spermine to lower the steady-state exogenous free Ca^{2+} concentration suggests that spermine does not interact at the Mg^{2+} site of the Ca^{2+} uniporter [67].

The main support for this proposal comes from the observation that spermine binding locks the Ca^{2+} uniporter into a conformational state that shifts the sigmoid kinetics of Ca^{2+} transport to a nearly hyperbolic curves. Indeed, spermine increases the apparent affinity for Ca^{2+} and decreases the cooperativity of uptake [76]. The same authors proposed that the allosteric activation by spermine is due to the binding to the same regulatory sites that are involved in the Ca^{2+}-induced activation [76].

It has also been reported that, besides enhancing electrogenic Ca^{2+} uptake, spermine inhibits Na^{+}-independent Ca^{2+} efflux if the rate of efflux is limited by dissociation of Ca^{2+} from the carrier [72]. This difference correlates with the phosphate concentration present in the medium in the different experiments, and was ascribed to the increase in phosphate accumulation by mitochondria induced by spermine [61]. At high phosphate concentrations, spermine strongly enhances phosphate transport into the matrix with a consequent inhibition of Ca^{2+} efflux [73].

Ca^{2+} transport in brain mitochondria has also been proposed to be regulated by adenine nucleotides, in particular by ADP, as well as by their translocase [51,96,97]. In the presence of ADP and spermine, brain mitochondria sequester Ca^{2+} down to the normal cellular free Ca^{2+} levels, suggesting that this effect is due to a modulation of the interactions among the surface positive charges of the adenine nucleotide translocator in its M-state (closed transition pore) and the membrane anionic phospholipids. The result is a stimulation of electrogenic Ca^{2+} uptake and, to a lesser extent, inhibition of electroneutral Ca^{2+} efflux. Spermine apparently exerts its modulatory action by acting on the surface charge of the adenine nucleotide translocator which enhances Ca^{2+} dissociation from its uniporter by favoring uptake, and the dissociation from the antiporter by favoring the retention [96].

The effect of spermine as a potent activator of mitochondrial Ca^{2+} uptake was also confirmed by elegant studies on rat heart, liver and kidney mitochondria by using the activities of the Ca^{2+}-sensitive intramitochondrial dehydrogenases (pyruvate, NADH isocitrate and 2-oxoglutarate dehydrogenases) as probes for matrix Ca^{2+} [95]. In this regard, it has been demonstrated that spermine leads to a decrease in the $K_{0.5}$ values for extramitochondrial Ca^{2+} in the activation of these enzymes.

It is important to note that studies performed by Kroner [94] confirmed the effects of spermine on Ca^{2+} uptake by liver mitochondria, but reported a lack of effect on heart mitochondria. This is at variance with other reports [50,95] that suggest that this is a widespread phenomenon in mammalian mitochondria. However, it must be stressed that some authors who had previously proposed the allosteric mechanism subsequently raised some doubt regarding this mechanism since the enhanced accumulation of Ca^{2+} was observed to be independent of the uptake rate [81]. These authors proposed that the inhibition or acceleration of Ca^{2+} uptake depends on the spermine load relative to the spermine concentration in the incubation medium.

Of special significance is the stimulation of the uniporter by Ca^{2+} itself [98–100], which was found to persist for minutes after removal of Ca^{2+} [98–100]. This "hysteretic" stimulatory behaviour was proposed to reflect a rebounding effect, and was shown to be blocked by spermine [76].

5.4. The polyamine binding site regulating Ca^{2+}

While disputed by some authors [81], numerous observations indicating an allosteric mechanism involved in the interactions between polyamines and the Ca^{2+} transport system raised interest in individualizing the regulatory site of this mechanism. All of the investigations regarding the role of polyamines in Ca^{2+} transport arrived at the circumstantial conclusion that spermine indirectly influences the operation of the Ca^{2+} uniporter by binding to low affinity sites [82,85]. Different laboratories have identified two groups of low affinity binding sites [56,57,82,83,85] which exhibit similar characteristics except for a markedly general low affinity and a more marked cooperativity when a K^+ medium was employed [85] instead of a sucrose-based medium [56,57,83].

These two groups of sites are proposed to display distinct structural requirements, with some authors suggesting that they are identifiable as phospholipid head groups [101,102], while other investigations proposed a proteinaceous structure [56,57,83]. As mentioned above, two types of specific polyamine binding sites, named S_1 and S_2, have been characterized, with spermine and spermidine binding to both sites [56,57], and putrescine binding only to S_2 [57].

A comparison between the free cytosolic spermine concentration and the reported affinities of these binding sites suggests that only the S_1 site operates under physiological conditions, while the S_2 site becomes available most likely when there is an increase in the polyamine concentration, for example as observed in neoplastic tissues [57]. These observations suggest that stimulation of Ca^{2+} uptake by spermine involves its binding to the S_1 site, which is characterized by very high hydrophilicity [57]. This peculiarity of the S_1 site, its localization in the bulk of the membrane thickness [62], and the typical saturation curves exhibited by bound polyamines [56,57] strongly suggest that the binding takes place on a proteinaceous structure. However, the dimensions of the spermine molecule and its accentuated flexibility [103] are compatible with the co-participation of the surface polar head groups of membrane phospholipids.

Fig. 1 reports the energy profile for spermine transport showing two asymmetric energy barriers with different heights. The S_1 site is located in the energy well between the two peaks, at 1/8 of the distance from the external side [62].

It was very recently reported that the reduction in the external Ca^{2+} concentration induced by spermine is reversed by the antitumor drug methylglyoxal-bis-(guanlyhydrazone) (MGBG) [104]. This study also demonstrated that the S_1 binding site for spermine is in close proximity to both Ca^{2+} transport systems [104]. Spermine most likely exhibits its effect on Ca^{2+} transport systems when the polyamine acts from the outside, i.e., in the direction of its uniporter channel. In fact, in order to exert its action, spermine must reach the S_1 binding site. The possibility that spermine can act from the inside, that is after its transport into the matrix, is less probable as it would have to overcome a very high

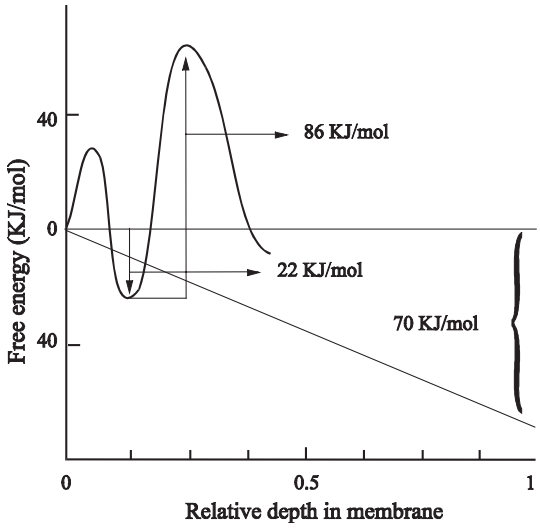

Fig. 1. Free energy of the spermine transport channel at 180 mV. The height of the first peak is not known; the height of the second peak was calculated from J_{max} per milligram of protein by use of the transition state theory and the number of high-affinity binding sites per milligram of protein determined in a previous report [56]. The depth of the well for the binding site was calculated from the K_m value at 180 mV. (Reprinted from Biochemistry [62] with permission from the American Chemical Society).

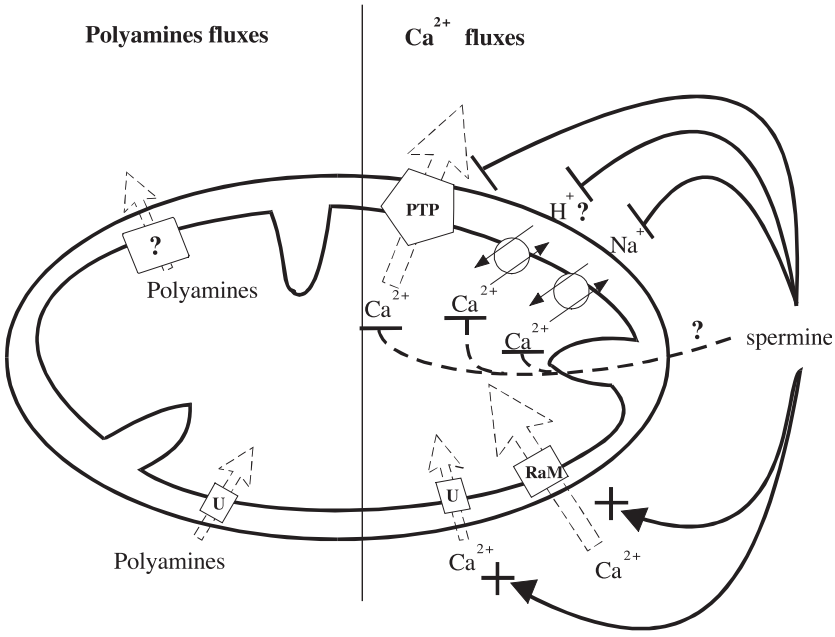

Fig. 2. Schematic representation of the Ca^{2+} and polyamine transport pathways in mitochondria. U, uniporter; rU, reversed uniporter; RaM, rapid uptake mode, Ex, electroneutral exchangers; PTP, permeability transition pore. The effect of spermine on Ca^{2+} transport is also depicted. In the figure we indicate RAM and the Ca^{2+} uniporter as separate transport pathways; however, it is not clear if they operate by separate molecular mechanisms. Polyamines efflux takes place by the reversed uniporter activity at low $\Delta\Psi$ values or by an electroneutral exchange mechanism [59,62,63]. Although not indicated in the figure, it is important to note that the S_1 binding site of polyamines, which is located in the uniporter and responsible for their effects on Ca^{2+} fluxes, is in close proximity to all the Ca^{2+} transporters.

energy barrier in this direction (see Figs. 1 and 2). However, massive electrophoretic accumulation of the polyamine and subsequent provocation of a decrease in $\Delta\Psi$ with a lowering of the energy barrier could trigger spermine efflux by the reversed uniporter, and thereby influence Ca^{2+} fluxes from within. Nevertheless, there is no evidence for this type of efflux. Instead, spermine efflux probably takes place by an electroneutral exchange mechanism [61] that does not involve the S_1 site. The study on MGBG also suggests that while S_1 does not represent the binding site for the drug, it is located on the same protein [105]. The reverse effect induced by MGBG has been explained as the result of an allosteric mechanism similar to that of spermine, which, however, inhibits both Ca^{2+} transporters [104,105].

This property supports the proposal that MGBG induces a deficiency in energy metabolism which seriously affects the activity of all the Ca^{2+}-dependent dehydrogenases.

5.5. Ca^{2+} uptake and MPT regulation

The intracellular concentration of spermine and its precursors has been estimated to be in the millimolar range [51,106–108]. However, binding to cytosolic macromolecules and interactions with subcellular organelles substantially reduce their free concentration to 25–100 μM [107]. Rustenbeck et al. [85] reported a total spermine concentration in hepatocytes of 460 μM. Other determinations measured similar total spermine concentrations in liver tissue, but estimated a lower limit of free spermine, i.e., 10 μM [109]. The concentration of spermine able to inhibit the permeability transition was found at best moderate at 1 mM and maximal at 20 mM, while the enhancement in Ca^{2+} accumulation was maximal below 1 mM [69]. Assuming that the above-reported concentrations of 10 and 460 μM represent the physiologically relevant free and total spermine concentrations in hepatocytes, the authors suggest that spermine may regulate the rate and extent of Ca^{2+} transport but is unlikely to act as a modulator of the MPT [85]. However, this very sound proposal must be considered in light of other investigations reporting different critical concentrations for spermine inhibition of the MPT. Lapidus and Sokolove [51], who utilized spermine under conditions more likely to yield an effect, reported an I_{50} value of 380 μM for inhibition of the MPT. Under similar conditions Tassani et al. [84] reported total MPT inhibition by 25 μM spermine and 100 μM spermidine. While these concentrations were calculated in the context of very low ionic strength media which favor spermine binding, participation of the polyamine in inhibiting the MPT cannot be completely excluded.

However, the considerations of Rustenbeck et al. [85] are in line with the proposal that, together with ADP, spermine binding may permit Ca^{2+} uptake by mitochondria during cytosolic Ca^{2+} oscillations and contribute to reset the physiological Ca^{2+} concentration without inducing the permeability transition. In this view, the presence of spermine would influence Ca^{2+} release from mitochondria induced by Ca^{2+} uptake, a phenomenon considered responsible for cytosolic Ca^{2+} oscillations [28,110].

One must also consider that when polyamine concentrations are elevated, such as in neoplastic tissues or regenerating liver [49,111], the S_2 site begins to become available [57]; under these conditions the protective effect of spermine and spermidine on MPT induction may assume importance. It is well known that the MPT is strictly correlated with the pro-apoptotic pathway due to the release of cytochrome *c* and AIF through the mitochondrial transition pore [112]. Interestingly, tumor cell mitochondria frequently have a high threshold for induction of the MPT [113]. The possibility that abnormally high polyamine concentrations in the cytosol can have an anti-apoptotic effect must be taken into account.

A particular consideration has to be made with regard to putrescine. At about 10 μM, the total putrescine level in liver is much lower than that of the other polyamines [108], and its free concentration is less than 1 μM. These values suggest that binding of putrescine to the S_2 site (the only site possible) cannot be physiologically relevant. However, putrescine binding may gain physiological meaning when ornithine decarboxylase, the enzyme that catalyzes the first step in polyamine synthesis, is overexpressed, as is observed in L1210 and hepatoma cell lines [114,115]. This results in a dramatic increase in cellular putrescine levels up to a concentration in the same range as its S_2 dissociation constant [57]. Putrescine may thereby function as a modulator of MPT induction as well as an activator of Ca^{2+} transport under these conditions. Fig. 2 reports a summarizing representation of the effect of spermine on Ca^{2+} transport.

5.6. Conclusions and future perspectives

The initial observation in 1984 [67] that spermine enhances Ca^{2+} accumulation in liver mitochondria was considered as a paradigmatic shift for several reasons. In fact, all of the many earlier investigations dealing with the transport of Ca^{2+} in mitochondria had emphasized that all species of isolated mitochondria are able to buffer the exogenous Ca^{2+} concentration to a level about one order of magnitude higher than that physiologically present in the cytosol. Thus, Ca^{2+} transport by mitochondria was considered as an interesting "in vitro" phenomenon with little physiological relevance if not to accumulate large amounts of the cation in the matrix. The fact that spermine is able to shift the exogenous Ca^{2+} concentration to a physiological level confers to mitochondria an important role in the regulation of the free cytoplasmic Ca^{2+} concentration in the cell and mitochondrial matrix. Until 1989, only a few reports had dealt with the effects of polyamines at the mitochondrial level and practically nothing was known about the constitutive presence of polyamines within the mitochondria, their binding to the membranes and transport into the matrix. In subsequent years, thanks to a series of technical developments and new conceptual information on both mitochondrial Ca^{2+} transport and polyamine–mitochondria interactions, the interest of a number of investigators was directed toward different aspects of this topic. In addition to studying the effect of polyamines as physiological regulators of cytosolic Ca^{2+} levels, their effects on mitochondrial Ca^{2+} transport were also evaluated in the light of other phenomena such as energy transduction, control of ion channels, and apoptosis. However, at present, none of the mitochondrial proteins involved in Ca^{2+} transport and polyamine binding have been purified, cloned, or expressed in large enough quantities for examination of their crystallographic structure. Taking into account the considerable interest revived in these years regarding the involvement of Ca^{2+} and polyamines in mitochondrial-mediated apoptosis, molecular comprehension of the process represents a logical future objective.

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