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Yeast Mitochondrial Calcium Uptake: Regulation by Polyamines and Magnesium Ions

Tatyana V. Votyakova,[1] Elena N. Bazhenova,[1] Renata A. Zvjagilskaya[1]

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Spermine, spermidine, and magnesium ions modulate the kinetic parameters of the Ca^{2+} transport system of *Endomyces magnusii* mitochondria. Mg^{2+} at concentrations up to 5 mM partially inhibits Ca^{2+} transport with a half-maximal inhibiting concentration of ~ 0.5 mM. In the presence of 2 mM $MgCl_2$, the $S_{0.5}$ value of the Ca^{2+} transport system increases from 220 to 490 μM, which indicates decreased affinity for the system. Spermine and spermidine exert an activating effect, having half-maximal concentrations of 12 and 50 μM, respectively. In the case of spermine, the $S_{0.5}$ value falls to 50–65 μM, which implies an increase in the transport system affinity for Ca^{2+}. Both Mg^{2+} and spermine cause a decrease of the Hill coefficient, giving evidence for a smaller degree of cooperativity. Spermine and spermidine enable yeast mitochondria to remove Ca^{2+} from the media completely. In contrast, Mg^{2+} lowers the mitochondrial buffer capacity. When both Mg^{2+} and spermine are present in the medium, their effects on the $S_{0.5}$ value and the free extramitochondrial Ca^{2+} concentration are additive. The ability of spermine and Mg^{2+} to regulate yeast mitochondrial Ca^{2+} transport is discussed.

KEY WORDS: Yeast mitochondria; calcium uptake; polyamines; magnesium ions.

INTRODUCTION

Ca^{2+} ions are known to regulate various processes in the cell. For this reason, the cell compartments participating in the intracellular Ca^{2+} transport are of great interest. Mitochondria possessing a system of active Ca^{2+} transport have attracted the close attention of several research groups (for a review, see Gunter and Pfeiffer, 1990). The relatively low affinity of the mitochondrial transport system for Ca^{2+} and the lack of evidence for activation of Ca^{2+} efflux by extracellular signals allowed some authors to conclude that the Ca^{2+} transport system of mitochondria was of local significance, i. e., that it was mainly involved in the regulation of the activity of Ca^{2+}-dependent dehydrogenase and electron flow rates (McCormack, 1985; Pietrobon *et al.*, 1990). However, there is convincing evidence that mitochondrial Ca^{2+} transport is considerably activated by naturally occurring polyamines (Nicchitta and Williamson, 1984; Lenzen *et al.*, 1986: Jensen *et al.*, 1987; Kröner, 1988; Rottenberg and Marbach, 1990a) and ADP (Rottenberg and Marbach, 1989, 1990b) present in physiological concentrations. These agents increase both the uniporter affinity for Ca^{2+} and the buffering capacity of animal mitochondria, thus suggesting that mitochondrial participation in animal cell Ca^{2+} homeostasis should be reevaluated. We have shown that coupled yeast mitochondria possess an effective energy-dependent Ca^{2+} transport system (Leikin *et al.*, 1987). Moreover, the system is very sensitive to spermine, the half-maximal concentration being 2–20 times lower than those causing the same effect on animal mitochondria (Votyakova *et al.*, 1990).

Mg^{2+} ions are known to play an important role in the regulation of membrane permeability (Masini *et al.*, 1983). In particular, Mg^{2+} can inhibit the Ca^{2+} uniporter in animal mitochondria (Robertson *et al.*, 1982; Favaron and Bernardi, 1985). At the present

[1] A. N. Bach Institute of Biochemistry, Russian Academy of Sciences, Moscow 117071, Russia.

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moment there are no relevant data on the Mg^{2+} action on yeast mitochondrial Ca^{2+} transport.

In this paper we report studies on the influence of spermine and Mg^{2+} on the Ca^{2+} uptake and distribution in yeast mitochondria, and discuss the mechanism of their action.

MATERIALS AND METHODS

Cultivation of *Endomyces magnusii* yeast cells, isolation of mitochondria, and measurements of respiration activities were carried out as described in (Leikin *et al.*, 1987). Ca^{2+} uptake by mitochondria was monitored with murexide as indicator (Scarpa, 1972) using a Hitachi-557 spectrophotometer. Initial rates of Ca^{2+} uptake and free Ca^{2+} concentrations were calculated on the basis of internal calibration obtained from additions of known amounts of Ca^{2+} to the sample. The presence of the polyamines did not affect this calibration. The incubation medium contained 0.3 M mannitol, 10 mM Tris-phosphate, 10 mM Hepes, pH 7.4, 16 mM pyruvate, 4 mM malate, 50 μM murexide, and 0.5 mg/ml mitochondrial protein. The protein was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1979). All the experiments were repeated 3–6 times using different mitochondrial preparations with similar results. The kinetic data were processed in terms of the Hill equation, and the parameters ($S_{0.5}$, V_{max}, H the Hill coefficient) were evaluated by the nonlinear regression method. The indicated values were obtained from the plots in which the correlation coefficients were not less than 0.96 and most usually 0.98. The parameters calculated for different preparations of yeast mitochondria showed the following deviations: $S_{0.5}$ was within 20%, H was within 10%, and V_{max} varied in 1.5–2 times. The computer program was kindly provided by Dr. D. R. Davydov.

RESULTS

According to our earlier studies (Leikin *et al.*, 1987; Votyakova *et al.*, 1990), the $S_{0.5}$ value for the Ca^{2+} yeast mitochondrial transport system is within the range of 150–300 μM, which determined the concentrations used in the present experiments.

Figure 1 illustrates plots of the initial rate of Ca^{2+} uptake by energized yeast mitochondria vs. the concentration of polyamines. Spermine shows the most

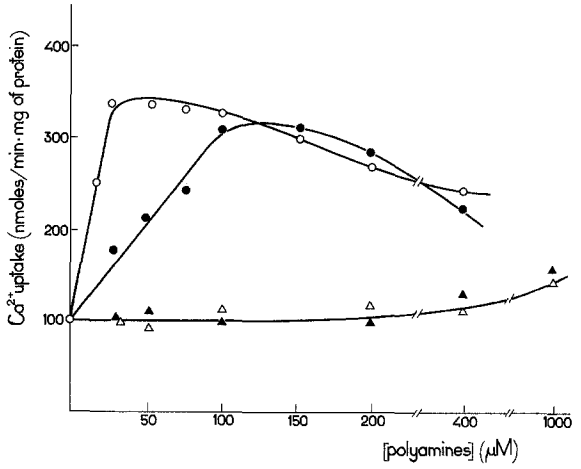

Fig. 1. Dependence of the initial rate of Ca^{2+} uptake by the yeast mitochondria on polyamine concentration in the incubation medium. For the incubation medium, see Materials and Methods. ○, spermine; ●, spermidine; △, putrescine; ▲, cadaverine. The result of a typical experiment is shown in this and in the following figures.

pronounced activation effect on Ca^{2+} uptake with half-maximal and maximal concentrations of 12 and 25–50 μM, respectively; at concentrations above 100 μM a partial inhibition of Ca^{2+} uptake takes place. Spermidine shows a half-maximal effect at about 50 μM. The maximum increase of the Ca^{2+} uptake rate in the presence of spermidine is almost the same as in the case of spermine, and a slight inhibition is also observed at concentrations exceeding 200 μM. Putrescine and cadaverine exert a slight influence on the transport, showing a 1.5-fold increase of the uptake rate at concentrations above 400 μM.

We earlier reported that *Endomyces magnusii*

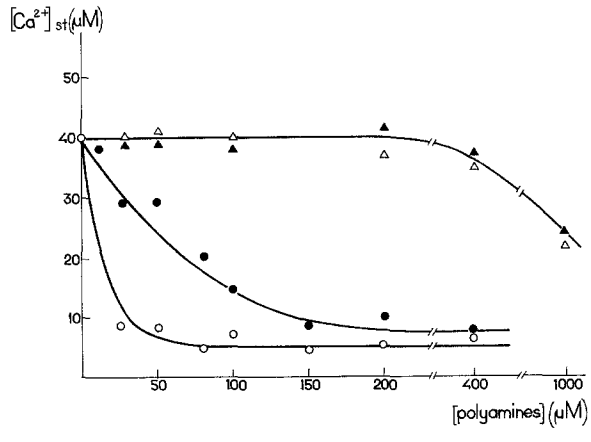

Fig. 2. Effect of polyamines on the steady-state level of free extramitochondrial Ca^{2+}. For the incubation medium, see Materials and Methods. ○, spermine; ●, spermidine; △, putrescine; ▲, cadaverine.

mitochondria can accumulate no more than 50–70% of the Ca^{2+} added at a protein concentration of 0.5 mg/ml without any effectors (Leikin *et al.*, 1987). Figure 2 shows the decreasing effect of the polyamines on the extramitochondrial steady-state Ca^{2+} concentration. Spermine at concentrations of 25–50 μM reduce the steady-state Ca^{2+} level to 5–9 μM. Spermidine at 100–150 μM is less effective, providing the 9–10 μM level of the steady-state [Ca^{2+}]. The effect of both polyamines is equal at concentrations about 400 μM. Putrescine and cadaverine decrease the steady-state Ca^{2+} level at concentrations higher than 400 μM, but even at a 1 mM concentration of the diamines the residual [Ca^{2+}] is not lower than 25 μM.

Figure 3 shows plots of the initial rate of Ca^{2+} uptake by yeast mitochondria vs. Mg^{2+} concentration. An increase in the Mg^{2+} concentration results in a gradual decrease of the initial rate of Ca^{2+} uptake, with the 50% inhibition at 0.5 mM and the maximal one at 2–5 mM. The initial rate of Ca^{2+} uptake decreased only by 50–70%. In the presence of spermine, Mg^{2+} also partially inhibits Ca^{2+} uptake (Fig. 3), the character of the plot of the initial rate of Ca^{2+} uptake vs. Mg^{2+} concentration remaining essentially unchanged, i.e., the effects of Mg^{2+} and spermine are additive.

The residual Ca^{2+} concentration increases from 50 to 90–100 μM upon Mg^{2+} addition, reaching a plateau at [$MgCl_2$] > 1 mM (Fig. 4). The addition of spermine lowers the residual [Ca^{2+}] in the whole range of the Mg^{2+} concentrations investigated.

We have studied the effect of Mg^{2+} and spermine

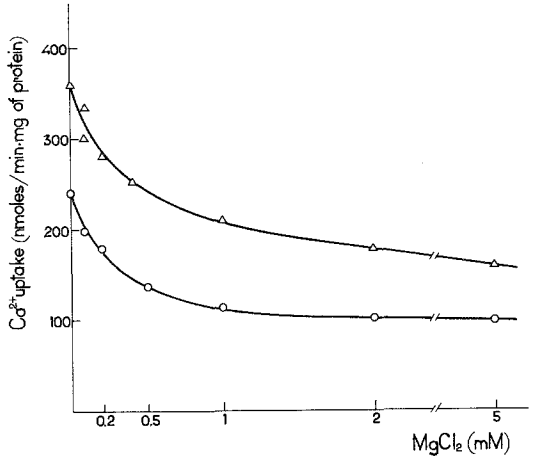

Fig. 3. Dependence of the initial rate of Ca^{2+} uptake on Mg^{2+} concentration. For the incubation medium, see Materials and Methods. ○, control mitochondria; △, 50 μM of spermine.

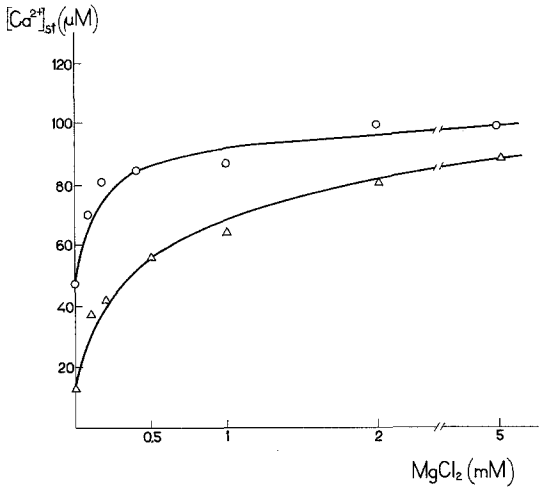

Fig. 4. Effect of Mg^{2+} on the steady-state level of free extramitochondrial Ca^{2+}. For the incubation medium, see Materials and Methods. ○, control mitochondria; △, 50 μM of spermine.

on the dependence of the initial rate of Ca^{2+} uptake on Ca^{2+} concentration. Since the plots were sigmoidal, the $S_{0.5}$, V_{max}, and H values were calculated using the Hill equation. It should be noted that the H values thus obtained are formal and can be considered as a qualitative estimation of the process cooperativity.

In the absence of spermine and Mg^{2+} (Fig. 5), Ca^{2+} uptake is slow at Ca^{2+} concentrations below 50 μM. An increase in Ca^{2+} concentrations results in a sharp rise of the initial rate of Ca^{2+} uptake, and the curve exhibits significant sigmoidicity (H about 3.0). The yeast Ca^{2+} transport system reveals low affinity for the ion, the $S_{0.5}$ and V_{max} values being 220 μM and 520 nmol/min/mg protein, respectively (Fig. 5). In *Endomyces magnusii* mitochondria, in contrast to animal mitochondria (Gunter and Pfeiffer, 1990), high calcium concentrations do not cause opening of nonspecific pores in the inner membrane (so-called Ca^{2+}-dependent permeability transition). In our previous experiments we observed release of accumulated calcium only after the addition of uncoupler or ionophore A23187 (Leikin *et al.*, 1987).

50 μM spermine shifts the plot toward lower Ca^{2+} concentrations, decreasing the $S_{0.5}$ value to 50–65 μM and thus increasing the affinity for Ca^{2+} ions. Moreover, the plot becomes less sigmoidal, with the Hill coefficient equal to ~ 2. An essential feature of spermine is its stimulating effect at Ca^{2+} concentrations lower than 300 μM, but at higher Ca^{2+} concentrations a moderate inhibition is observed (Fig. 5). $MgCl_2$ (2 mM) reduces the initial

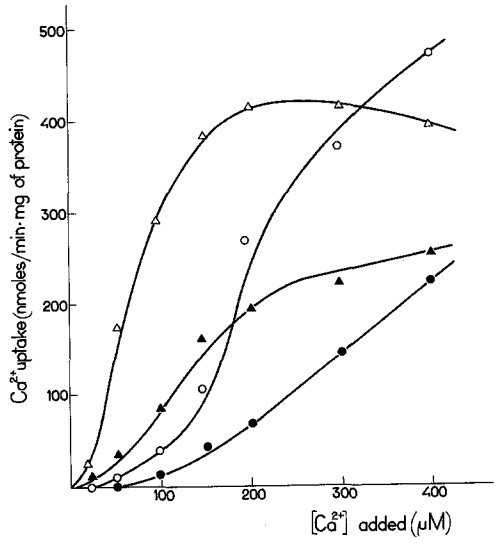

Fig. 5. Dependence of the initial rate of calcium uptake by the mitochondria on Ca^{2+} concentration in the incubation medium. For the incubation medium, see Materials and Methods. ○, control mitochondria ($S_{0.5} = 220\,\mu M$, $H = 3.2$, $V_{max} = 520$ nmol/min/mg protein); △, 50 μM of spermine ($S_{0.5} = 65\,\mu M$, $H = 2.3$, $V_{max} = 450$ nmol/min/mg protein); ●, 2 mM $MgCl_2$ ($S_{0.5} = 490\,\mu M$, $H = 2.4$, $V_{max} = 630$ nmol/min/mg protein); ▲, 50 μM of spermine and 2 mM of $MgCl_2$ ($S_{0.5} = 135$, $H = 2.1$, $V_{max} = 275$ nmol/min/mg protein).

rates of Ca^{2+} uptake. The corresponding sigmoidal plot does not reach its inflection point even at maximal Ca^{2+} concentration. Thus, the parameters calculated for this curve (Fig. 5) can be considered as tentative approximations. The low transport rate and the high value of $S_{0.5}$, however, provide evidence for a decrease in the system's affinity for Ca^{2+} ions. When both Mg^{2+} ions and spermine are present in the medium, the plot appears between those for the modulators operating separately (Fig. 5).

The influence of Mg^{2+} and spermine upon the residual extramitochondrial Ca^{2+} concentration as a function of the initial Ca^{2+} concentration is shown in Fig. 6. In the absence of these agents, the dependence is biphasic. Mitochondria maintain the concentration of residual Ca^{2+} at about 20 μM, when the added amount of the ion is lower than 100 μM, and a linear rise of residual [Ca^{2+}] takes place at higher initial concentrations. Spermine lowers the steady-state [Ca^{2+}] to 5–8 μM at initial Ca^{2+} concentrations up to 200 μM. This suggests that in the presence of spermine and at Ca^{2+} concentrations up to 200 μM, yeast mitochondria may be an effective buffer for Ca^{2+} ions. At higher concentrations of Ca^{2+} the mitochondria are not able to maintain a low Ca^{2+} level in the medium.

In the presence of 2 mM $MgCl_2$ the residual [Ca^{2+}] gradually increases from 40 to 150 μM, reflecting the lowering of the buffer capacity of mitochondria. Under these conditions mitochondria are able to take up only 30–55% of the calcium added. Nevertheless, Mg^{2+} ions fail to prevent the generation of Ca^{2+} membrane gradient, since ionophore A23187 releases accumulated Ca^{2+} (data not shown). The effect of Mg^{2+} on the parameters of Ca^{2+} transport system cannot be explained by variations in the degree of oxidative phosphorylation coupling, since the value of respiratory control is not affected at $MgCl_2$ concentrations up to 12 mM (Zvjagilskaya *et al.*, 1987).

Upon addition of both spermine and Mg^{2+} the plot resembles the dependence when only Mg^{2+} is present, but the Ca^{2+} steady-state level is lower, indicating the formation of a higher gradient of calcium concentrations on the yeast mitochondrial membrane.

DISCUSSION

The Ca^{2+} transport system of *Endomyces magnusii* mitochondria, despite its lower affinity for the ion, has many common features with that of animal mitochondria, which are much more extensively studied to date (e.g., see Gunter and Pfeiffer, 1990). Therefore, in our

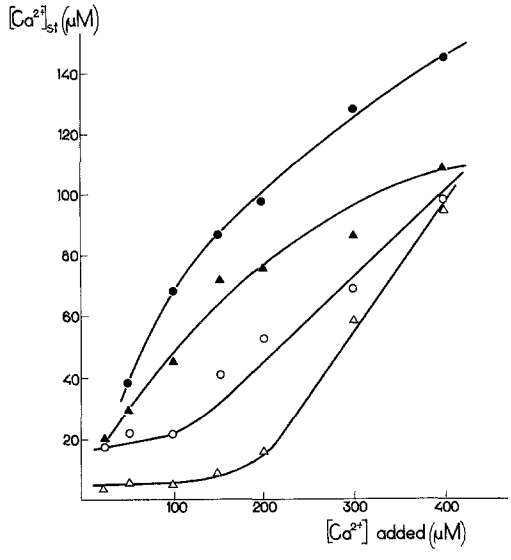

Fig. 6. Dependence of the steady-state free extramitochondrial Ca^{2+} on initial Ca^{2+} concentration in the incubation medium. For the incubation medium, see Materials and Methods. ○, control mitochondria; △, 50 μM of spermine; ●, 2 mM $MgCl_2$; ▲, 50 μM of spermine and 2 mM of $MgCl_2$.

opinion it is proper to compare Ca^{2+} transport systems of these taxonomically diverse and unrelated groups.

We have studied the influence of aliphatic polyamines and Mg^{2+}, both obligatory components of all types of cells, on Ca^{2+} transport in yeast mitochondria. Among polyamines, spermine, possessing 4 amino groups, shows the most profound activation effect on the yeast Ca^{2+} transport system. The triamine spermidine is less effective, and the diamines cadaverine (C_5) and putrescine (C_4) have little effect on the parameters studied. Polybasicity seems to be the main structural feature of amines possessing activation properties at low concentrations. The effect of spermine and spermidine on the ion uptake rate of the yeast mitochondria is the same at a concentration of 100 μM or higher; i.e., at high concentrations of polyamines the tetrabasicity of spermine is of minor importance. Variations in dimensions and shapes of the molecules may also play a certain role.

The activating effect of spermine and spermidine on the yeast mitochondria is essentially higher as compared with the action on animal mitochondria, the half-maximal effective concentration being 4 (Lenzen *et al.*, 1986) or even 10 (Nicchitta and Williamson, 1984; Kröner, 1988) times lower. Spermidine was less active than spermine even at the maximal concentration used (Kröner, 1988). We observed a 3–3.5-fold rise of the Ca^{2+} influx rate as a result of spermine action, whereas in many papers on animal mitochondria (Lenzen *et al.*, 1986; Rottenberg and Marbach, 1990) only 1.5–2-fold enhancement was reported. The data by Kröner (1988) is a notable exception: this author showed a tenfold increase in Ca^{2+} uptake rate at high spermine concentration (1.2 mM).

Presently, it is assumed that polyamines can be effectively bound to the membrane surface and/or Ca^{2+} carrier acid groups (Rottenberg and Marbach, 1990a; Gunter and Pfeiffer, 1990). Toninello *et al.* (1988) quantitatively estimated spermine binding with rat liver mitochondrial membrane. Such binding can substantially change the calcium influx rate either as a result of changes in uniporter activity or as a consequence of charge-screening effects. Kröner was the first who reported the action of spermine as an allosteric regulation. His suggestion resulted from a decrease in the K_m and H values (Kröner, 1988). However, the spermine concentrations used in his experiments exceeded the calcium concentration several times; therefore, the affinity of spermine for the assumed regulatory site would be low, a property not typical of most allosteric regulators (Kurganov, 1982). Furthermore, spermine is known to infuence various processes, such as peroxidation of acid lipids (Tadolini, 1988), lateral mobility of membrane proteins (Schindler *et al.*, 1980), activity of lipid-dependent proteins (Wojcinkiewicz and Fain, 1988, Smith and Snyderman, 1988; Moruzzi *et al.*, 1987), etc., thus exhibiting rather nonspecific effects.

On the other hand, Rottenberg suggested (1990a) that at low (micromolar) concentrations of Ca^{2+} the rate-determining step of the ion transport is diffusion of adsorbed calcium toward the uniporter along the membrane surface. According to this hypothesis, spermine being adsorbed on the surface facilitates Ca^{2+} diffusion. By treating the decrease in the K_m as an increase of local [Ca^{2+}] available for the uniporter, this model fails to explain the observed decrease in the Hill coefficient and inhibition of calcium efflux pathways (Karadzhov *et al.*, 1988; Rottenberg and Marbach, 1990a). At the concentrations used in our work, when a sufficient amount of calcium is present in the bulk phase, surface diffusion could hardly be regarded as a limiting factor.

This brief analysis of the literature and our data allowed us to conclude that the polyamines studied modify the membrane rather nonspecifically due to the adsorption. Nevertheless, the effect, being allosteric in nature, is not a result of interaction with a specific polyamine regulatory site.

The considerably higher sensitivity of the yeast mitochondrial Ca^{2+} transport system to polyamines observed in our experiments might be due to the different structure of the surface parts of the carrier. Since ruthenium red, interacting with the surface of the animal mitochondrial uniporter, fails to suppress the yeast mitochondrial Ca^{2+} transport (Leikin *et al.*, 1987), the difference between the surface structure of the animal and yeast mitochondria uniporters might be quite substantial.

Although many reports were devoted to the inhibition of Ca^{2+} transport by magnesium ions (for a review, see Diwan, 1987) the mechanism of this phenomenon is unclear. Mg^{2+} can be transported into the heart mitochondria by a separate pathway independently from Ca^{2+} (Brierly *et al.*, 1987), although Mg^{2+} influx in liver (Kun, 1976) and brain (Rugolo and Zoccarato, 1984) mitochondria is suppressed by specific Ca^{2+}-uniporter inhibitors. Therefore, the mechanism of Mg^{2+} inhibition of Ca^{2+} transport is tissue specific, being formally competitive in brain and liver mitochondria, and noncompetitive in heart mitochondria. The characteristic

feature of magnesium action is incomplete inhibition of Ca^{2+} uptake, which varies from 50 to 80% depending on the tissue (Favaron and Bernardi, 1985).

Our experiments show that Mg^{2+} ions at physiological concentrations partially suppress Ca^{2+} uptake (about 60%) by *Endomyces magnusii* mitochondria. Analysis of the kinetic curve indicates that Mg^{2+} ions reduce the affinity transport system for Ca^{2+}, due likely to Ca^{2+} displacement from the carrier. A high value of the calculated V_{max} may provide evidence for a competitive mode of inhibition.

Under simultaneous action of Mg^{2+} and spermine, their effects on the rate of Ca^{2+} uptake by yeast mitochondria are additive. Some authors supposed that these effectors compete for the same regulatory site on the uniporter (Jensen *et al.*, 1989). However, taking into account that Mg^{2+} acts at millimolar concentrations, it is unlikely that Mg^{2+} is a specific allosteric regulator of the Ca^{2+} uniporter.

An important feature of spermine and Mg^{2+} is their influence on the steady-state Ca^{2+} level. In agreement with the literature data (e.g., see Nicchitta and Williamson, 1984), spermine improves the buffer capacity of yeast mitochondria, whereas magnesium acts as an antagonist. These changes in residual Ca^{2+} concentration may be caused both by thermodynamic and kinetic reasons. The action of spermine on rat liver mitochondria is accompanied by a slight but reliable rise in the membrane potential (about 2–3 mV) (Nicchitta and Williamson, 1984; Lenzen *et al.*, 1986). We also observed a similar effect for yeast mitochondria (data not shown). For animal mitochondria, the inhibition of Ca^{2+} efflux pathways (Karadzhov *et al.*, 1988; Rottenberg and Marbach, 1990a) in the presence of spermine has also been shown, which also led to diminution of external stationary $[Ca^{2+}]$. At present, we can rely only on data concerning Ca^{2+} influx, since no effective inhibitors of yeast mitochondrial Ca^{2+} uptake have been found to date.

On the other hand, it was shown that Mg^{2+} reduced the mitochondrial membrane potential both in the absence and in the presence of spermine and decreased the rate of Ca^{2+} uptake, thus deteriorating the buffer capacity (Lenzen *et al.*, 1986).

Considering the physiological role of spermine and Mg^{2+} ions in the yeast mitochondrial Ca^{2+} transport system, we can speculate that spermine promotes a rapid lowering of the free Ca^{2+} concentration in the cytoplasm and ensures a low steady-state level of Ca^{2+}. Magnesium ions seem to play a protective role, preventing accumulation of excess amounts of Ca^{2+} and membrane deenergizing upon mitochondria overload. Thus, the spermine-induced activation of Ca^{2+} uptake by mitochondria and Mg^{2+} inhibition of this process might be regulatory mechanisms of Ca^{2+}-dependent metabolisms of the yeast cell.

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