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ACCELERATED PUBLICATION

The Ca^{2+}-Transport System of Yeast (*Endomyces magnusii*) Mitochondria: Independent Pathways for Ca^{2+} Uptake and Release

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Abstract—Some features of the Ca^{2+}-transport system in mitochondria of the yeast *Endomyces magnusii* are considered. The Ca^{2+} uniporter was shown to be specifically activated by low concentrations of physiological modulators such as ADP, NADH, spermine, and Ca^{2+} itself. The Na^+-independent system responsible for Ca^{2+} release from Ca^{2+}-preloaded yeast mitochondria was characterized. The rate of the Ca^{2+} release was proportional to the Ca^{2+} load, insensitive to cyclosporin A and to Na^+, inhibited by La^{3+}, TPP^+, P_i, and nigericin, while being activated by spermine. We conclude that Ca^{2+} release from preloaded *E. magnusii* yeast mitochondria is mediated by a Na^+-independent pathway, very similar to that in mitochondria from nonexcitable mammalian tissues. A scheme describing an arrangement of the Ca^{2+} transport system of yeast mitochondria is proposed.

Key words: yeast, *Endomyces magnusii*, mitochondria, calcium transport, regulation, ADP, NADH, calcium ions, Na^+-independent calcium release

Ca^{2+} plays an important role in the control of basic physiological functions by serving as an intracellular second messenger [1, 2]. The Ca^{2+}-transport system is implicated in the signal transduction pathway from the cytoplasm to the mitochondrial matrix to stimulate respiration and oxidative phosphorylation [3, 4]. This transport system is now receiving increasing attention as playing a central role in the mechanism of cell apoptosis [5].

The calcium transport system of mammalian mitochondria is composed of two distinct components: 1) the Ca^{2+} uniporter, responsible for both energy-dependent Ca^{2+} uptake into the mitochondrial matrix [6, 7] and Ca^{2+} release caused by a drop in the transmembrane potential [8] or by a decrease in the Ca^{2+} concentration in incubation medium [9]; 2) specific pathways for Ca^{2+} release known as the Na^+-dependent pathway (typical for mitochondria from excitable tissues) and the Na^+-independent pathway (functional in mitochondria from nonexcitable animal tissues) [6, 8]. The Ca^{2+}-uptake system is specifically inhibited by ruthenium red (RR) [10, 11] and lanthanides [10] and activated by various natural modulators including ADP [12], polyamines [13, 14], and Ca^{2+} itself [15]. The Na^+-dependent pathway for Ca^{2+} efflux is inhibited by Mg^{2+}, Mn^{2+}, diltiazem, RR, and TPP^+; the Na^+-independent pathway is sensitive to Sr^{2+}, Mn^{2+}, and TPP^+ [6].

Until recently, it was a general belief that yeast mitochondria, in contrast to mammalian mitochondria, lack an efficient Ca^{2+}-transport system of any physiological relevance [16, 17]. However, in our laboratory we found that tightly coupled *E. magnusii* mitochondria have a highly efficient system for energy-dependent Ca^{2+} uptake by a uniport mechanism [18]. Later we showed that the kinetic properties of the Ca^{2+}-transport system could be substantially changed in the presence of polyamines and Mg^{2+} [19, 20]. This study presents, in concise form, data on the regulation of the Ca^{2+}-uptake system of yeast mitochondria by various natural modulators and on some features of the Ca^{2+}-release system.

MATERIALS AND METHODS

BSA, EDTA, EGTA, sorbitol, mannitol, spermine, murexide, $CaCl_2$, ADP, ATP, Tris, pyruvate, and malate were purchased from Sigma (USA); Coomassie G-250, safranine O, and NADH were from Serva (Germany); A23187 was

Abbreviations: RR) ruthenium red; CCCP) carbonyl cyanide *m*-chlorophenylhydrazone; TAN) translocase of adenine nucleotides; TPP^+) tetraphenylphosphonium; CsA) cyclosporin A.

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from Boehringer Mannheim (Germany); dithiothreitol was from Reanal (Hungary). Other reagents of highest quality available were obtained from domestic suppliers.

The yeast *E. magnusii*, strain VKM Y-261, was grown as described earlier [21]. Mitochondria were isolated by a method developed in our laboratory and described previously [22]. Ca^{2+} uptake by mitochondria was assayed with a Hitachi-557 spectrophotometer using dual-wavelength photometry (507-540 nm) with murexide as a metallochromic indicator [23]. The incubation medium contained 0.6 M mannitol, 2 mM Tris-phosphate or 20 mM Tris-acetate, pH 7.4, 20 mM Tris-pyruvate, 5 mM Tris-malate, 50 μM murexide, and mitochondria corresponding to 0.5 mg protein/ml. Mitochondrial protein was assayed by the method of Bradford [24] with BSA as standard.

RESULTS AND DISCUSSION

Our first step was to search for natural modulators of the Ca^{2+}-uptake system in yeast mitochondria. Low, close to physiological concentrations of ADP (15-25 μM) were found to specifically (other examined mono-, di-, and trinucleotides were ineffective) enhance Ca^{2+} accumulation by yeast mitochondria. The half-maximal effect was attained at 3 μM, which is comparable with the K_m value for ADP binding to the translocase of adenine nucleotides (TAN) [12]. ADP is known as an inhibitor of the nonspecific permeabilization (pore) of the mammalian inner mitochondrial membrane; it acts by interacting with the TAN and locking it in the "M"-conformation, which is favorable for cation accumulation by mitochondria [12]. To test these two possibilities, we examined the rate of Ca^{2+} uptake as affected by atractyloside, a competitive inhibitor of the TAN, locking it in the "C"-conformation, and by cyclosporin A (CsA), a seemingly specific inhibitor of the Ca^{2+}-dependent pore. Atractyloside totally prevented, while CsA had no impact on the stimulatory effect of ADP. We concluded therefore that the specific stimulatory effect of ADP was due to the locking of the TAN in the "M"-conformation. However, a direct effect of ADP on the Ca^{2+} uniporter could not be excluded [25].

Then we showed that NADH specifically stimulated Ca^{2+} uptake in yeast mitochondria. A peculiarity of the yeast respiratory chain is that it contains a dehydrogenase that oxidizes exogenous NADH. NADH, as we have demonstrated previously [26], is the main inducer of reverse electron transport, which determines the redox state of the mitochondrial pyridine nucleotides and, ultimately, the ability of mitochondria to accumulate and maintain large amounts of Ca^{2+}. Yeast mitochondria after a short-term incubation with NADH displayed very high (record) rates of Ca^{2+} uptake and gained the capacity to retain the Ca^{2+} taken up. NADH may influence the transporter either directly or via modification of SH-groups of membrane proteins that play a crucial role in the ion-transport process.

Ca^{2+} itself was found to activate the Ca^{2+} uptake by yeast mitochondria. A short-term preincubation of the mitochondrial suspension with low Ca^{2+} concentrations accelerated the Ca^{2+} uptake and considerably improved the buffering properties of mitochondria due to an increased affinity of the system for Ca^{2+}. It seems plausible that the Ca^{2+}-induced stimulatory effect was due to allosteric activation of the uniporter as a result of interaction of the cation with regulatory sites of the uniporter [15].

Added together, all physiological modulators examined, along with spermine, whose stimulatory effect on Ca^{2+} uptake by yeast mitochondria was shown by us earlier [20, 21, 25], provided significantly enhanced rates of Ca^{2+} uptake (Fig. 1a, *2*) and the ability of yeast mito-

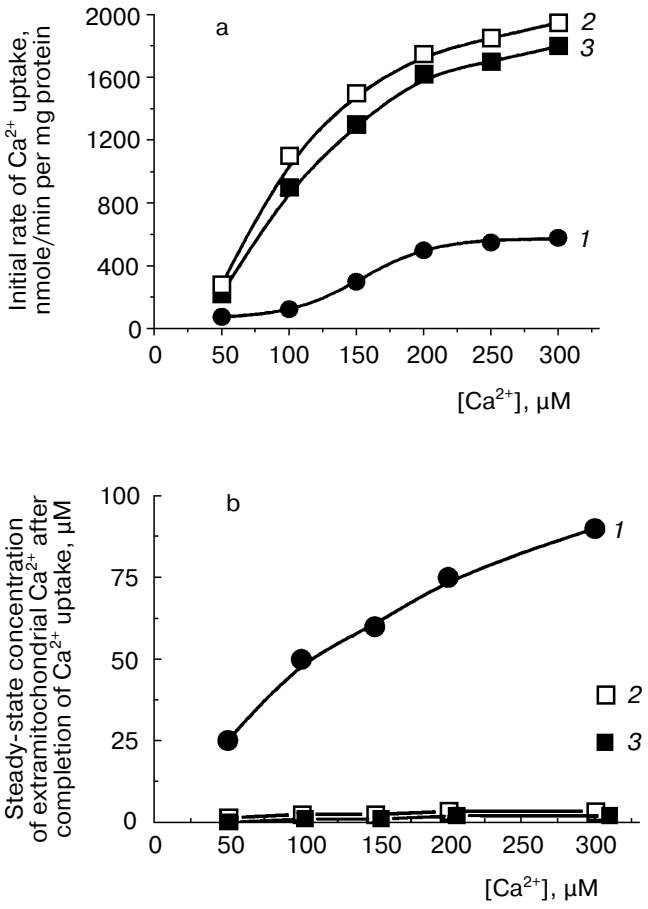

Fig. 1. The combined effect of ADP, spermine, and preincubation of mitochondrial suspension with Ca^{2+} and NADH on the initial rate of Ca^{2+} uptake by yeast mitochondria (a) and steady-state concentration of Ca^{2+} in the incubation medium (b). The incubation medium contained 0.6 M mannitol, 2 mM Tris-phosphate, pH 7.4, 20 mM pyruvate, 5 mM malate, 50 μM murexide, and mitochondria corresponding to 0.5 mg protein/ml: *1*) control; *2*) in the presence of 25 μM ADP, 25 μM spermine and after a 1-min preincubation of mitochondrial suspension with 20 μM Ca^{2+} and 4 mM NADH; *3*) the experimental conditions were as in (*2*) except that the incubation medium was supplemented with 0.5 mM $MgSO_4$, 5 mM NaCl, and 100 mM KCl.

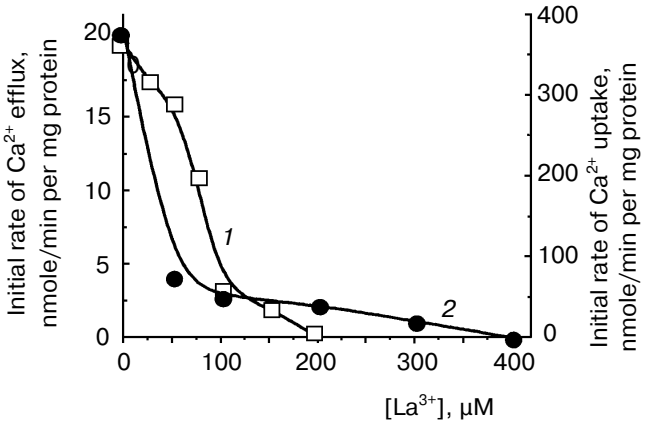

Fig. 2. Effect of La^{3+} on initial rates of Ca^{2+} uptake (*1*) and Ca^{2+} efflux (*2*) in *E. magnusii* mitochondria. The incubation medium contained: 0.6 M mannitol, 20 mM Tris-acetate, pH 7.4, 20 mM pyruvate, 5 mM malate, 50 μM murexide, and mitochondria corresponding to 0.5 mg protein/ml.

chondria to maintain a set-point at extramitochondrial Ca^{2+} concentrations comparable to the lower limit of sensitivity of the Ca^{2+} assay by the murexide technique (Fig. 1b, *2*).

The stimulatory effect of modulators remained in the presence of concentrations of Mg^{2+}, NaCl, and KCl mimicking the ionic composition of the cytoplasm (Fig. 1, *3*). Thus, it was shown that *E. magnusii* mitochondria have a highly efficient system for Ca^{2+} uptake that is under the control of low concentrations of physiological modulators. This suggests its role in regulation of intramitochondrial Ca^{2+} and, ultimately, of mitochondrial oxidation, as was postulated for animal mitochondria [3, 4].

Indeed, we showed that NAD-dependent isocitrate dehydrogenase located in the mitochondrial matrix was activated by submicromolar Ca^{2+} concentrations, with a half-maximal effect at 100 nM Ca^{2+}, which is comparable with the Ca^{2+} concentration in the matrix of animal mitochondria [6, 8].

However, to postulate a role of a Ca^{2+}-transport system in regulation of oxidative cellular metabolism, one should show the functioning of Ca^{2+}-uptake-independent pathways for Ca^{2+} release from mitochondria. This was the goal of the second part of the study. Inorganic phosphate (P_i) is known as an inhibitor of Ca^{2+}-uptake-independent pathways for Ca^{2+} efflux [27, 28]. Therefore, Ca^{2+} release from Ca^{2+}-loaded mitochondria was examined using acetate in the incubation medium instead of phosphate as a permeant anion. In the acetate-containing medium, a spontaneous Ca^{2+} efflux ensued after uptake of about 75% of the added Ca^{2+}. The rate of the Ca^{2+} release was proportional to the "Ca^{2+} load". Spermine enhanced Ca^{2+} efflux. La^{3+}, a competitive inhibitor of the Ca^{2+}-uptake system, decreased the rate of both Ca^{2+} efflux (Fig. 2, *1*) and Ca^{2+} uptake (Fig. 2, *2*), albeit with distinct concentrations required for half-maximal and maximal inhibitory effects. This supports the view that reversed activity of the Ca^{2+} uniporter was not responsible for the Ca^{2+} release observed. CsA, an inhibitor of the Ca^{2+}-dependent pore, did not affect Ca^{2+} release. This implies that the Ca^{2+} release from yeast mitochondria was not due to induction of the pore or reversed activity of the Ca^{2+} uniporter. In animal mitochondria, efflux of Ca^{2+} occurs via two specific Ca^{2+} release pathways, i.e., the Na^+-dependent and the Na^+-independent ones [6, 8]. Of these, the first could be definitely excluded in yeast mitochondria, as Ca^{2+} release was not stimulated by Na^+. The Ca^{2+}-efflux pathway in yeast mitochondria may thus be similar to the Na^+-independent mechanism in animal mitochondria. This is supported by the fact that Ca^{2+} release was inhibited by TPP^+ (Fig. 3a), a specific inhibitor of the Na^+-independent pathway for Ca^{2+}

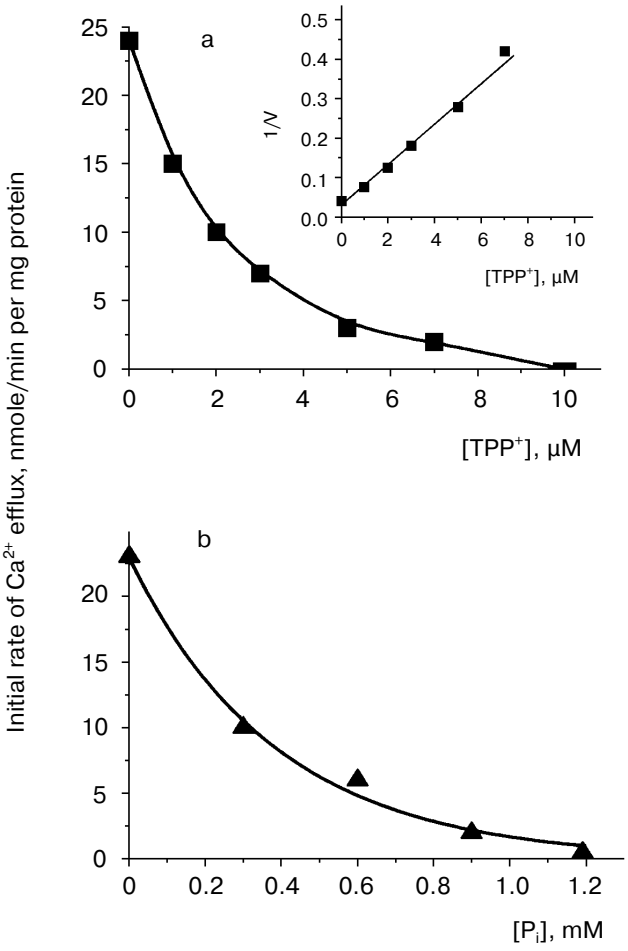

Fig. 3. Effect of TPP^+ (a) and P_i (b) on Ca^{2+} efflux from *E. magnusii* mitochondria. The experimental conditions were as described in the legend to Fig. 2.

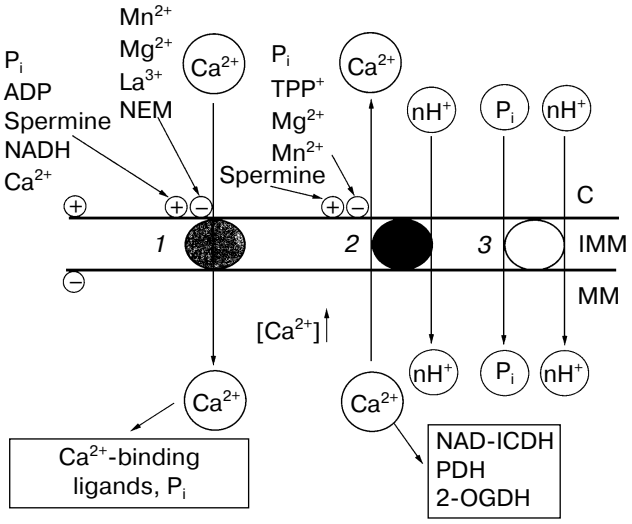

Fig. 4. Scheme describing properties of the Ca^{2+} transport system in *E. magnusii* mitochondria and their modulation. Designations: *1*) Ca^{2+} uniporter; *2*) Ca^{2+}/nH^+ antiporter; *3*) P_i/nH^+ symporter; NAD-ICDH) NAD-dependent isocitrate dehydrogenase; PDH) pyruvate dehydrogenase complex; 2-OGDH) 2-oxoglutarate dehydrogenase complex; C) cytoplasm; IMM) inner mitochondrial membrane; MM) mitochondrial matrix; +) activation; –) inhibition.

release from animal mitochondria [8], with K_i of about 2 μM, which is close to the K_i value obtained for animal mitochondria.

Two main mechanisms are currently known for the Na^+-independent Ca^{2+} release, i.e., a passive one driven by the ΔpH component of the membrane potential [28], and an active one using Δψ as a driving force [29]. To obtain more information about the mechanism of the Ca^{2+} efflux from yeast mitochondria, we performed a series of experiments with the uncoupler CCCP, as well as with the K^+ ionophores nigericin (decreasing ΔpH across the membrane) and valinomycin (decreasing Δψ across the membrane). The Ca^{2+} efflux was only insignificantly inhibited by CCCP, activated by valinomycin, and completely inhibited by nigericin. These results suggest that the Ca^{2+} release from the yeast mitochondria was primarily driven by the ΔpH. This suggestion was substantiated by the fact that the Ca^{2+} release was inhibited by low concentrations of P_i (Fig. 3b).

The data presented demonstrate, to our knowledge for the first time, that mitochondria from a yeast species are endowed with not only an efficient Ca^{2+}-uptake system which is under the control of low (physiological) concentrations of natural modulators, but also with a Ca^{2+}-release system, most probably a $Ca^{2+}/2H^+$ exchanger, very similar to that in mitochondria from nonexcitable mammalian tissues [8].

Figure 4 depicts a hypothetical scheme summarizing our results on the main principles of the arrangement of the Ca^{2+}-transport system of yeast mitochondria. According to this scheme, the sequence of processes at the level of the inner mitochondrial membrane is the following: 1) an increase in the cytosolic Ca^{2+} would activate the Ca^{2+} uniporter, and this would establish a higher concentration of free Ca^{2+} in the mitochondrial matrix; 2) a rise of matrix Ca^{2+} concentrations would trigger Ca^{2+} efflux; 3) concerted operation of both the Ca^{2+}-uptake and Ca^{2+}-efflux systems would provide a certain steady-state concentration of Ca^{2+} in the matrix required to activate Ca^{2+}-sensitive dehydrogenases of the Krebs cycle (notably, NAD-dependent isocitrate dehydrogenase), thus supplying more NADH and activating oxidative cellular metabolism.

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