Biochemistry (Moscow), Vol. 69, No. 1, 2004, pp. 91-102. Translated from Biokhimiya, Vol. 69, No. 1, 2004, pp. 114-127.

REVIEW

Mitochondrial Calcium Transport Systems: Properties, Regulation, and Taxonomic Features

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Received November 11, 2002
Revision received April 24, 2003

Abstract—Currently available information on properties and regulation of mitochondrial Ca^{2+} transporting systems in eukaryotic cells is summarized. We describe in detail kinetic properties and effects of inhibitors and modulators on the energy-dependent Ca^{2+} uptake through the Ca^{2+} uniporter, as well as on Na^+-dependent and Na^+-independent pathways for Ca^{2+} release in mammalian mitochondria. Special emphasis is placed on Ca^{2+} transport systems (for ion uptake and release) in mitochondria of higher plants, algae, and yeasts. Potential physiological implications of mitochondrial Ca^{2+} fluxes (influx and efflux), e.g., regulation of activity of Ca^{2+}-dependent enzymes of the Krebs cycle, maintaining of cellular Ca^{2+} homeostasis, and engagement in pathophysiological processes, are discussed.

Key words: mitochondria, calcium transport, Ca^{2+} uniporter, Na^+-dependent and Na^+-independent pathways for Ca^{2+} release, apoptosis

Calcium (Ca^{2+}) controls a diverse range of cellular processes [1], serving as a second intracellular messenger in signal transduction from the ambient medium into the cell [2, 3]. The Ca^{2+} gradient between the cytoplasm and the ambient medium reaches four orders of magnitude [4, 5]; it is maintained by binding to specific proteins [6, 7], by Ca^{2+} sequestration in intracellular organelles (endoplasmic reticulum, vacuoles, mitochondria) endowed with specific membrane Ca^{2+} transport systems and due to operation of Ca^{2+} channels [8-11]. Kinetic parameters, molecular structure and modulators of Ca^{2+}-ATPases of reticular and plasma membranes [10, 12], the Na^+/Ca^{2+}-transporter of the plasmalemma of excitable tissues [13], the Ca^{2+}/H^+-exchanger of the vacuolar membrane [14], Ca^{2+}-channels of the plasmalemma [15], and the Ca^{2+}-transport system of the inner mitochondrial membrane have been extensively investigated and characterized (see reviews [8, 16-18]). An enormous amount of experimental material has been accumulated on the properties, regulation, and physiological implications of the Ca^{2+}-transport system of vertebrate mitochondria, which is due to the great interest in Ca^{2+} transport due to its involvement in triggering of apoptotic processes [19-21]. At the same time, properties of the similar transport systems in mitochondria of higher plants and lower eukaryotes (yeasts and algae) have not been adequately explored. This review fills this gap and describes the taxonomic features and possible physiological role of mitochondrial Ca^{2+} transport systems in these organisms in comparison with the thoroughly studied mammalian mitochondrial system.

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THE Ca^{2+} TRANSPORT SYSTEM OF VERTEBRATE MITOCHONDRIA

The study of the Ca^{2+} transport system in animal mitochondria began more than 45 years ago. In his pioneering work, Chance showed that addition of Ca^{2+} to liver mitochondria caused a reversible uncoupling of respiration [22]. In the early 60s, Vashington and Murphy established that Ca^{2+} was taken up by mitochondria in an energy-dependent fashion [23]. Later, it was ascertained that ion accumulation is driven by a gradient of the electrochemical potential on the inner mitochondrial membrane ($\Delta\mu_{H^+}$) generated by the respiratory chain [24], or by the imposed potassium ion gradient (so-called "diffusion potential") [25]. The energy-dependent Ca^{2+} uptake was found to occur by a uniport mechanism, i.e., regardless of other ion movement [26], and therefore the mitochondrial system responsible for unidirectional Ca^{2+} accumulation was named the uniporter. Ca^{2+} uptake was greatly enhanced in the presence of penetrable anions (acetate, phosphate, bicarbonate, β-hydroxybutyrate)

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(see review [27]). Ca^{2+} entry into mitochondria was associated with a parallel increase in proton ejection and reversible decrease in the electric component of the membrane potential ($\Delta\Psi$).

In the course of further studies, a number of problems arose concerning calculation of the Ca^{2+} gradient across the mitochondrial membrane. Taking as a background that free cytoplasmic Ca^{2+} concentrations ($[Ca^{2+}]_c$) in resting cells are of 0.1-1 μM and the membrane potential values of 180 mV, a steady-state concentration of free calcium in the mitochondrial matrix ($[Ca^{2+}]_m$) was calculated to be equal to 0.1-1 M, which is at least an order of magnitude higher than the value of 0.1-1 mM obtained experimentally in early studies [28]. Later, $[Ca^{2+}]_m$ in heart and liver mitochondria loaded with specific Ca^{2+}-probes (*fura-2AM* and *indo-1*) to ensure measurements of low, submicromolar Ca^{2+} concentrations, was found within 1 μM [29, 30], while $[Ca^{2+}]_c$ in intact resting cells varied from 100 to 240 nM [31, 32]. It was concluded that the Ca^{2+} concentration gradient on the mitochondrial membrane is rather high. Later, the fact that $[Ca^{2+}]_m$ is out of equilibrium with $[Ca^{2+}]_c$ was explained by simultaneous operation of distinct pathways for Ca^{2+} influx and efflux, compensating the Ca^{2+} accumulation and establishing a "set point" for extramitochondrial Ca^{2+}. In the 1970s, pathways for Ca^{2+} release were identified as the Na^+-dependent (or a Na^+/Ca^{2+} exchanger [33, 34]) and the Na^+-independent [35, 36] pathways.

Table 1. Kinetic properties of the Ca^{2+} uniporter in mammalian mitochondria

Mitochondria	$C_{0.5}$, μM	V_{max}, nmol Ca^{2+}/min per mg protein	Reference
Rat liver	~7	250	[45] (1)
	~10	900-1400	[25] (2)
	15.6	520	[42] (3)
	60 ± 10	480-780	[40] (4)
Rat heart	6.7	385	[46] (5)
	75	—	[47] (5)
	—	833	[48] (1, 4)
Rabbit heart	12-15	84	[49] (6)
	55	~300	[50] (4)
Dog heart	189	1750	[48] (1, 4)

Note: Numbers in parentheses indicate the methods for Ca^{2+} measurements: 1) with metallochromic dye Arsenazo III; 2) with the K^+ diffusion potential as a driving force for Ca^{2+} uptake; 3) with $^{45}Ca^{2+}$ in the presence of Ca^{2+} buffers; 4) with murexide dye; 5) with $^{45}Ca^{2+}$; 6) with a Ca^{2+}-selective electrode.

PATHWAYS FOR Ca^{2+} UPTAKE

The Ca^{2+} uniporter. Ca^{2+} enters mitochondria unidirectionally, the uptake process being driven by the electrochemical potential gradient (see reviews [8, 16-18, 27]) depending on ion concentrations in the incubation media. Additionally to Ca^{2+}, the uniporter transports other divalent cations such as Sr^{2+}, Mn^{2+}, Ba^{2+}, Fe^{2+}, lanthanides (see reviews [27, 37]), and Zn^{2+} [38]. The assessment of kinetic properties of the uniporter is complicated by the fact that the rate of Ca^{2+} uptake by mitochondria is limited by the rate of proton ejection from the respiratory chain. This brings about serious divergences in V_{max} and K_m (more correct to use a half-maximal efficient Ca^{2+} concentration, $C_{0.5}$) values of the transport process [39]. Azzone et al. [25] in an attempt to solve this problem used K^+-diffusion potential as a driving force for Ca^{2+} uptake by deenergized mitochondria and maintained the constant extramitochondrial Ca^{2+} concentration in the presence of Ca^{2+}-buffers. For rat liver mitochondria, the V_{max} and $C_{0.5}$ were found equal to 1400 nmol/min per mg protein and <10 μM, respectively. Kinetic constants of the Ca^{2+} transport system may vary considerably (Table 1) depending on the tissue type, the methodology employed, modulators of the uniporter included in incubation media, and energy suppliers (oxidation of respiratory substrates, ATP hydrolysis, or electrochemical gradient of K^+) (see [27, 37]).

Ca^{2+} uniporter activity, as already mentioned, depends on the cation concentration in incubation medium. In the majority of studies, the dependence of the initial rate v_0 of cation uptake versus its concentrations was found to be sigmoidal in character, thus indicating a positive cooperativity of the process, with saturation at high Ca^{2+} concentrations [40-42]. Addition of non-permeable divalent cations (Mg^{2+} and Li^{2+}), as well as a decrease in temperature or changes in the ionic composition of incubation medium can make this sigmoidicity of the dependence v_0 on [S] more pronounced [25], which is known to be typical for allosteric enzymes. The existence of an allosteric activating center was proved in experiments showing reversed activity of the heart mitochondrial uniporter, providing that mitochondria were incubation with Ca^{2+} chelators or ruthenium red, an inhibitor of the Ca^{2+} uptake process [43, 44]. The allosteric centers were found to be located on the cytosolic face of the inner mitochondrial membrane and regulate the rate of both Ca^{2+} uptake and release through the uniporter. Difficulties in determination of the real maximal activity of the uniporter pose some problems for Hill coefficient (h) calculations. Hill coefficients of 1.7 to 2.0, depending on the experimental conditions, were obtained [40, 41]. The value $h = 2.0$ implies that either two calcium ions are bound to the uniporter regulatory site or that only one of the calcium ions

interacts with the transport center, while the other one is bound to the regulatory site, thus increasing affinity of the transporter for Ca^{2+} [37]. According to other data available, the dependence of the initial rate of cation uptake on its concentrations is hyperbolic, but it becomes sigmoidal in the presence of Mg^{2+} and Li^{2+}, inhibitors of the Ca^{2+} transport, at lowered temperature, or with changed ionic composition of the incubation medium [25, 51, 52]. The cooperativity model advanced by Kasparinsky and Vinogradov [45] for the Ca^{2+}-transport system in liver mitochondria reconciles the seemingly contradictive data on kinetics of the Ca^{2+} uniporter. These authors found that the initial rate of the Ca^{2+} uptake through the uniporter increased ~10 times after preincubation of deenergized mitochondria in the presence of low (2-5 μM) Ca^{2+} concentrations. The dependence of v_0 on the cation concentration was hyperbolic in nature, while in control experiments (without preincubation with Ca^{2+}) it was sigmoidal. An apparent constant for first-order rate for the Ca^{2+}-dependent activation of the uniporter was found equal to 0.5 min^{-1}, which is several orders of magnitude less than estimated turnover numbers ($2 \cdot 10^5$ min^{-1}) of the uniporter in its active state. Consequently, activation of the Ca^{2+} transporter is a slow process. According to the authors' data, the Ca^{2+} uniporter in the absence of exogenous Ca^{2+} is in an inactive form and fails to efficiently transport cations (not only Ca^{2+}, but also other ions of di- and monovalent metals) and protons (Fig. 1, *1*). Binding of Ca^{2+} to specific transporter sites is accompanied by slow activation of the uniporter, parallel to oligomerization of its protomers (Fig. 1, *2* and *3*), thus forming an active gated channel that is sensitive to ruthenium red and lanthanides (Fig. 1, *4*). It is reasonable to suggest that hyperbolic dependence of the initial rate of Ca^{2+} uptake on its concentration in the incubation medium found by some researches may be attributed to natural Ca^{2+} leakage (release) from mitochondria, thus activating the uniporter. The described model for the uniporter functioning postulates the inactive state of the Ca^{2+} uniporter as an important physiological factor, preventing mitochondria from nonspecific transport of cations and protons, challenging undesirable consequences (futile uncoupling, for instance). At the same time, the low level of the cytosolic Ca^{2+} remains a single factor supporting the uniporter in a physiologically active conformation.

Uniporter activity is defined also by the membrane potential value [53]. The uniporter is inactive when the potential is diminished even if the cation concentration gradient reaches 1 : 8 [54]; $\Delta\mu_{H^+}$ maintains the carrier in a conformation competent for transport [54]. When the membrane potential is reduced due to addition of an uncoupler [55] or when Ca^{2+} is chelated by EGTA [44], the Ca^{2+} uniporter may exhibit "reversed" activity, causing efflux of the accumulated cation. Rapid Ca^{2+} release by this mechanism is characterized by partial sensitivity to ruthenium red [56], is accompanied by phenomena typically arising from nonspecific membrane permeability (Permeability Transition Pore, PTP) [56], and is inhibited by the pore inhibitor cyclosporin A [57]. At present the phenomenon of uniporter reversed activity is in doubt (see review [8]). It seems very likely that the main function of the uniporter is the Ca^{2+} influx (uptake), but not its release, even when mitochondria are almost totally deenergized.

The uniporter is under the control of a set of positive modulators with adenine nucleotides [58], polyamines [42, 59], Ca^{2+} [40, 41, 43, 45, 46], and high levels of reduced intramitochondrial pyridine nucleotides [60] being foremost among them. Litsky and Pfeiffer [55], studying reversed activity of the Ca^{2+} uniporter on Sr^{2+}-loaded rat liver mitochondria, i.e., under conditions excluding the PTP induction, advanced a model for regulation of the Ca^{2+} uniporter by adenine nucleotides, Mg^{2+}, and polyamines. According to this model, the Ca^{2+} uniporter is considered as a gated channel with varying conformation depending on the $\Delta\mu_{H^+}$ value on the inner mitochondrial membrane, the presence of modulators, and the status of the modulator-binding sites. When Ca^{2+},

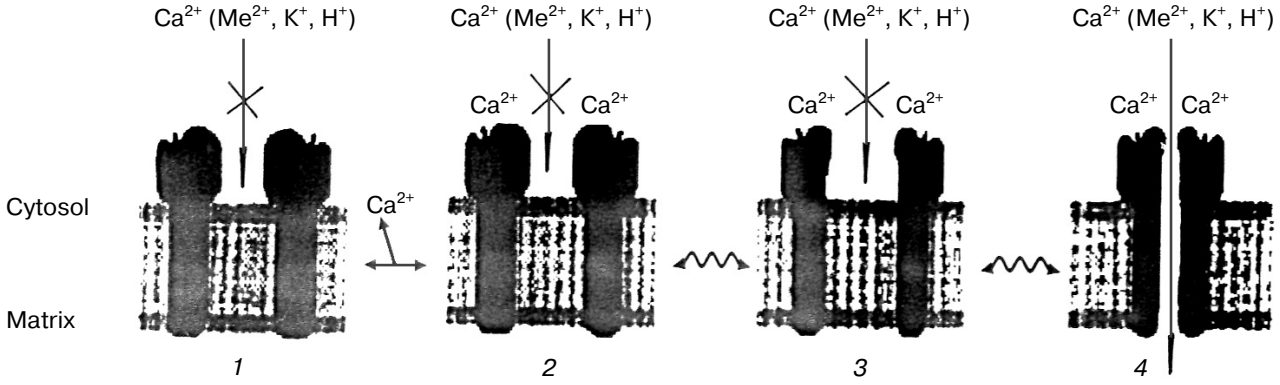

Fig. 1. Scheme describing mechanism of energy-dependent Ca^{2+} uptake by animal mitochondria (see [45]). See text for details.

Sr^{2+}, and, possibly, other divalent cations are bound to specific regulatory sites of the uniporter in energized mitochondria, the channel is open and transports cations into the mitochondrial matrix. A decrease in $\Delta\mu_{H^+}$ changes the conductivity of the channel. Binding of Mg^{2+} quickly closes the channel. ATP (to a lesser extent ADP and AMP) acts like Mg^{2+}, but from the inner face of the channel, providing its closing. Spermine favors the opening state of the channel, while P_i makes it closed. Along with the model of Kasparinsky and Vinogradov [45], this model can be used to critically comprehend and systematize the enormous data available on characteristics, regulation, and structure of the Ca^{2+} uniporter. Nevertheless, it is still rather difficult to reconcile the inhibitory effect of adenine nucleotides on the open state of the channel proposed by Litsky and Pfeiffer with numerous data claiming just the opposite effect of adenine nucleotides on the channel [58]. There are other hypotheses on regulation of the uniporter by natural modulators. So, Rottenberg and Marbah [58] suggested that stimulating effect of adenine nucleotides on Ca^{2+} uptake by brain mitochondria relates to the change in the conformation state of the adenine nucleotide translocator, while activation by spermine is associated with its effect on the surface charge of the mitochondrial membrane, thus improving Ca^{2+} binding to its translocation system [61].

Several inhibitors of the Ca^{2+} uniporter are known. Ruthenium red, a hexavalent inorganic complex of Ru^{3+}, is a powerful inhibitor of the transport process (see reviews [27, 37]). Reed and Bigrave [62] showed that ruthenium red is a noncompetitive inhibitor of the transport with $[I]_{50}$ = 30 nM. Other ruthenium-containing compounds, for example Ru-360, a derivative of Ru^{2+} [63, 64], also inhibit the Ca^{2+} uptake process. Lanthanides and lanthanum salts represent another class of inhibitors; they are less specific competitive inhibitors with $[I]_{50}$ = 20-50 nM [62]. Inhibition by these compounds decreases in the series: $La^{57} > Nd^{60} > Sm^{62}$ [65]. Divalent metal ions (Sr^{2+}, Mn^{2+}, Ba^{2+}), being transported via the uniporter, are also competitive inhibitors of the Ca^{2+} transport system (see reviews [8, 27]). Compounds of this type act by binding to the transport or regulatory sites of the uniporter.

Some attempts have been made to identify the component responsible for Ca^{2+} uptake by mitochondria. Sottocasa et al. [66] in their pioneering work isolated from ox liver a glycoprotein with some properties (K_d, sensitivity to inhibitors) resembling those of the Ca^{2+}

Table 2. Kinetic properties of the Na^+-dependent pathway for Ca^{2+}-release from mitochondria

Mitochondria	V_{max}, nmol Ca^{2+}/min per mg protein	K_m for Na^+, mM	K_m for Ca^{2+}	Reference
Rat liver	1.4	—	—	[75] (1, 2)
	1.6	—	—	[78] (1)
	2.6 ± 0.5	9.4 ± 0.6	8.1 ± 1.4 nmol Ca^{2+} per mg protein	[83] (1)
Rat heart	3.0	—	—	[75] (1, 2)
	10.8 ± 2.1	4	5.7 ± 0.8 μM	[89] (3)
	14.3	8	—	[33] (4)
			—	[34] (4)
Rat brain	12	9		
	16	—	—	[77] (1)
Rabbit skeletal muscle	4-5	7	—	[34] (4)
Rabbit smooth muscle	4	8	—	[34] (4)
Bull saliva gland	4-5	8	—	[34] (4)
Rat brown fat cells	5.5	10	—	[90] (2)
Rat lungs	2.4	—	—	[77] (1)

Note: Numbers in parentheses indicate methods for Ca^{2+} measurements: 1) with $^{45}Ca^{2+}$; 2) with metallochromic dye Arsenazo III; 3) with metallochromic dye Antypyrylazo III; 4) with the Ca^{2+}-selective electrode.

transporter. Antibodies against this glycoprotein inhibited Ca^{2+} uptake by intact rat liver mitochondria and by mitoplasts. A glycoprotein specifically binding Ca^{2+} was isolated from beef heart mitochondria by Mironova's group [67]. According to these authors, the Ca^{2+} transporter contained not only glycoprotein (40 kD), serving as a Ca^{2+} receptor, but also a low-molecular-weight channel-forming peptide with molecular mass of 2000 daltons [68]. This complex, being incorporated into artificial lipid membranes, highly selectively transports Ca^{2+}, and this transport is inhibited by ruthenium red and polyclonal antibodies against the glycoprotein with molecular mass of 40 kD [69]. Another research group [70], using ^{103}Ru-360, an inhibitor of the Ca^{2+} transport process, isolated an 18-kD protein with Ca^{2+}-transporting activity. Recently, a ryanodine receptor with molecular mass of 600 kD with properties very similar to those of the Ca^{2+} transporter has been identified in rat heart mitochondria [71]. Addition of ryanodine to isolated mitochondria inhibited Ca^{2+} transport and high-amplitude swelling of mitochondria; however, specific Ca^{2+} transport activity was not observed. It was suggested that mitochondrial ryanodine receptor may play an essential role in dynamics of Ca^{2+} distribution. At present, the nature of the mitochondrial Ca^{2+} uniporter is still uncertain.

"Rapid Ca^{2+} uptake." *In vivo* in the cytoplasm there are cyclic changes in $[Ca^{2+}]_c$. With these data as a background, Gunter et al. [72, 73], using pulses of physiological Ca^{2+} concentrations (~400 nM), observed in liver and heart mitochondria cation accumulation far exceeding the expected net cation accumulation via the uniporter and named this phenomenon RaM (Rapid Mode of Ca^{2+} uptake). This type of Ca^{2+} translocation takes place at $[Ca^{2+}]$ below 200 nM; it is inhibited with increasing $[Ca^{2+}]$, possibly at the level of outer-faced regulatory sites. V_{max} of this process reached 7 nmol/min per mg protein. The transport process is inhibited by high concentrations of ruthenium red, while being insensitive to Mg^{2+}, and stimulated by ADP and spermine. It was suggested that RaM occurs via an independent Ca^{2+} transporter (carrier) or a Ca^{2+} channel and that this type of Ca^{2+} uptake operates simultaneously with the Ca^{2+} uniporter.

Pathways for Ca^{2+} release from Ca^{2+}-loaded mitochondria. *The Na^+-dependent pathway for Ca^{2+} release* was revealed in mitochondria from excitable tissues [13, 27, 34, 74-76]; it functions with lower efficiency in mitochondria from liver [77], kidney, and lungs [78]. The initial rate of Ca^{2+} release through this pathway depends on ionic composition of the medium, its tonicity [75, 77], temperature [79], pH [80], as well as on the presence of permeable anions [75]. The highest stimulation of the Na^+-dependent pathway occurs in the presence of acetate, whereas phosphate inhibits this process by reducing $[Ca^{2+}]_m$ [81]. Kinetics of the Na^+-dependent pathway for Ca^{2+} release varied depending on the tissue type and

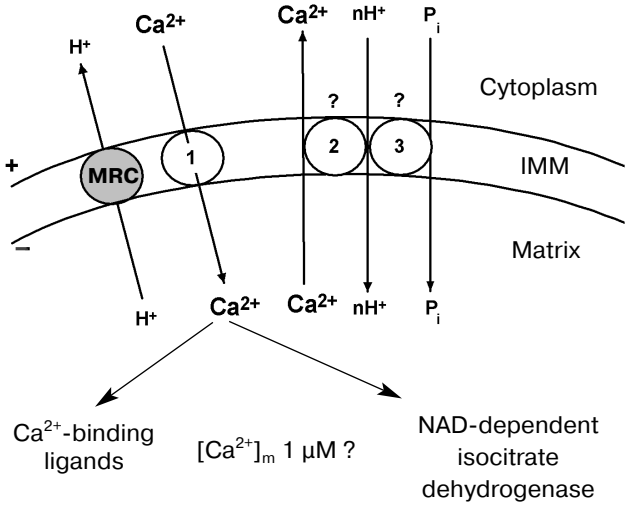

Fig. 2. Schematic representation of the arrangement of Ca^{2+} transport systems in plant mitochondria (according to [138, 139]): 1) the Ca^{2+} uniporter; 2) the Ca^{2+}/nH^+-exchanger providing Na^+-independent Ca^{2+} release; 3) nH^+/P_i-symporter. IMM, inner mitochondrial membrane; MRC, mitochondrial respiratory chain.

methods for Ca^{2+} determination (Table 2). The dependence of the initial rate of Ca^{2+} release on the Na^+ concentration in the medium is sigmoidal [34], suggesting that two or more Na^+ ions are exchanged for Ca^{2+}, *h* values varying from 2.8-3.1 [33] to 1.4-2.0 [82, 83]. Recently, Griffiths [76] using cardiomyocytes has shown that activity of the Na^+/nCa^{2+}-exchanger can be reversed under hypoxic conditions and when a decay of $\Delta\Psi$, the electric component of the electrochemical gradient, or inactivation of the Ca^{2+} uniporter takes place. Gunter and coauthors [84] established that Ca^{2+} efflux via the Na^+/nCa^{2+}-exchanger of beef heart mitochondria occurs electrogenically, against the concentration gradient, which was found to exceeds 15-100 times that for the passive $Ca^{2+}/2\ Na^+$-exchange (Fig. 2). Taking into consideration the data obtained by Crompton and coauthors [85] that the Na^+-dependent pathway for Ca^{2+} release is inhibited by uncouplers, Jung et al. [86] suggested that this process is driven by the electrochemical proton gradient ($\Delta\mu_{H^+}$). At present the hypothesis of active Ca^{2+}/nNa^+-exchange (Fig. 2) dominates; however, the stoichiometry of the process is still debated (see review [8]). Ca^{2+}/nNa^+-exchanger is inhibited by Mg^{2+}, Mn^{2+}, diltiazem, the inhibitor of Ca^{2+} channels ruthenium red, tetraphenylphosphonium (see reviews [8, 27]), and CGP-37157 (a derivative of benzothiazepine) [87]. Garlid et al. [88] isolated from beef heart mitochondria a protein with molecular mass of 110 kD that was sensitive to diltiazem and tetraphenylphosphonium and, being incorporated into liposomes, was able to catalyze a Ca^{2+}/Na^+-exchange. Antibodies against this protein inhibited

Ca^{2+}/Na^+-exchange in mitochondria. At present, this is the only carrier known to be responsible for the Na^+-dependent release of Ca^{2+} from mitochondria.

The Na^+-independent pathway for Ca^{2+} release was described for mitochondria from non-excitable tissues [27, 34, 37, 81]. This pathway is less active in heart mitochondria [75]. V_{max} for Ca^{2+} efflux from liver mitochondria was 0.8-1.2 nmol/min [91, 92], while in heart mitochondria it reached 2.0 nmol/min per mg protein [75]. The *h* value for liver mitochondria was found to be 1.9 [92]. The Na^+-independent pathway for Ca^{2+} release is promoted in the presence of ruthenium red, thus indicating that this pathway does not depend on the Ca^{2+} uniporter, and is accompanied by exchange of Ca^{2+} for nH^+ supposedly through a Ca^{2+}/nH^+-exchanger (see reviews [8, 27, 37]) (Fig. 2). In addition to Ca^{2+}, this system also transports Sr^{2+}, Ba^{2+}, and Mn^{2+} (see review [41]) and is activated by hypotonicity [75]. The mechanism of this Ca^{2+} release pathway is rather complicated because it is characterized by properties similar to those of the PTP (for instance, it is stimulated in the presence of oxidants of glutathione [93] and pyridine nucleotides [94]); therefore, it is sometimes rather difficult to discriminate these two processes. In their attempts to solve this problem, i.e., to differentiate the two processes, Gunter et al. [95] used conditions excluding the PTP induction. They showed that the Na^+-independent pathway catalyzes Ca^{2+} efflux against the Ca^{2+} gradient, which far exceeded the theoretically calculated gradient value for the electroneutral Ca^{2+}/nH^+-exchange. Therefore, they concluded that the Na^+-independent pathway for Ca^{2+} release, like the Na^+-dependent pathway for Ca^{2+} release, is an active, electrogenic mechanism, with $n > 2$, driven by $\Delta\mu_{H^+}$. The Na^+-independent pathway for Ca^{2+} release is inhibited by Sr^{2+} [96], Mn^{2+} [97], tetraphenylphosphonium, and tetramethylphosphonium (see review [37]).

Ca^{2+}-dependent nonspecific permeability of the inner mitochondrial membrane. In addition to specific pathways for Ca^{2+} release, not disturbing membrane integrity of energized mitochondria, there is a nonspecific pathway for Ca^{2+} release, a Ca^{2+}-dependent nonspecific permeabilization of the inner membrane (PTP, the "membrane pore"), which is accompanied by the uncontrolled, increased permeability of the membrane for compounds with molecular mass <1500 kD, leading to massive release of the accumulated Ca^{2+}, Mg^{2+}, adenine nucleotides, and matrix proteins, $\Delta\Psi$ decay, and high-amplitude swelling of mitochondria (see reviews [8, 19-21]). PTP induction requires the presence of Ca^{2+} and triggering agents, amongst which the most known are P_i, heavy metals, inhibitors and uncouplers of mitochondrial respiration, SH-reagents, and organic hydroperoxides. Ca^{2+} accumulation in the matrix is required for PTP production [37]. The Ca^{2+}-dependent pore is defined as a protein complex comprised of a potential-dependent anion channel of the outer mitochondrial membrane (porin), the translocator of adenine nucleotides, and cyclophilin D. Upon binding of Ca^{2+} with this complex under pathological conditions (oxidative stress, depletion of intracellular ATP pools), an open membrane pore with a diameter of 2.6-2.9 nm is formed [18, 19]. The most important characteristic of the PTP is its reversibility observed in the presence of Ca^{2+} chelators and ruthenium red, inhibitors of the Ca^{2+} transport [27, 37]. PTP induction is inhibited by high ratio of reduced intramitochondrial pyridine nucleotides [37], high $\Delta\Psi$ values [57], and acidification of the matrix [98]. The immunosuppressor cyclosporin A is the potent highly specific inhibitor of the PTP [99] with a K_i of ~5 nM. The physiological role of this phenomenon is discussed below.

MITOCHONDRIAL Ca^{2+}-TRANSPORTING SYSTEM OF PLANTS AND ALGAE

Ca^{2+} uptake by plant mitochondria. Ca^{2+} accumulation supported by substrate oxidation or ATP hydrolysis in mitochondria from seedlings of corn (*Zea mays* L.) was first demonstrated by Hodges and Hanson in 1965 [100]. Then efficient Ca^{2+} transport systems were found in mitochondria from sweet potato tubers [101], corn coleoptiles and leaves, pumpkin hypocotyls, cultured carrot cells [102], Jerusalem artichoke tubers [103], and corn seedlings [104]. In all cases low affinity of the system for Ca^{2+}, high degree of energy-independent Ca^{2+} binding to mitochondria, and an absolute requirement for phosphate were observed. Ruthenium red did not affect Ca^{2+} uptake in mung bean mitochondria [105], while inhibiting this process in mitochondria from Jerusalem artichoke [103]. Kinetic properties of Ca^{2+} transport systems in plant mitochondria are listed in Table 3. The plot of v_0 of Ca^{2+} uptake versus extramitochondrial Ca^{2+} concentrations was sigmoidal with saturation at 120 μM Ca^{2+} and $h = 2.22$ (in mitochondria from etiolated corn seedlings [106]). $[Ca^{2+}]_m$ in mitochondria from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers was found equal to 400-600 nM (the florescent Ca^{2+}-indicator *fura-2* was used) [107]. The apparent affinity of the system for Ca^{2+} turned out to be greatly above earlier reported values (Table 3); it was therefore concluded that the Ca^{2+} transport systems in plant mitochondria are similar to those of animal mitochondria. Recently, Ca^{2+}-uptake activity has been demonstrated in mitochondria from trigonella (*Trigonella foenum-graecum* L.) seedlings [108], this activity increasing after treatment of the plant with selenium and mimosine.

Thus, rather limited data on Ca^{2+}-transporting capacity of plant mitochondria indicate that this varies from the failure of mitochondria to accumulate Ca^{2+} in an energy-dependent fashion to the high-affinity, high-capacity system. The main feature of the studied mitochondrial Ca^{2+}-transporting system of plants is their

Table 3. Kinetic parameters of Ca^{2+} uptake by plant mitochondria

Mitochondria	$C_{0.5}$, μM	V_{max}	Reference
Mung bean	—	5 nmol Ca^{2+}/min per mg protein	[105] (1)
(*Phaseolus aureus* Roxb.)	—	435-635 nmol Ca^{2+}/min per mg protein	[110] (2)
Corn coleoptiles	31	140 nmol Ca^{2+}/min per mg protein	[106] (3)
(*Zea mays* L.)	250	63 nmol Ca^{2+}/min per mg protein	[111] (4)
	300	31-604 μmol Ca^{2+} per mg mitochondrial N*	[100] (4)
Apple fruit (*Malus pumula* Mill. var. *domestica* Fuji)	350	1.7 nmol Ca^{2+}/min per g fresh biomass	[112] (2)
Jerusalem artichoke tubers	0.15	—	[107] (5)
(*Heliantus tuberosus* L.)	—	60 nmol Ca^{2+}/min per mg protein	[103] (4)
Sweet potato tubers (*Impomoea batatas* L.)	—	46 nmol Ca^{2+}/min per mg protein	[100] (4)
Pumpkin hypocotyl (*Cucurbita pepo* L.)		8.4 nmol Ca^{2+}/min per mg protein	[100] (4)

Note: Number in parentheses indicate methods for Ca^{2+} measurements: 1) with metallochromic dye Arsenazo III; 2) flame atomic spectrometry; 3) Ca^{2+}-selective electrode; 4) with $^{45}Ca^{2+}$; 5) with fluorescent probe *fura-2*.

* In experiments, 0.1 mg mitochondrial nitrogen was used as a protein equivalent.

absolute requirement for phosphate. In most cases, the studied systems are slow, with low affinity for Ca^{2+}, and with low, if any sensitivity to ruthenium red and lanthanides, which are specific inhibitors of animal Ca^{2+} transport systems.

Pathways for Ca^{2+} release from cation-loaded plant mitochondria. Information about independent pathways for Ca^{2+} release from plant mitochondria is scarce. In corn mitochondria, an uncoupler-induced release of the accumulated Ca^{2+} was observed by Siliva et al. [104]. The Ca^{2+} release process was sensitive to mersalyl, an inhibitor of the phosphate carrier. Mersalyl promoted Na^+-insensitive Ca^{2+} efflux, thus showing direct participation of phosphate in regulation of Ca^{2+} fluxes. The authors concluded that corn seedling mitochondria possess a system for Ca^{2+} release that is similar to the Na^+-independent pathway for Ca^{2+} release in animal mitochondria, and they offered an extended scheme describing the arrangement of Ca^{2+} transport systems in the inner mitochondrial membrane of plant mitochondria (Fig. 2). According to this scheme, calcium ions enter plant mitochondria in an energy-dependent process, synergistically with P_i, while exiting mitochondria through the $Ca^{2+}/2H^+$-exchanger.

Ca^{2+} transport by alga mitochondria. Ca^{2+} accumulation supported by oxidation of succinate and lactate and sensitive to ruthenium red and lanthanides was revealed in euglena (*Euglena gracilis* L.) mitochondria [109]. The initial rate of Ca^{2+} uptake is enhanced in the presence of phosphate, while inhibitors of the respiratory chain do not affect this process.

THE Ca^{2+}-TRANSPORTING SYSTEM OF YEAST MITOCHONDRIA

Ca^{2+} uptake. Anraku and coauthors [113] using the florescent probe *fura-2* have found that the concentration of free cytoplasmic Ca^{2+} in resting *Saccharomyces cerevisiae* cells is 0.1 μM (it is close to the value found in animal cells), but this level can be greatly increased upon pheromone action [114, 115]. Similar data were obtained for dimorphic fungus *Candida albicans* [116] when the growth of filaments was initiated. In spite of appreciation of an important role of Ca^{2+} in regulation of cellular process, all attempts in the early 70s to reveal Ca^{2+} uptake in yeast mitochondria were unsuccessful. Carafoli and coauthors [117] did not observed Ca^{2+} stimulated respiration in mitochondria from two yeast species, *S. cerevisiae* and *Torulopsis* (*Candida*) *utilis*. Other researchers showed that *S. cerevisiae* mitochondria accumulate Ca^{2+} passively, by adsorbing it on the membrane [118]. Therefore, it was concluded that yeast mitochondria lack a Ca^{2+} transport system. Later, it was established that *S. cerevisiae* and *C.*

utilis mitochondria do actively accumulate high (1-10 mM) concentrations of Ca^{2+}, Sr^{2+}, and Mn^{2+} [119]. The cation accumulation was accompanied by proton extrusion into the incubation medium and was inhibited by respiratory inhibitors and uncouplers. Unlike plant mitochondria, yeast mitochondria transported Ca^{2+} in the absence of phosphate, though its addition enhanced the net Ca^{2+} uptake. Unlike animal mitochondria, yeast mitochondria could not use energy derived from ATP hydrolysis for Ca^{2+} transport. Later, Uribe and coauthors [120], with *S. cerevisiae* mitochondria loaded with the florescent probe *fluo-3*, observed a ruthenium red-insensitive increase in $[Ca^{2+}]_m$ in response to cation addition and a decrease in $[Ca^{2+}]_m$ in response to EGTA inclusion. Ca^{2+} accumulation occurred by a concentration gradient mechanism with the exceedingly low rate of 2 to 7 nmol $^{45}Ca^{2+}$/min per mg protein. The low affinity of the system for Ca^{2+} and low initial rates of Ca^{2+} uptake have cast some doubt on the possible physiological role of this process and promoted the transient loss of interest in this phenomenon.

However, in 1983 Zvyagilskaya and coauthors [121, 122] showed that tightly-coupled mitochondria from the yeast *Endomyces magnusii* are capable of accumulating Ca^{2+} via an energy-dependent uniporter mechanism. Ca^{2+} uptake was specific (other divalent cations such as Mn^{2+}, Sr^{2+}, Ba^{2+}, Mg^{2+} at concentrations <1 mM were not accumulated by mitochondria) and the net uptake process was enhanced in the presence of permeable anions and inhibited by respiratory inhibitors and uncouplers. The plot of Ca^{2+} uptake versus Ca^{2+} concentrations in the medium was sigmoidal with saturation at ~300 μM Ca^{2+}. V_{max} was calculated as 1000 nmol/min per mg protein, while the K_m value appeared to be rather high, 150-180 μM. Later, it was found that the kinetic characteristics of the Ca^{2+}-transport system in *E. magnusii* mitochondria could be considerably improved in the presence of natural modulators, including polyamines (spermine and spermidine) [123], ADP, high intramitochondrial NADH/NAD ratio, and low Ca^{2+}-concentrations [124-126]. When all the mentioned regulators were added together, the initial rate of Ca^{2+} accumulation reached record values, i.e., up to 2000 nmol/min per mg protein, and mitochondria gained an ability to take up almost all Ca^{2+} from the incubation medium [124-126]. Unlike animal mitochondria, yeast mitochondria turned out to be particularly sensitive to low concentrations of effectors. The observed activation of Ca^{2+} accumulation by low Ca^{2+} concentrations suggests the existence of specific Ca^{2+}-binding regulatory sites on the uniporter. Ca^{2+} uptake was inhibited by Mg^{2+}, Mn^{2+}, and La^{3+} [127-129] and slightly activated by ruthenium red [130].

Pathways for Ca^{2+} release from Ca^{2+}-loaded mitochondria. *E. magnusii* mitochondria were proved to harbor a pathway for Ca^{2+} release which was independent of the Ca^{2+} uniporter [128, 129]. When mitochondria were incubated in medium containing acetate as permeable anion, we observed a spontaneous release of Ca^{2+} after accumulation of ~75% of the added cation. The Ca^{2+} release process was insensitive to cyclosporin A and Na^+. The rate of the process decreased upon addition of La^{3+}, Mn^{2+}, Mg^{2+}, tetraphenylphosphonium, and nigericin (in the presence of KCl), being stimulated by spermine and hypotonicity. We concluded that the system responsible for Ca^{2+} release from Ca^{2+}-loaded *E. magnusii* mitochondria is similar to the active Na^+-independent pathway for Ca^{2+} release in mitochondria of non-excitable animal tissues. Recently, Pfeiffer and coauthors [131] showed that in *S. cerevisiae* mitochondria artificially loaded with Ca^{2+} in the presence of the specific ionophore ETH 129, operates a novel $Ca^{2+}/2H^+$-antiporter, activated by fatty acids and catalyzing Ca^{2+} efflux via an electroneutral mechanism. It is rather complicated to evaluate the role of this pathway in Ca^{2+} release of *S. cerevisiae* mitochondria, which are known to lack any efficient natural system for cation accumulation. The Ca^{2+} uniporter and independent pathways for Ca^{2+} release would generate a steady Ca^{2+} circuit on the mitochondrial membrane, finely responding to alterations in the physiological states of the cell, and the lack of one of the components of this ensemble hampers the process as a whole.

Analysis of the data available shows that the only proved, effectively controlled Ca^{2+}-transport system in yeast mitochondria is the ruthenium red insensitive, high-capacity Ca^{2+}-transport system of *E. magnusii* mitochondria, displaying a relatively low affinity for Ca^{2+}, that however is greatly increased in the presence of low (physiological) concentrations of natural modulators.

PHYSIOLOGICAL ROLE OF MITOCHONDRIAL Ca^{2+} TRANSPORTING SYSTEMS

At present, there exist three main hypotheses about the physiological role of mitochondrial Ca^{2+} transporting systems.

Regulation of $[Ca^{2+}]_m$. According to this hypothesis, the mitochondrial Ca^{2+} transport system comprised of independent Ca^{2+} influx and efflux pathways is responsible for setting the $[Ca^{2+}]_m$ in a range (from submicromolar to low micromolar concentrations) thought to regulate activities of Ca^{2+}-sensitive enzymes of the Krebs cycle, resulting in increased NADH production and ATP synthesis [8, 16, 132, 133]. At present, at least four matrix enzymes are known to be activated by submicromolar Ca^{2+} concentrations: NAD-dependent isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, glycerol-3-phosphate dehydrogenase, and pyruvate dehydrogenase complex. In the latter, the Ca^{2+}-dependent enzyme is a phosphatase, transforming the complex to the active form, while for other dehydrogenases Ca^{2+} is an allosteric activator, decreasing K_m values for substrates. Recently,

with stimulated animal cells it has been shown that the agonist-dependent increase in $[Ca^{2+}]_c$ causes prolonged increased level of NADH [134] and ATP [135], thus confirming the idea that calcium ions are responsible for the long-term activation of oxidative metabolism. Of other Ca^{2+}-dependent intramitochondrial processes related to the mitochondrial Ca^{2+} transport system are the regulation of ATPase activity [136] and the Ca^{2+}-dependent changes in the mitochondrial volume [137].

Several enzymes in plant mitochondria are also sensitive to Ca^{2+}, in particular, NAD-dependent glutamate dehydrogenases of mitochondria from turnip (*Brassica rapa* L.) [138] and from tobacco (*Tobacco nicotiana* L.) calluses [139]. However, high (1-10 μM) Ca^{2+} concentrations required for half-maximal activation of the enzyme are incompatible with the physiological importance of this phenomenon. Instead, as postulated by Silva and coauthors [104], the Ca^{2+} transport system of plant mitochondria participates in intracellular P_i redistribution.

Mitochondrial pyrophosphatase in the yeast *S. cerevisiae* was shown to be inhibited by low concentrations of Ca^{2+}-pyrophosphate with the K_d value of ~1.9 μM. It was concluded that small Ca^{2+} fluxes control pyrophosphate activity and concentration in this yeast species [140]. NAD-dependent isocitrate dehydrogenase in the mitochondrial matrix of *E. magnusii* is also Ca^{2+}-sensitive [124, 126]. This suggests that the mitochondrial Ca^{2+}-transport system of yeasts, like the similar system of animal mitochondria, can control $[Ca^{2+}]_m$.

Regulation of $[Ca^{2+}]_c$. In spite of popularity of the first hypothesis, it is impossible to exclude a role of mitochondria (along with the endoplasmic reticulum and vacuole) in regulation of $[Ca^{2+}]_c$ under certain physiological conditions (see reviews [8, 16]). This type of regulation is typical for contractile animal tissues. Deposition (sequestration) of significant amounts of the cytosolic Ca^{2+} occurs during repeated synaptic activations, when $[Ca^{2+}]_c$ is high and mitochondria can temporarily reserve the cation [13, 141]. In cancer tissues, there also exists a temporary Ca^{2+} redistribution first from the endoplasmic reticulum into the cytosol and then, after activation of specific receptors, into mitochondria [142]. It is conceivable that mitochondria are involved in both types of regulation, depending on physiological conditions and tissue type.

Regulation of pathophysiological processes. Participation of Ca^{2+} transport systems of animal mitochondria in some pathological cellular reactions, mainly apoptotic and necrotic ones, is well known and actively debated (see reviews [19-21]). Under pathological conditions with oxidative stress as in ischemia–reperfusion, the $[Ca^{2+}]_c$ is progressively increased, thus triggering a Ca^{2+} cycle on the mitochondrial membrane via participation of independent Ca^{2+} influx and efflux systems and ultimately increasing $[Ca^{2+}]_m$ up to the threshold value of 1-3 μM [19]. These conditions promote the PTP induction accompanied by high-amplitude swelling of mitochondria, disturbing the integrity of the outer mitochondrial membrane thus releasing from the intermembrane space into the cytosol proapoptotic factors such as cytochrome *c*, apoptosis-inducing factor (AIF), a number of procaspases that are involved directly in the apoptosis signal pathways [20, 21]. Recently, other factors have been described also, i.e., Smac/DIABLO factor, further promoting apoptosis by acting as an inactivator of inhibitors of apoptotic proteins [143-145]. Thereby, Ca^{2+} overload on mitochondria in oxidative stress conditions and deficit of cellular ATP can bring about the most dramatic consequences at the level of the intact cell.

Figure 3 presents schematically physiological and pathological process related to the mitochondrial Ca^{2+} as exemplified on animal mitochondria (adapted from [19]). Functioning of independent pathways for Ca^{2+} influx and efflux under physiological condition results in establishing a certain $[Ca^{2+}]_m$ level required for activation of key matrix dehydrogenases of the Krebs cycle. Under pathological conditions, in the presence of Ca^{2+} and triggers (for instance, P_i) the opening of the PTP occurs, initiating apoptotic reactions and cell death.

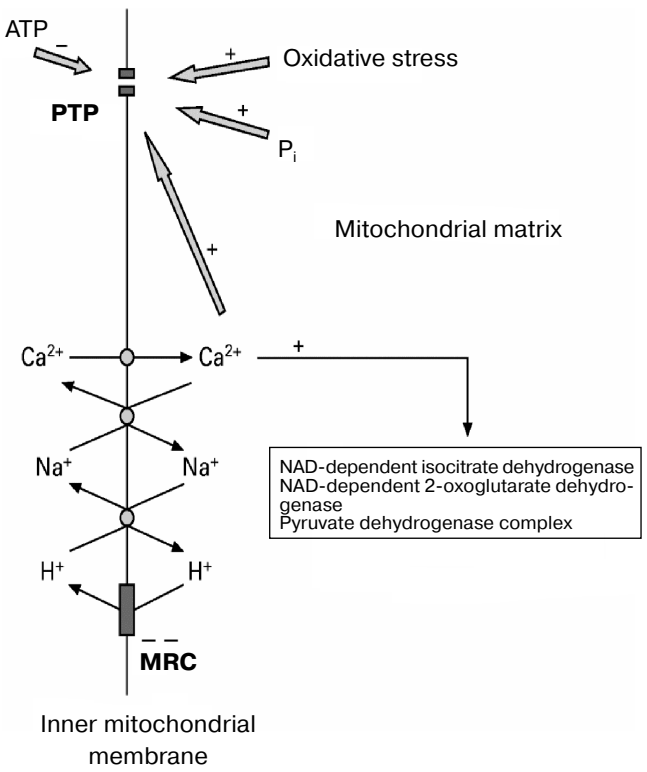

Fig. 3. A generalized scheme describing physiological and pathological effects of mitochondrial Ca^{2+} in animal mitochondria (adapted from [19]). For details, see the text. MRC, mitochondrial respiratory chain.

Gunter and coauthors [72, 73], discoverers of the phenomenon of rapid Ca^{2+} uptake, suggested that under physiological conditions the RaM pathway is mainly responsible for Ca^{2+} accumulation in large amounts and that it is a key mechanism in determining the $[Ca^{2+}]_m$ and, ultimately, in activating Ca^{2+}-sensitive dehydrogenases of the Krebs cycle, while the Ca^{2+} uniporter is responsible only for hormone signal transduction from the cytosol into mitochondria and for apoptosis.

Analysis of the data available shows that not only animal mitochondria, but also mitochondria from yeasts and algae possess efficient Ca^{2+} transport systems. Slow and low-affinity mitochondrial Ca^{2+} transport systems were revealed in a few of plants, while the pathway for Ca^{2+} release functioning independently of the Ca^{2+} uptake system was found only in corn mitochondria. Survey of Ca^{2+} transport activities of yeast mitochondria allows us to postulate that the highly active mitochondrial system possibly participating in regulation of oxidative metabolism can be ascribed only to the yeast *E. magnusii*. A role of mitochondrial Ca^{2+} transport systems in pathological processes in plants and yeast is now a nearest prospect.

Mitochondrial Ca^{2+} transport has been studied already more than 45 years, but the structure of the proteins responsible for this process, their arrangement in the mitochondrial membrane, and mechanisms underlying their functioning still remain uncertain. There is no graceful concept on how Ca^{2+} fluxes are organized *in vivo* and maintain the Ca^{2+} homeostasis in the cell. The Ca^{2+} transport system of yeast mitochondria with its taxonomic features and flexible regulation may serve as a promising model to solve these problems.

This study is supported by the Russian Foundation for Basic Research (grant Nos. 02-04-06641mas and 02-04-49717).

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