Slow Ca^{2+}-induced inactive/active transition of the energy-dependent Ca^{2+} transporting system of rat liver mitochondria: clue for Ca^{2+} influx cooperativity

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Abstract Rat liver mitochondria essentially free of endogenous Ca^{2+} show low initial rate of energy-dependent Ca^{2+} uptake. Preincubation of mitochondria under de-energized conditions in the presence of small amounts of external Ca^{2+} results in a 8–10-fold time-dependent increase of energy-dependent Ca^{2+} uptake. Ca^{2+}-dependent activation of the Ca^{2+}-transporting system follows first-order kinetics ($t_{1/2}$ ~1 min in the presence of 5 μM Ca^{2+} at 20°C). Ca^{2+}-activated mitochondria demonstrate a simple hyperbolic initial rate–Ca^{2+} concentration dependence, whereas strong apparent cooperativity is observed in the velocity–substrate curves for Ca^{2+}-depleted mitochondria. It is concluded that apparent cooperativity of the energy-dependent Ca^{2+} uptake is due to slow (as compared with the 'turnover number') activation of a Ca^{2+}-specific uniporter which is inactive in the absence of external Ca^{2+}.

Key words: Ca^{2+} transport; Cooperative kinetics; Rat liver mitochondria

1. Introduction

The important role of Ca^{2+} as an effective regulator for extra- and intramitochondrial metabolism is well recognized. The intracellular distribution of Ca^{2+} is largely dependent on the kinetic (K_m and V_{max}) and thermodynamic (quasi-equilibration and capacity) properties of various membrane-bound systems participating in Ca^{2+} sequestering and release. Energy-dependent accumulation of Ca^{2+} in mitochondria was discovered more than 30 years ago [1,2] and numerous studies on various aspects of Ca^{2+} movement across the inner mitochondrial membrane have been published (see review [3]). The kinetic parameters of Ca^{2+} uptake by isolated mitochondria from different tissues have been determined under different experimental conditions with different methods, and it is not unexpected that considerable quantitative and qualitative variations of the data have appeared in the literature [3]. The earliest studies on Ca^{2+} uptake velocity versus Ca^{2+} concentration using rapid filtration technique [4] or direct spectral stopped flow measurements with murexide [5] revealed strong sigmoidicidy of the curves. The model for the mitochondrial bivalent cation transporting system with an 'activating' site was proposed to explain the kinetic cooperativity of Ca^{2+} and Mn^{2+} uptake [5]. The methodological difficulties of in vitro studies of Ca^{2+} transport [6] have prompted several authors to reinvestigate kinetic parameters of Ca^{2+} uptake by liver mitochondria by monitoring the valinomycin-induced K^+ efflux-driven Ca^{2+} uptake, in order to exclude $\Delta\bar{\mu}_{H^+}$ generation as the limiting factor [7,8]. It was concluded that sigmoidal dependence of the uptake rate on Ca^{2+} concentration 'can be attributed to factors which are extraneous to the Ca^{2+} transport system per se' [8]. However, further evidence for an activating site have come independently from three different laboratories. The kinetics of Ca^{2+} uniporter in mitochondria has shown to be changed from strongly sigmoidal to near hyperbolic after transitory loss of the membrane potential [9], thus suggesting activation of the system upon binding of released Ca^{2+} at the activating site. It was further shown that chelation of external Ca^{2+} greatly inhibits the uncoupler-induced efflux of Ca^{2+} from Ca^{2+}-preloaded mitochondria [10,11]. Thus, Ca^{2+}-binding site (located on the cytoplasmic side of the inner mitochondrial membrane) responsible for induction of bivalent cation permeability, which seems to be identical to an 'activating' site postulated earlier [5], has been identified.

An important factor which may contribute significantly to Ca^{2+} uptake kinetics is the possible time-dependence of the Ca^{2+}-induced activation phenomenon. In this report we will show that, indeed, an increase of the Ca^{2+}-transporting system activity by Ca^{2+} is a slow process (as compared with the activated uniporter 'turnover'). A model is proposed which explains apparent controversies regarding mitochondrial Ca^{2+} transport kinetics. The possible physiological significance of Ca^{2+}-induced Ca^{2+} transport activation is briefly discussed.

2. Materials and methods

Mitochondria (respiratory control ratio of 5 : 7 with succinate) were isolated from rat liver homogenates (0.25 M sucrose and 1 mM EDTA, pH 7.4) by the conventional procedure [12] and washed in 0.25 M sucrose treated to remove contaminating Ca^{2+}. The stock suspension was stored in a polyethylene vial (80–100 mg/ml) in 0.25 M sucrose and 10 mM HEPES, pH 7.4. The endogenous Ca^{2+} content determined as the amount of Ca^{2+} released after addition of uncoupler (FCCP) was 4–6 nmol/mg of the mitochondrial protein. The standard reaction mixture contained: 0.1 M sucrose, 75 mM KCl, 1 mM potassium phosphate, 10 mM HEPES (pH 7.4) and 1 μM rotenone. Sucrose (0.25 M) and the reaction mixture (except for rotenone) were passed through an Amberlite IRC-718 column and contained less than 0.1 μM Ca^{2+}. Succinate oxidase activity was measured with an oxygen electrode. Ca^{2+} uptake was measured as a difference in absorption at 662 and 692 nm with 50 μM arsenazo III as metallochromic indicator [13]. The membrane potential change was recorded as a difference in absorption of 5 μM safranine O at 555 and 523 nm [14]. An 'in-house' produced dual-wavelength photometer equipped with constant mixing device was used. The time response of the instrument calibrated by the additions of dyes was not more

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Abbreviations: EGTA, [ethylene*bis*(oxyethylenenitrilo)]tetraacetic acid; FCCP, carbonylcyanide-4-trifluoremethoxyphenylhydrazone; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid

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than 0.5 s. The mitochondrial protein content was determined by the biuret procedure [15].

Rotenone, safranine 0 and Amberlite IRC-718 were from Serva (Germany), arsenazo Ill and HEPES were from Sigma (USA), FCCP was from Fluka (Switzerland), other reagents were of the purest grade commercially available.

3. Results

Fig. 1 depicts examples of the actual tracing of Ca^{2+} uptake by rat liver mitochondria. When the reaction was started by the addition of Ca^{2+} followed by almost immediate energization by succinate, the time-course of Ca^{2+} uptake was triphasic (Fig. 1A): the initial rate was very low; it gradually increased within about 10 s and finally decreased upon external Ca^{2+} accumulation. Remarkably, the initial rate of Ca^{2+} uptake started by the second addition of Ca^{2+} was much higher. It was shown in the separate samples without Ca^{2+} additions that the membrane potential increase induced by succinate as measured with safranine 0 showed no lag in the response and it was the same when succinate was added 2 or 12 min after mitochondria. When the same experiments were conducted except that mitochondria were preincubated with externally added Ca^{2+} for 10 min the initial rate of Ca^{2+} uptake was about 10 times higher and it was further slightly increased by the second Ca^{2+} addition (Fig. 1B). Fig. 2 shows the time-dependence of the initial Ca^{2+} uptake rate on the time interval between Ca^{2+} addition to the non-energized mitochondria and

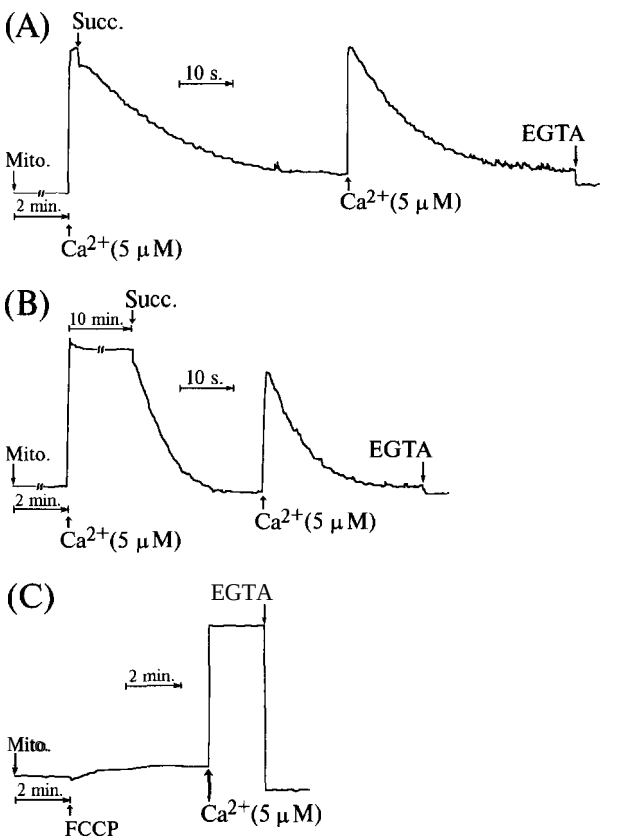

Fig. 1. Time-course of Ca^{2+} uptake by rat liver mitochondria. The standard reaction mixture contained 50 μM Arsenazo Ill and the absorption difference at 662 nm and 692 nm was recorded. Mitochondria (0.2 mg/ml), succinate (5 mM), $CaCl_2$, EGTA (20 μM), and FCCP (1 μM) were added where indicated. C: Control tracing for the instrument calibration.

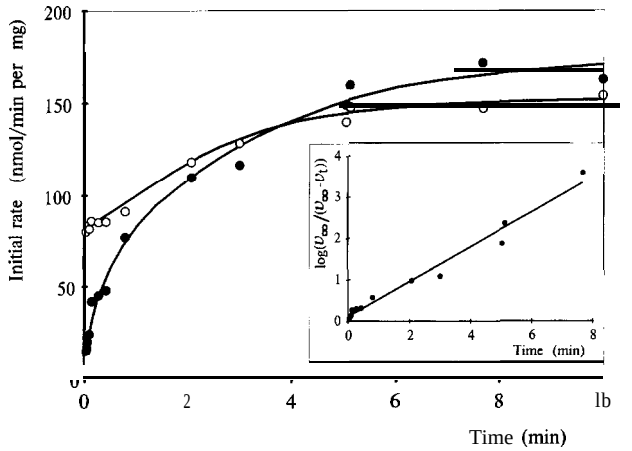

Fig. 2. Time-dependence of the initial Ca^{2+} uptake rate. Experiments were performed as described in Fig. 1 and the time interval between the addition of Ca^{2+} and energization of mitochondria by the addition of succinate was varied. Initial rate values for first (●) and second (○) additions of 5 μM $CaCl_2$. Insert: semilogarithmic plot of the data shown by the closed symbols. v_∞ and v_t, initial rates measured after 12 and t min preincubation of the de-energized mitochondria with 5 μM $CaCl_2$ (see Fig. 1B).

the addition of succinate. The semi-logarithmic plot (Fig. 2, insert) gives the apparent first-order rate constant for the external Ca^{2+}-induced activation of Ca^{2+} uniporter of 0.5 min^{-1}; this value is about $4{\cdot}10^5$ times less than a 'turnover number' of the activated Ca^{2+} uniporter at 5 μM Ca^{2+} ($\sim 2{\cdot}10^5$ min^{-1} assuming a content of the uniporter of 10^{-3} nmol/mg of the mitochondria protein based on the Ruthenium Red sensitivity of Ca^{2+} transport titer [16]).

It was obviously of interest to compare the standard kinetic parameters of the energy-dependent Ca^{2+} uptake for the conditions where the reaction was started by addition of succinate immediately (2 s) after Ca^{2+} and those for the Ca^{2+}-activated

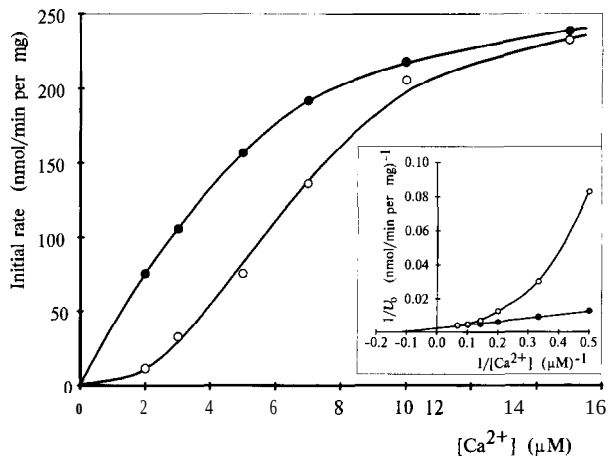

Fig. 3. Dependence of the initial Ca^{2+} uptake rate on the added Ca^{2+} concentration. (○), Ca^{2+} uptake was initiated by addition of succinate 2 s after the addition of Ca^{2+} as shown in Fig. 1A; (●), the reaction was started by addition of succinate 10 min after preincubation of the de-energized mitochondria in the presence of 2 μM externally added Ca^{2+}. Insert: double-reciprocal plot of the data. The parameters of the succinate-supported energy-dependent Ca^{2+} uptake derived from the double-reciprocal plot for the activated Ca^{2+}-specific uniporter are: $K_m^{Ca^{2+}} = 9\mu M, V_{max} = 0.36$ μmol/min per mg, 20°C, pH 7.4).

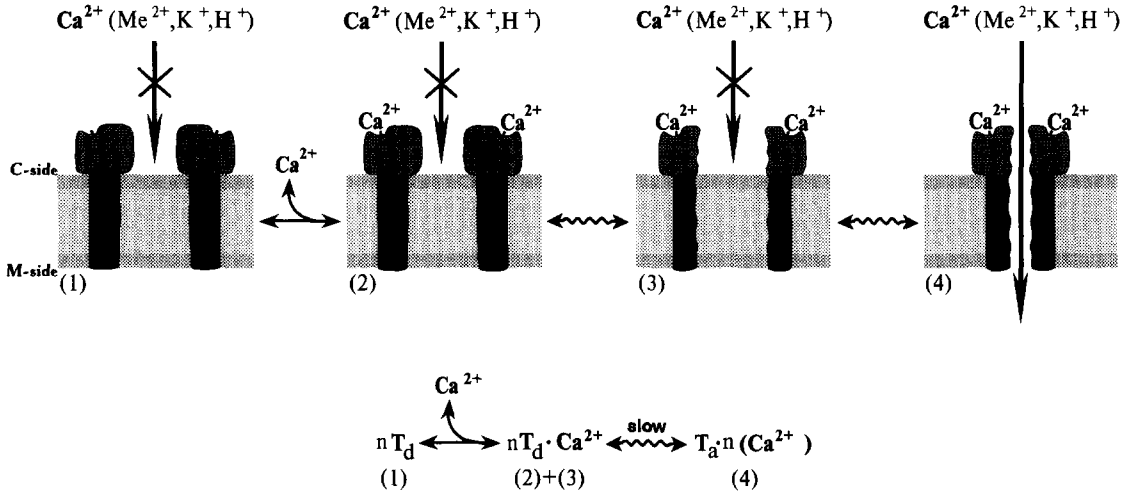

Fig. 4. A scheme to illustrate the proposed mechanism of the energy-dependent Ca^{2+} transport in mitochondria. The external Ca^{2+}-dependent formation of active uniporter (4) is shown as an oligomerization of the protomers (1) resulting in a formation of the ruthenium- and lanthanides-sensitive gated channel [20,21]. For the sake of simplicity two protomers are shown although more subunits may participate in the active uniporter formation. The kinetic description of the proposed mechanism includes rapid binding of 'activating' Ca^{2+} to n de-activated protomers (T_d) followed slow formation of active uniporter. The experimental results do not permit to specify the slow step as either Ca^{2+}-induced conformational change of the single protomer ((2) → (3) transition), or as an association step ((3) → (4) transition). High and low conductivity of the gated channel for Ca^{2+} and other cations (Me^{2+}, K^+, H^+) respectively is emphasized.

uniporter. Fig. 3 shows strong apparent sigmoidicity of the velocity–substrate curve for the reaction initiated by succinate immediately after Ca^{2+} and a simple hyperbolic curve for the Ca^{2+}-preactivated transport induced by the delayed (12 min) addition of succinate.

4. Discussion

The data presented in this report show unambiguously that the Ca^{2+} uniporter is present in the inner mitochondrial membrane in de-activated form (in the absence of external Ca^{2+}) and it is *slowly* (as compared with the 'turnover' of the active state) activated by Ca^{2+}. Our findings are in line with previous reports on Ca^{2+}-induced Ca^{2+} release from de-energized mitochondria [10,11] and those on external Ca^{2+}-induced modification of the transport kinetic parameters [9]. In addition to providing new information on the slow interconversion between de-activated and active forms of Ca^{2+}-uniporter our data seem to explain the contradictory reports on the sigmoidal [4,5] or simple hyperbolic [7,8] dependence of the reaction rate on Ca^{2+} concentration. In addition to obvious differences in experimental conditions employed in different laboratories (mitochondria from different tissues, ionic composition of the reaction mixture, the Ca^{2+} measurement methods) the initial state of the uniporter appears to be a crucial factor. The latter is expected to be dependent, in particular, on protein concentration: when a relatively high content of the mitochondria is used in the experiments [7,8] the release of endogenous Ca^{2+} before membrane energization would result in activation of the uniporter and hyperbolic kinetics would be observed.

The scheme in Fig. 4 describes a model for the Ca^{2+} uniporter operation in the inner mitochondrial membrane. We believe that the existence of Ca^{2+}-transporting system in the de-activated state in the absence of cytoplasmic Ca^{2+} is an important factor for cell physiology. Since no absolute specificity for the substrate exists, the active uniporter in the absence of Ca^{2+} in the energized membrane would result in a permanent accumulation of cations in the mitochondrial matrix and/or in futile uncoupling, if H^+ would travel through the channel designed for Ca^{2+} uptake. Observations on the Ruthenium Red-sensitive uncoupling of mitochondria at acid pH [17] and an increase of coupling capacity of submitochondrial particles by lanthanides [18] are relevant in supporting this hypothesis. On the other hand, a low level of cytoplasmic Ca^{2+} which would give rise to the active form of bivalent cation uniporter, may serve as a transitory regulator for the physiologically controlled 'mild uncoupling' of mitochondria (thermogenesis, acceleration of NADH oxidation, regulation of oxygen concentration [19]).

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