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FUNCTIONAL ACTIVITY OF PERMEABILITY TRANSITION PORE IN ENERGIZED AND DEENERGIZED RAT LIVER MITOCHONDRIA

O. V. AKOPOVA✉, *L. I. KOLCHINSKAYA*, *V. I. NOSAR*

Bogomoletz Institute of Physiology, National Academy of Sciences of Ukraine, Kyiv;
✉*e-mail: ov_akopova@ukr.net*

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Permeability transition pore (mPTP) opening was studied under energized and deenergized conditions in rat liver mitochondria, and the effect of membrane depolarization on mPTP activity was evaluated. To assess mPTP activity, cyclosporine-sensitive swelling and cyclosporine sensitive Ca^{2+} *efflux from mitochondria was studied using light absorbance techniques. In energized mitochondria, mPTP opening in sub-conductance states, at* $[Ca^{2+}] \leq K_a$*, contributed positively to the rate of respiration, without affecting* $\Delta\Psi_m$*. Threshold* Ca^{2+} *concentrations were found, which excess resulted in fast mitochondrial depolarization upon mPTP opening. An estimate of mPTP activity by cyclosporine-sensitive* Ca^{2+} *transport under energized and deenergized conditions showed that membrane depolarization by protonophore CCCP essentially increased initial rate (*V_0*), at simultaneous decrease of the half-time (*$t_{1/2}$*) of* Ca^{2+} *efflux, which indicated mPTP activation, as compared to energized mitochondria. However, only partial release of* Ca^{2+} *via mPTP upon membrane depolarization was observed. With the use of selective blockers of* Ca^{2+} *uniporter and mPTP, ruthenium red (RR) and cyclosporine A (CsA), partial contribution of* Ca^{2+} *uniporter and mPTP in* Ca^{2+} *transport was found. "Titration" of* Ca^{2+} *transport by adding RR at different times from the onset of depolarization showed that depolarization dramatically reduced "life span" of mPTP as compared to energized mitochondria, which agreed with the kinetic characteristics of CsA-sensitive* Ca^{2+} *transport after the abolition of* $\Delta\Psi_m$*.* Ca^{2+} *added from the outer side of mitochondrial membrane produced dual effect on mPTP activity: activation at the onset of depolarization, but consequent promotion of mPTP closure. Based on the experiments, it was concluded that mitochondrial energization was required for prolonged mPTP functioning in sub-conductance states, whereas membrane depolarization promoted the transition of mPTP to inactive state during calcium release from mitochondria.*

Keywords: rat liver mitochondria, calcium, permeability transition pore, Ca^{2+} *transport, membrane potential.*

Disclosure of the molecular composition of mitochondrial Ca^{2+} uniporter (MCU) [1] and novel data on the molecular nature of mitochondrial permeability transition pore (mPTP) [2] started new wave of research interest to mitochondrial Ca^{2+} transport and its functions in living cells. Mitochondria play important role in the regulation of cellular calcium homeostasis and exhibit high plasticity to the fast fluctuations of cytosolic Ca^{2+}, which is maintained by mitochondrial Ca^{2+} transporting machinery [3-5]. As it is known, mitochondrial Ca^{2+} transporting system basically encompasses Ca^{2+} uptake by MCU, 'rapid Ca^{2+} uptake mode' and mitochondrial ryanodine receptors, and Ca^{2+} efflux via Na^+/Ca^{2+}- and H^+/Ca^{2+}-exchangers [3, 5]. Basically, Ca^{2+} uptake via MCU is counterbalanced by

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Ca^{2+} efflux via Na^+/Ca^{2+}- and $H+/Ca^{2+}$-exchangers, which operate together forming Ca^{2+} cycle in energized mitochondria.

In the past decade plenty of evidence was obtained, which confirmed earlier knowledge that the impairments of mitochondrial Ca^{2+} handling, both uptake and efflux, constitute molecular basis of several cardiovascular, neurodegenerative diseases, and other pathophysiological conditions [6-8].

Mitochondrial Ca^{2+} overload is known to induce the opening of permeability transition pore (mPTP) and promote cellular apoptosis [9, 10]. On functional basis, mPTP is a non-selective megachannel composed of the proteins of inner and outer mitochondrial membrane. For more than three decades several proteins were proposed as core components of this multiprotein complex: VDAC, ANT, cyclophylline D, mitochondrial peripheral benzodiazepine receptor, hexokinase II, and others (reviewed in details e.g. in [11]), and recently, F_0F_1 ATP synthase [2]. However, rigorous studies based on the genetic deletion of putative mPTP subunits challenged proposed hypotheses on the molecular composition of mPTP, which in spite of the efforts of several research groups, still remains undisclosed [12]. Interestingly that each of the proteins proposed as mPTP constituent has its specific well described cellular function not related to mPTP, and none yet was found specifically related to mPTP formation.

In spite of lasting discussions about molecular architecture of mPTP, it's biophysical and biochemical properties are relatively well studied. It is known that mPTP undergoes a number of transitions between low- and high-conductance states, and about six of such sub-conductance states were described [13, 14]. It is well known that mPTP opening in high conductance states results in the collapse of mitochondrial membrane potential ($\Delta\Psi_m$), loss of vital matrix proteins and solutes, induction of cellular apoptosis and necrosis [9, 10, 15]. Meanwhile, mPTP functioning in low conductance states is much less studied. The main issue is whether mPTP opening in low conductance states can accomplish any functions relevant to normal physiological conditions. In the literature, it was hypothesized that mPTP opening in sub-conductance states could take part in Ca^{2+} signaling [4, 16] and the modulation of metabolism [17], while mPTP opening in high conductance states was definitely considered as the trigger of cell death [15, 16].

As we have shown earlier, in low-conductance states mPTP releases Ca^{2+} in exchange for protons [18], takes part in Ca^{2+} cycling and oxygen consumption without much affecting $\Delta\Psi_m$ in rat liver mitochondria [19]. Based on our previous studies, reversible mPTP opening in low conductance states in liver mitochondria contributed to ROS production and caused mild uncoupling of Oxphos [19, 20].

It is generally accepted that mPTP can help mitochondria get rid of the excess of accumulated Ca^{2+} [4], however, in the literature it was doubted whether mPTP can operate as Ca^{2+} efflux system of mitochondria [21]. Under energized conditions MCU works as RR-sensitive Ca^{2+} uptake system, and RR-insensitive Ca^{2+} efflux from mitochondria occurs via Na^+/Ca^{2+}- or Ca^{2+}/H^+-exchangers [3, 5, 21]. As it is known, mPTP too releases Ca^{2+} by RR-insensitive mechanism. Based on our earlier study [18], RR-insensitive and CsA-sensitive release of Ca^{2+} occurs in exchange for stoichiometric uptake of protons, so we hypothesize that CsA-sensitive Ca^{2+} efflux cannot be considered as simple diffusion via concentration gradient. However, the possible function of mPTP as Ca^{2+} efflux system in energized mitochondria did not receive much attention in the literature.

Contrary to energized conditions, mitochondrial depolarization triggers massive RR-sensitive release of Ca^{2+}, presumably via MCU operating in a 'reversal' mode [3]. As it is known, mPTP is highly susceptible to activation by mitochondrial membrane depolarization [22]. So, to assess the role of mPTP in the release of Ca^{2+} from deenergized mitochondria, combined function of mPTP and MCU should be considered, and partial contribution of mPTP in Ca^{2+} transport should be evaluated.

So, for the purpose to assess the properties of mPTP as Ca^{2+} transporting system in rat liver mitochondria, the aim of this work was to compare the kinetics of cyclosporine-sensitive Ca^{2+} efflux under energized and deenergized conditions, and to find partial contributions of mPTP and MCU in Ca^{2+} efflux from deenergized mitochondria.

Materials and Methods

Mitochondrial preparations. The work has been carried out in accordance with "Guide for the Care and Use of Laboratory Animals" 8th ed. Washington, DC: National Research Council of the National Academies: The National Academic Press, 2011 approved by the Ethics Commission on Animal Experiments of Bogomoletz Institute of Physiology, NAS of Ukraine. Adult Wistar-Kyoto female rats with 180-200 g mean body weight were used. Liver was washed by cold 0.9% KCl solution (4°C), minced and homogenized in 1:5 volume of the isolation medium: 250 mM sucrose, 1 mM EDTA, 1 mg/ml BSA, 20 mM Tris-HCl buffer, 4°C (pH 7.2). Mitochondria were isolated by centrifugation for 7 min at 1000 g (4°C) and after the pellet have been discarded; supernatant was centrifuged again for 15 min at 12 000 g (4°C). Final pellet was resuspended in a small volume of isolation medium without EDTA and stored on ice. The protein content was determined by the Lowry method.

Absorbance assay. mPTP opening was monitored spectrophotometrically based on the absorbance decrease at 520 nm due to mitochondrial swelling starting from the additions of mitochondria at 0.5 mg/ml to standard incubation medium: 120 mM KCl, 5 mM Tris-HCl-buffer (pH 7.4), 5 mM Na glutamate, 1 mM KH_2PO_4.

Calcium transport. Ca^{2+} transport was studied spectrophotometrically in the presence of 70 µM of Ca^{2+} indicator arsenazo III in standard incubation medium. Absorbance was monitored by double wavelength technique at 654 and 700 nm. To remove trace amounts of Ca^{2+}, 5 µM EGTA was routinely added to the incubation medium. Mitochondria were added at ~ 0.3 mg/ml protein.

Oxygen consumption assay. Oxygen consumption was studied polarographically in 1 cm^3 closed termostated cell at 26°C with platinum electrode at constant stirring in the same standard incubation medium. Mitochondria were added at 1.5-2.0 mg/ml protein.

The estimate of mitochondrial membrane potential: $\Delta\Psi_m$ was assessed by potentiometric method using TPP^+-sensitive electrode [23]. Measurements were carried out in standard incubation medium in a closed 1 cm^3 cell at room temperature and constant stirring. TPP^+ was added at 10 µM. The response of TPP^+ electrode to TPP^+ additions was calibrated by adding the aliquots of TPP^+ to the incubation medium. The amount of TPP^+ taken up by mitochondria was found from the calibration curves. TPP^+ concentration in the matrix was estimated based on the known matrix volume of 1.6 µl/mg protein for liver mitochondria [24]. $\Delta\Psi_m$ calculated from the Nernst equation constituted −170 ± 5 mV in our preparations. High energy state of native liver mitochondria was confirmed by the high RCR (about 7-8) which reflected tight coupling of mitochondrial preparations.

Chemicals. All reagents were from Sigma-Aldrich, USA. Deionized water was used for medium preparations.

Statistical analysis. The data were expressed as mean ± S.E. of 4-6 independent experiments. Statistical analysis was performed using paired Student's *t*-test; $P < 0.05$ was taken as the level of significance.

Results and Discussion

At present, there are no means to assess biophysical properties of mPTP directly in isolated mitochondria. In our work mPTP activity was assessed based on cyclosporine-sensitive swelling caused by water uptake, which increased with the increase in Ca^{2+} concentration (Fig. 1, *A*, *B*). Based on the literature, increase in swelling amplitude indicated increase in mPTP activity and its conductance as nonselective channel. Apparent activation constant K_a found from the concentration dependence (Fig. 1, *B*) constituted 35 ± 5 µM Ca^{2+}. Hill coefficient 2 found from linearized plot (not shown) indicated allosteric activation of mPTP by Ca^{2+} ions, and two types of Ca^{2+} binding sites for mPTP acticvation, which agreed with the literature [25].

From the literature, it is known that mPTP can operate in sub- and high conductance states [16], and mPTP switch from low- to high conductance state triggers the transition from physiological conditions to cellular apoptosis and necrosis [15, 16]. However, how much Ca^{2+} is required for mPTP transition from low- to high conductance state(s) in isolated mitochondria, is dependent on a variety of conditions, such as mitochondrial energy state, redox state of NADH pool and of thiol groups involved in mPTP functioning [26], redox state of Q-pool [27], ROS formation [28], membrane fluidity [29], and others. So, we assumed that Ca^{2+} additions that allowed mitochondria to retain stable $\Delta\Psi_m$ upon mPTP opening, corresponded to reversible mPTP operation in low-conductance state(s), which blocking by the addition of CsA fully restored mitochondrial functions. Unlike this, it is known that mPTP opening in high conductance states results in fast mitochondrial depolarization caused by the outer membrane rupture, release of cytochrome c, of vital matrix solutes, and irreversible collapse of mitochondrial functions. So, to find the threshold amounts of Ca^{2+} needed for mPTP transition from low to high conductance states, we monitored the response of mitochondrial $\Delta\Psi_m$ to the additions of Ca^{2+} aliquots to mitochon-

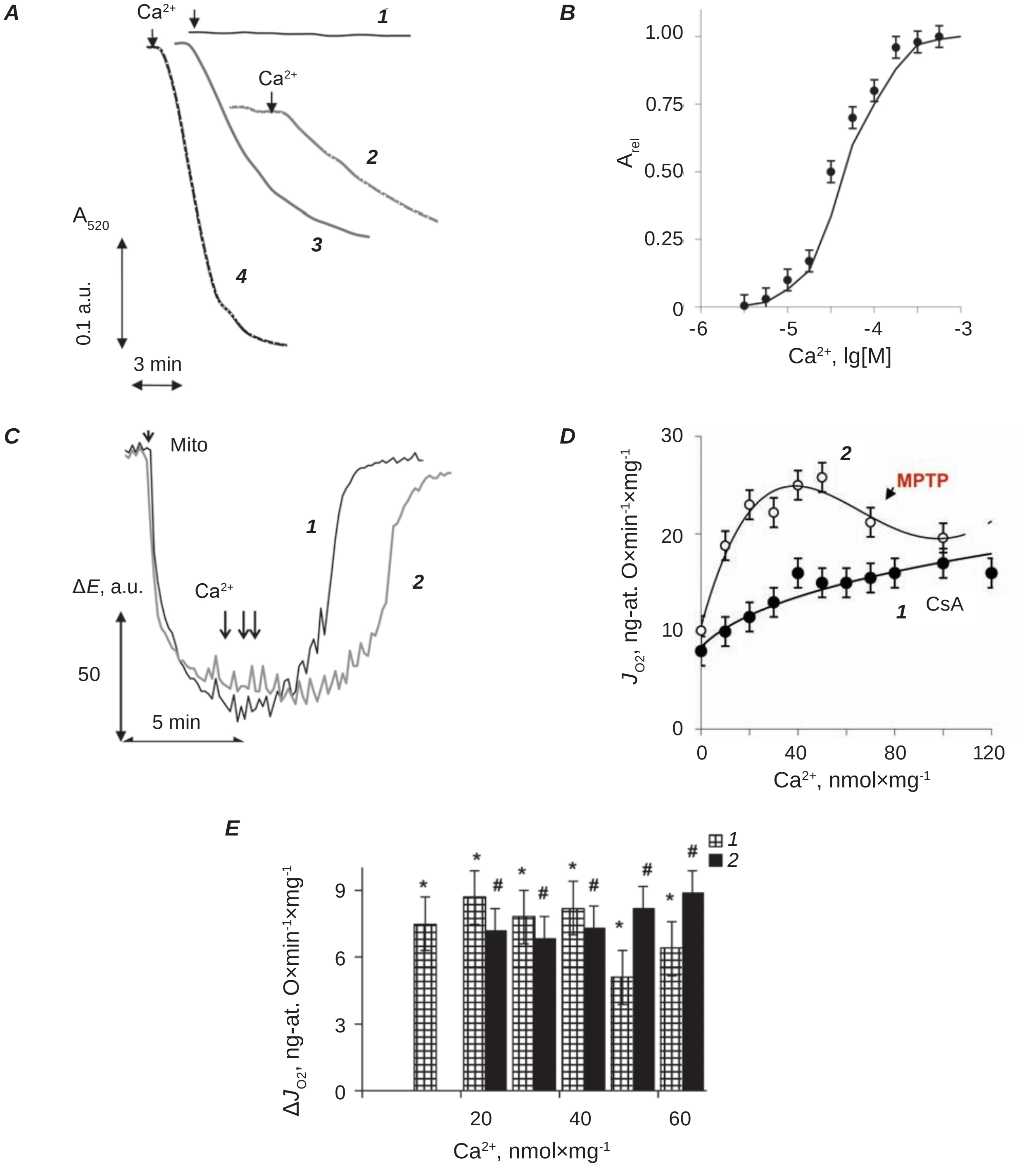

*Fig. 1. The effect of Ca^{2+} on mitochondrial swelling (**A**, **B**), membrane potential (**C**) and respiration (**D**, **E**). **A**: representative traces of mitochondrial swelling; **B**: normalized amplitude of cyclosporine-sensitive swelling. **C**: Titration of mitochondrial membrane potential (**C**) by the additions of Ca^{2+} (arrows; not all additions were shown for clarity); **D**: dependence of the rate of respiration on added Ca^{2+}; **E**: cyclosporine-sensitive (1) and RR-sensitive (2) changes in the rate of respiration (absolute values). M ± m, n = 6. *$P < 0.05$ (as compared to control in the presence of CsA); $^{\#}P < 0.05$ (as compared to control without RR)*

drial suspensions in the presence and the absence of cyclosporine A (Fig. 1, *C*).

'Titration' of $\Delta\Psi_m$ by the additions of Ca^{2+} showed that mPTP opening promoted Ca^{2+}-dependent depolarization and reduced Ca^{2+} accumulating capacity of mitochondria (Fig. 1, *C*, *1*). In the absence of CsA threshold Ca^{2+} concentration required for membrane depolarization constituted ~180 μM, which was near the plateau on the concentration dependence (Fig. 1, *B*). mPTP blocking by CsA increased threshold $[Ca^{2+}]$ up to ~360 μM. Worth notion that, regardless of the activation of mPTP and large amplitude swelling (Fig. 1, *A*), mitochondria were capable of maintaining stable $\Delta\Psi_m$ in a wide interval of Ca^{2+} concentrations (Fig. 1, *B*).

Parallel recording of oxygen consumption showed that mPTP opening at low $[Ca^{2+}]$ ($\le K_a$) increased the rate of state 4 respiration, as compared to the rates of respiration in the presence of CsA (Fig. 1, *D*). The 'titration' of mitochondrial respiration by CsA and RR added alternately to mitochondrial suspensions showed equal contribution of mPTP and MCU to the Ca^{2+} cycle at low $[Ca^{2+}]$ (Fig. 1, *E*, *1*, *2*). So, mPTP opening in low-conductance states reduced Ca^{2+} accumulating capacity (Fig. 1, *B*) and contributed to the rate of respiration (Fig. 1, *D*, *E*) without dramatic effects on mitochondrial energy state. However, to answer the issue whether mPTP can operate as Ca^{2+} transporting mechanism of mitochondria, and contribute to mitochondrial Ca^{2+} transport, Ca^{2+} transporting properties of mPTP should be estimated. So, with the aim to assess the role of mPTP in mitochondrial Ca^{2+} transporting system, we examined the kinetics of cyclosporine sensitive Ca^{2+} efflux from energized and deenergized mitochondria.

Cyclosporine-sensitive Ca^{2+} efflux was studied after the accumulation of aliquots of added Ca^{2+}. When required, RR was added to block MCU. For mitochondrial depolarization CCCP was added simultaneously with RR. Representative traces of Ca^{2+} transport in the absence and the presence of cyclosporine A are shown on Fig. 2, *A*, *B*. Fig. 2, *C*, *D* shows kinetic characteristics of cyclosporine-sensitive Ca^{2+} efflux from energized and deenergized mitochondria obtained after the blocking of MCU with RR.

In the absence of CsA spontaneous efflux of Ca^{2+} was observed after Ca^{2+} accumulation, which indicated mPTP opening (Fig. 2, *A*, *1*). At Ca^{2+} concentrations below threshold values (Fig. 1, B) Ca^{2+} efflux occurred without depolarization and was blocked by CsA, which testified functional activity of mPTP in energized mitochondria. Addition of RR visibly increased the rate of Ca^{2+} efflux (Fig. 2, *A*, *B*, *1*). Meanwhile, RR-insensitive Ca^{2+} transport was almost completely blocked by CsA, which means that the activity of other than mPTP Ca^{2+} efflux pathways (such as H^+/Ca^{2+}-exchanger) was negligible as compared to mPTP activity (Fig. 2, *B*, *1*, *2*). This indicated that in energized mitochondria the rate of Ca^{2+} efflux was mainly the resultant of the rates of Ca^{2+} uptake via MCU and Ca^{2+} efflux via mPTP. The blocking of MCU with RR resulted in the shift of this equilibrium towards Ca^{2+} efflux via mPTP. This allowed the estimation of mPTP activity based on the initial velocities of RR-insensitive and CsA-sensitive Ca^{2+} efflux.

Also, the rate of Ca^{2+} uptake under steady state conditions related to state 4 respiration, primarily was the resultant of the differences between the rates of Ca^{2+} uptake via MCU and Ca^{2+} efflux via mPTP. This could explain the decrease of Ca^{2+} uptake caused by mPTP opening, even at stable level of $\Delta\Psi_m$ (Fig. 2, *A*).

Membrane depolarization resulted in the reversal of MCU, which caused RR-sensitive Ca^{2+} efflux from mitochondria. For this reason, kinetic characteristics of cyclosporine-sensitive Ca^{2+} transport under energized and deenergized conditions were obtained in the presence of MCU blocker RR. In energized mitochondria mPTP activity was estimated from initial rates of CsA-sensitive Ca^{2+} efflux after the addition of RR. For depolarization conditions, $\Delta\Psi_m$ was simultaneously abolished by the addition of CCCP. CCCP and RR were added after the accumulation of added Ca^{2+}. Ca^{2+} was added at 30 μM, i.e. around K_a and below the threshold values, which caused irreversible impairments of mitochondrial functions upon mPTP opening (Fig. 1, *B*).

As showed the experiments, time dependences of cyclosporine-sensitive Ca^{2+} efflux were well approximated by exponential dependences, which agreed with first order kinetics of the transport process: $P = P_{max}(1 - e^{-kt})$. In energized mitochondria V_0 of cyclosporine-sensitive Ca^{2+} transport found directly from the plots constituted 45.0 ± 7.0 nmol $Ca^{2+} \cdot min^{-1} \cdot mg^{-1}$, which increased to 71.0 ± 8.5 nmol $Ca^{2+} \cdot min^{-1} \cdot mg$ under depolarization conditions (Fig. 2, *C*).

Rate constants and the half-times k and $t_{0.5}$ found from linearized exponential dependences for

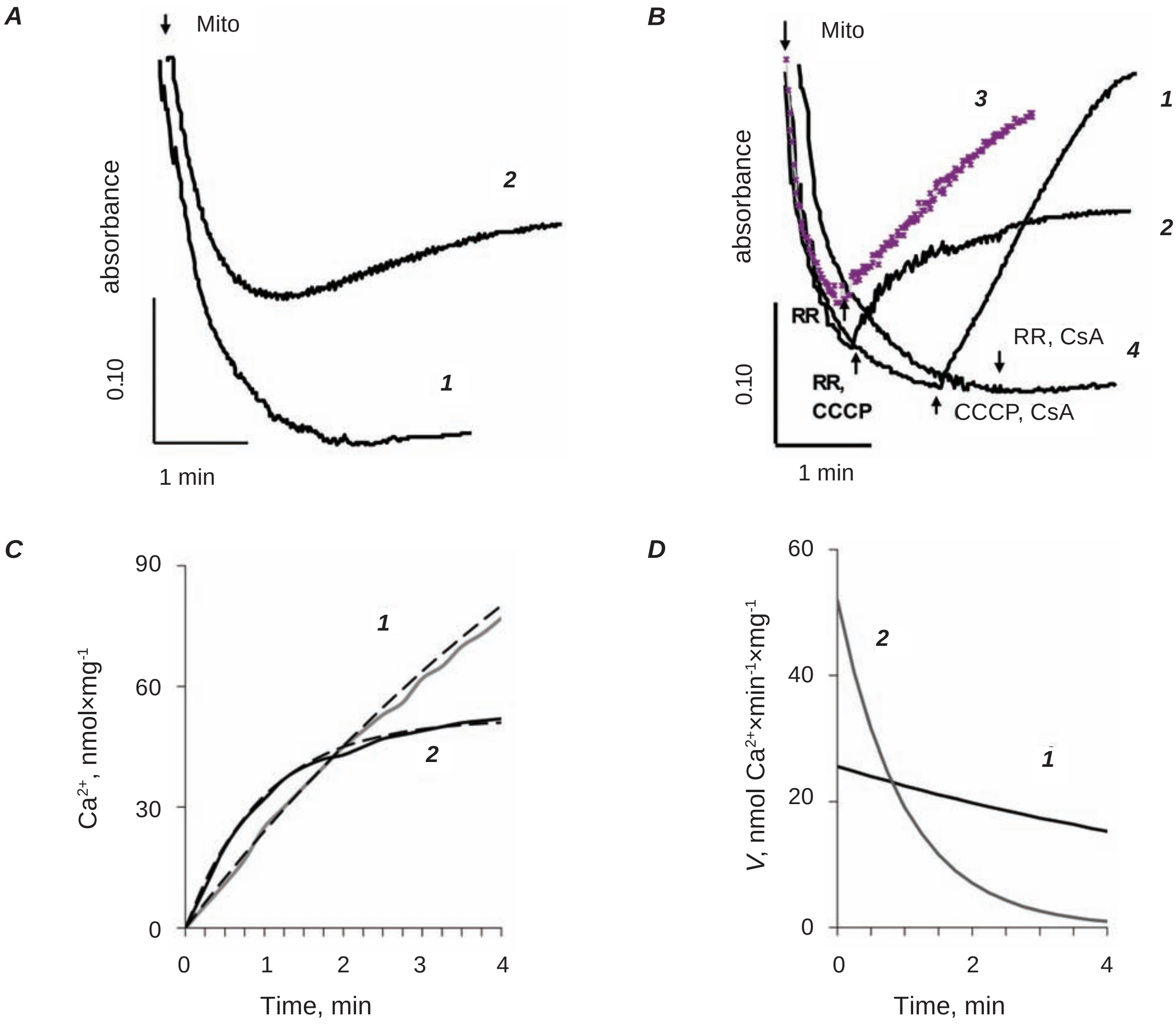

Fig. 2. The effect of mPTP opening on Ca^{2+} transport in rat liver mitochondria. ***A**, **B**: typical time courses of the changes in absorbance of Ca^{2+}-arsenaso III complexes in the absence and the presence of cyclosporine A in native mitochondria (**A**, 1, 2) and after the additions of CCCP (**B**, 1, 2, 4), RR (**B**, 2-4), and CsA (**B**, 1, 4) as shown by the arrows. **C**: RR-insensitive and cyclosporine-sensitive Ca^{2+} efflux from energized (1) and deenergized (2) mitochondria; time courses (solid lines) were approximated by exponential dependences (dashed lines, detailed in the text). **D**: instantaneous velocities of RR-insensitive Ca^{2+} transport in energized mitochondria (1) and after the depolarization (2). Ca^{2+} was added at 30 µM (100 nmol/mg), CsA at 1µM, CCCP at 1 µM, RR at 10 µM*

cyclosporine-sensitive Ca^{2+} transport (not shown), constituted 0.128 min^{-1} and 5.4 min (Fig. 2, C, 1) in energized mitochondria vs. 1 min^{-1} and 0.69 min (Fig. 2, *C*, *2*) under depolarization. Increase in V_0, rate constant and the reduction of the half-time of Ca^{2+} efflux showed the activation of mPTP upon the abolition of $\Delta\Psi_m$, which agreed with biophysical properties of mPTP as potential-dependent channel [13, 22]. Worth notion that under energized conditions relatively slow CsA-sensitive Ca^{2+} efflux almost completely released added Ca^{2+} from the matrix (Fig. 2, *C*, *1*). Unlike this, membrane depolarization essentially limited Ca^{2+} release from mitochondria (Fig. 2, *C*, *2*). Incomplete release of Ca^{2+} from deenergized mitochondria allowed an assumption of mPTP transition to inactive state.

To test this hypothesis, we set a goal to find the timeframes of mPTP functioning in active state after the abolition of $\Delta\Psi_m$. For this purpose we compared the changes with time of the rates of CsA-sensitive

Ca^{2+} efflux from energized and deenergized mitochondria. Instantaneous velocities of Ca^{2+} transport (V_t) were found from the equation: $V_t(t) = P_{max}\cdot k\cdot e^{-kt}$ obtained by differentiation of kinetic curves of CsA-sensitive Ca^{2+}-efflux (Fig. 2, *D*). Obtained time dependences indicated slow decay of instantaneous velocity of CsA-sensitive Ca^{2+} transport in energized mitochondria (Fig. 2, *D, 1*). In deenergized mitochondria, initial activation of mPTP based on the obtained V_t values at the onset of depolarization was observed followed by fast decay of CsA-sensitive pathway, as compared to the same in energized mitochondria (Fig. 2, *D*, *2*). In line with the limited release of Ca^{2+} from the matrix (Fig. 2, *C*, *2*), fast decay of the rate of CsA-sensitive Ca^{2+} transport to zero too indicated the termination of mPTP functioning shortly after depolarization. To examine this observation in more detail, it was of interest to find partial contribution of mPTP to mitochondrial Ca^{2+} transport under deenergized conditions.

For this purpose, Ca^{2+} efflux from deenergized mitochondria was 'titrated' by RR added at different times after membrane depolarization and the onset of transport process (Fig. 3, *A*). As previously, mPTP activity was assessed as RR-insensitive and CsA-sensitive Ca^{2+} transport. Each time as RR was added to mitochondrial suspension, the amount of Ca^{2+} released since the addition of RR was compared to the amount of Ca^{2+} simultaneously released in parallel probes without RR (Fig. 3, *A*). Part of RR-insensitive and CsA-sensitive Ca^{2+} efflux was expressed in percents of the amount of Ca^{2+} released by CCCP without RR.

RR addition at 'zero time' showed that mPTP was capable of the release of about ~50% of added Ca^{2+} since the onset of depolarization (Fig. 3, *A*, *1*, *2*). However, at about ~60 s Ca^{2+} efflux was completely blocked by RR, which indicated termination of CsA-sensitive Ca^{2+} transport, in spite that only part of accumulated Ca^{2+} was released from mitochondria (Fig. 3, *A*, *3*). Incomplete release of $Ca^{2+}$by CsA-sensitive pathway was confirmed by the addition of Ca^{2+} ionophore A23187, which fully released Ca^{2+} from the matrix (Fig. 3, *B*, *1*). Addition of external Ca^{2+} did not prevent the release of Ca^{2+} by A23187 (Fig. 3, *B*, *2*), so we ruled out an assumption that mPTP could be blocked by the transmembrane Ca^{2+} gradient built up gradually in the course of Ca^{2+} efflux and directed from the outer side of mitochondrial membrane to the matrix because of Ca^{2+} accumulation in the medium.

So, 'titration' of Ca^{2+} transport by RR confirmed our previous observations of the fast decay of CsA-sensitive pathway of Ca^{2+} efflux from deenergized mitochondria, which allowed us draw a conclusion of mPTP transition to the closed state (Fig. 3, *C*). In deenergized mitochondria, mPTP closure takes place much earlier than the completion of transport process, and still much earlier than the completion of Ca^{2+} efflux by CsA-sensitive pathway from energized mitochondria (Fig. 2, *C*, *D*). Our results agree with the published data of mPTP activation by mitochondrial depolarization. Meanwhile, we obtained convincing evidence that membrane depolarization promoted the transition of mPTP to closed (inactive) state.

As showed the representative curves of Ca^{2+} transport, addition of Ca^{2+} at the onset of depolarization changed the kinetics of CsA-sensitive Ca^{2+} efflux (Fig. 3, *B*, *1*, *2*). From the literature, it is known that Ca^{2+} binding to the outer sites of mitochondrial membrane results in allosteric regulation of Ca^{2+} transporting systems of mitochondria, particularly MCU and mPTP [25, 30, 31]. Our data too indicated allosteric regulation of mPTP activity by Ca^{2+} (Fig. 1, *B*). So, we studied the effect of extra-mitochondrial Ca^{2+} on mPTP activity under the conditions of mPTP activation by membrane depolarization. For this purpose, CsA-sensitive swelling and CsA-sensitive Ca^{2+} efflux were recorded after membrane depolarization by CCCP. RR was added for complete blocking of MCU, and the aliquots of Ca^{2+} were added immediately after CCCP and RR. Under these experimental conditions Ca^{2+} was capable of binding only to the outer sites of mitochondrial membrane.

As showed the experiments, additions of Ca^{2+} from the outer side of mitochondrial membrane similarly affected kinetic characteristics of both CsA-sensitive swelling and Ca^{2+} efflux (Fig. 4). From the literature, it is known that Ca^{2+} binding to the outer sites of mitochondrial membrane inhibited mPTP activity [25]. However, as showed the data of Fig.4, Ca^{2+} additions at the onset of depolarization resulted in concentration-dependent increase of the amplitude of CsA-sensitive swelling and the amount of Ca^{2+} released by mPTP (Fig. 4, *A*, *D*). This was in line with simultaneous increase in V_0 of swelling and Ca^{2+} efflux (Fig. 4, *B*, *E*) and the reduction of the half-times of both processes (Fig. 4, *C*, *F*), which testified mPTP activation. Meanwhile, earlier termination of swelling under the action of added Ca^{2+},

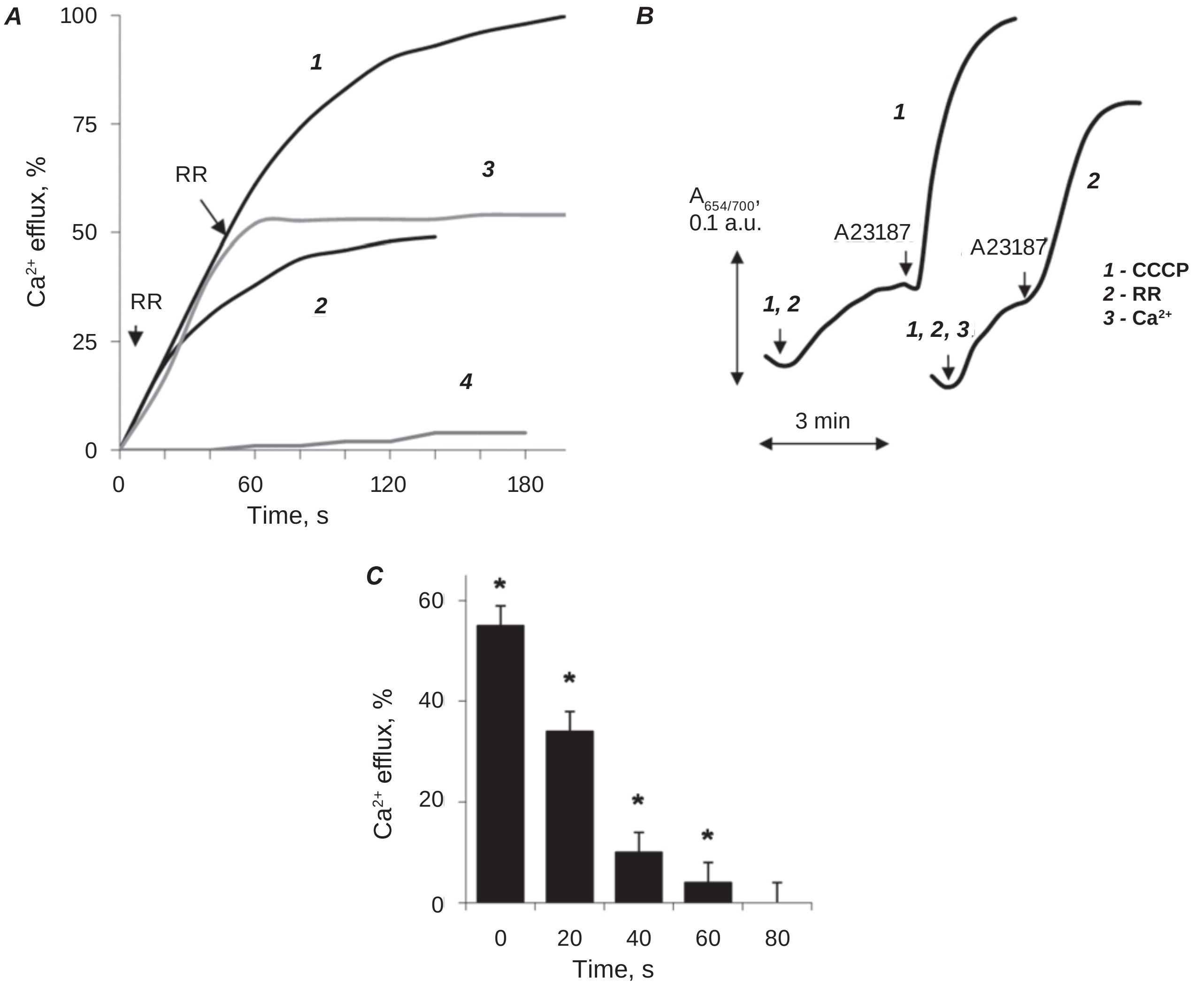

Fig. 3. The effect of RR on CCCP-induced Ca^{2+} efflux. ***A***: *Ca^{2+} efflux in the absence (1) and the presence of RR (2-4); RR was added initially (2, 4) and at 60 s time interval (3) in the absence (2, 3) and the presence of CsA (4).* ***B***: *Ca^{2+} efflux caused by Ca^{2+} ionophore A23187 (additions are shown by the arrows, Ca^{2+} was added at 30 µM).* ***C***: *partial contribution of RR-insensitive Ca^{2+} transport to CCCP-induced Ca^{2+} efflux; RR was added at times indicated on the abscissa axis. For Ca^{2+} uptake, 100 µM Ca^{2+} was added to the medium; total Ca^{2+} released by CCCP in the absence of RR was taken for 100%. M ± m, n = 4. *$P < 0.05$ (as compared to control, in the absence of RR)*

which was coincident with earlier termination of Ca^{2+} efflux (Fig. 4, *A*, *D*), strongly indicated the termination of mPTP functioning. mPTP closure under these conditions was routinely confirmed by the addition of Ca^{2+}-ionophore, which showed the release of Ca^{2+} after the completion of CsA-sensitive Ca^{2+} efflux (Fig. 3, *B*). Thus, we observed concentration-dependent mPTP activation by Ca^{2+} at the onset of depolarization, and Ca^{2+}-dependent mPTP inhibition at larger time intervals.

To ascertain inhibitory effect of extramitochondrial Ca^{2+} on mPTP activity, 20 µM aliquots of Ca^{2+} were added at different times since mitochondrial depolarization. Each time starting from the onset of depolarization, swelling amplitude found without added Ca^{2+} (Fig. 5, *A*, *1*, control) was taken for 100% and compared to the same value found after the additions of Ca^{2+} (Fig. 5, *A*, 2-5). Experiments showed contrary effects of Ca^{2+} on mPTP activity, dependent on the time of Ca^{2+} addition, i.e. mPTP activation at 'zero time', but faster mPTP inhibition with the increase of time interval since mitochondrial depolarization (Fig. 5, *B*). Thus, we observed dual effect of extramitochondrial Ca^{2+} on mPTP activity: Ca^{2+} was

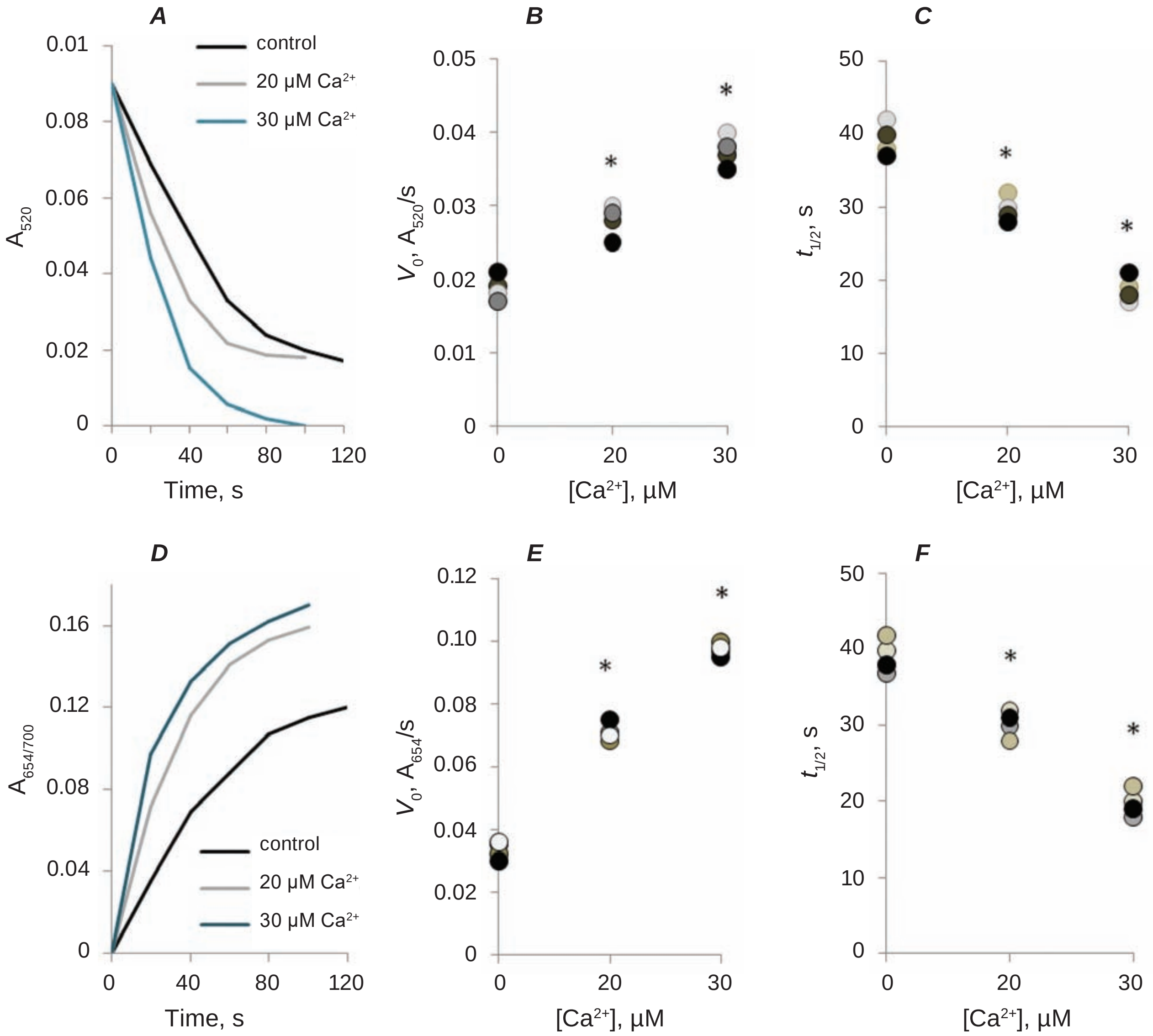

Fig. 4. The effect of extramitochondrial Ca^{2+} on mPTP activity at the onset of depolarization. ***A-C****: the effects of Ca^{2+} added at 'zero' time on the time courses (****A****), V_0 (****B****) and half-times (****C****) of CsA-sensitive swelling;* ***D-F****: the effects of Ca^{2+} on the kinetics (****D****), V_0 (****E****), and the half-times (****F****) of CsA-sensitive Ca^{2+} efflux.* ***B, C, E, F****: the data are represented as M ± m, n = 4; *$P < 0.05$*

capable of concentration-dependent mPTP activation at the onset of depolarization, but promoted mPTP transition to the closed state.

Release of Ca^{2+} from mitochondria by CsA-sensitive pathway was shown in numerous studies, but whether mPTP could be considered as Ca^{2+} transporting system, was doubted in the literature, and Na^+/Ca^{2+} (H^+/Ca^{2+}) exchangers were proposed as key Ca^{2+} efflux pathways [21]. However, based on the experiments on rat liver mitochondria, we observed that CsA-insensitive Ca^{2+} efflux, which could be ascribed to H^+/Ca^{2+} exchanger activity, was negligible as compared to CsA-sensitive one (Fig. 2, *B*). The sensitivity of major part of Ca^{2+} efflux to acknowledged mPTP blocker CsA observed in our work, allowed us consider mPTP operating in sub-conductance mode as key Ca^{2+} efflux pathway in energized rat liver mitochondria. Based on the knowledge of mPTP properties as potential-dependent channel, which is activated by membrane depolarization [13, 14, 22], we compared the kinetics of CsA-sensitive Ca^{2+} efflux from mitochondria under energized and deenergized conditions and studied the effect of membrane depolarization on

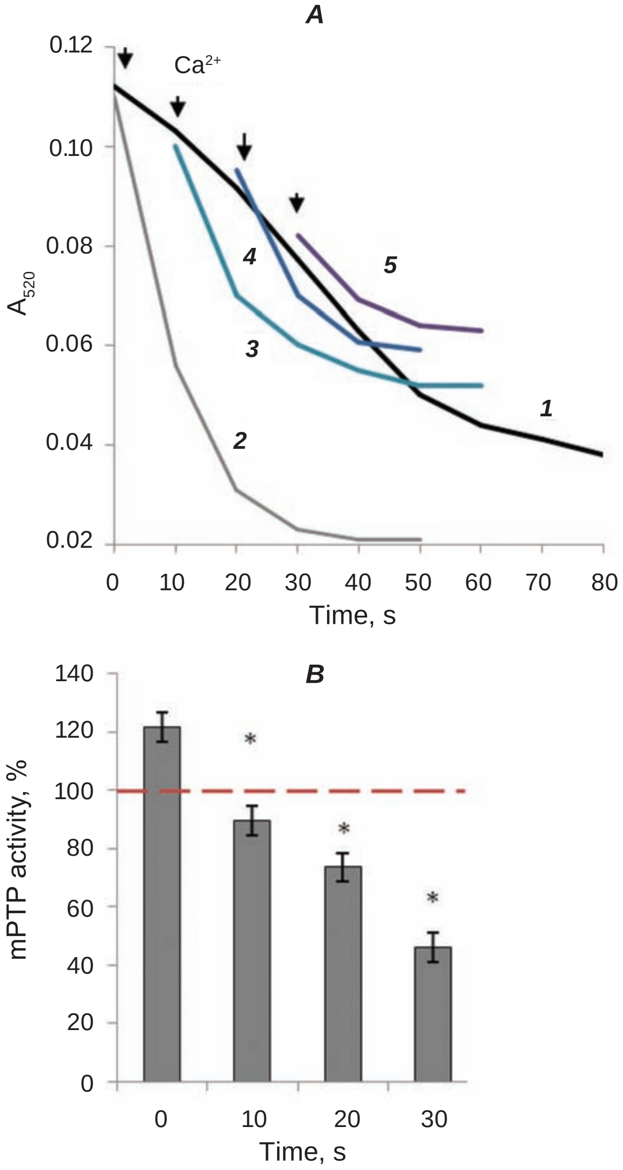

Fig. 5. The time-dependent effects of extramitochondrial Ca^{2+} on mPTP activity. ***A****: (1) – no added Ca^{2+} (control); (2-5) – Ca^{2+} (20 µM) was added at time intervals starting from the onset of depolarization (shown by the arrows).* ***B****: mPTP activity as compared to control (dotted line); the data are represented as M ± m, n = 4; *P < 0.05*

mPTP functioning as Ca^{2+} transporting system in rat liver mitochondria. mPTP activity was assessed as CsA-sensitive swelling and CsA-sensitive Ca^{2+} efflux, which were recorded after the accumulation of Ca^{2+} added to the incubation medium.

As showed the results of the experiments, energized state of mitochondria enabled sustained mPTP operation as Ca^{2+} transporting mechanism, but mitochondrial depolarization dramatically terminated mPTP functioning and promoted mPTP transition to the closed state. Also, we obtained evidence that mPTP activity was regulated by Ca^{2+} ions from the outer side of mitochondrial membrane, which principally agreed with the literature [25]. However, as it was shown in our study, the occupation of the outer low affinity regulatory site(s) by Ca^{2+} ions had dual effect on mPTP activity. Added at the onset of depolarization and transport process, Ca^{2+} produced concentration-dependent activation effect, but at larger time intervals, it promoted mPTP closure. Limited release of added Ca^{2+} via mPTP after depolarization, in spite that large part of Ca^{2+} remained in the matrix, strongly indicated the transition of mPTP to inactive (closed) state.

Unfortunately, the limitations of our experimental approach did not enable us to decide on the mechanism of mPTP closure after mitochondrial depolarization. In the literature, it was shown that mPTP was blocked by Ca^{2+} from the outer side of mitochondrial membrane [25]. Also, mPTP is known to be highly sensitive to the blockage by protons entering the matrix in exchange for Ca^{2+} [16]. However, an assumption that under depolarization conditions mPTP was blocked by Ca^{2+} released from the matrix, or, differently, by protons from the matrix side, should be ruled out because in energized mitochondria, unlike deenergized ones, mPTP was capable of complete release of Ca^{2+} taken up from the medium, and mPTP activity as well, was not blocked by larger decrease of matrix pH caused by Ca^{2+} efflux.

Thus, from the experiments it became evident that mitochondrial energization was required for sustained mPTP functioning in sub-conductance states. To explain observed phenomenon, we hypothesized that the release of Ca^{2+} by CsA-sensitive pathway (which, as we have shown earlier, occurred in stoichiometric exchange for protons [18]), could be not purely electroneutral, but energy requiring process, similarly to mitochondrial H^{+}/Ca^{2+} exchange [32]. Thus, abolition of $\Delta\Psi_m$ resulted in fast decay of mPTP operation as CsA-sensitive Ca^{2+} transporting pathway.

Another assumption is that, as mPTP activity is highly sensitive to mitochondrial ROS [28, 33], ROS production by the respiratory chain is required for sustained mPTP operation in sub-conductance states, which occurs without dramatic effects on mitochondrial functions. Under such conditions mPTP can function as Ca^{2+} transporting system of mitochondria capable to take part in mitochondrial Ca^{2+}

cycling and respiration. Contrary to this, transition of mPTP to high conductance states should result in the blockage of electron transport, membrane depolarization and collapse of mitochondrial bioenergetics, which eventually would promote blocking of mPTP activity. It needs to be considered that protonophore CCCP was used in our work to cause mitochondrial depolarization. Based on literary data [34], we assume that ROS production could be suppressed under such conditions, which eventually resulted in the termination of mPTP functioning.

Worth notion that mPTP properties described in our work differ from the properties of mPTP channel operating in sub- and high conductance states described in the literature [16]. Classical mPTP opening in sub-conductance state (“flickering”) described in the literature occurs without matrix swelling, and mPTP operating in ‘flickering’ mode is permeable only to ions (Ca^{2+}, H^+, K^+) and small molecules. Flickering mode of mPTP functioning is highly sensitive to the blockage by protons entering the matrix in exchange for Ca^{2+}, which explains “flickering” phenomenon, i.e. alternate mPTP opening and closure [16]. However, it worth mention that between classical sub- and high-conductance states, there is a number of intermediate states of mPTP operation. In our work mPTP was opened by much higher Ca^{2+} concentrations and, based on observed large amplitude swelling (Fig. 1, *A*), exhibited elevated activity as compared with ‘flickering’ mode. So, to explain differences in mPTP properties observed in our work and literary data, we assume that intermediate conductance substates of mPTP channel can be less sensitive to protons than ‘flickering’ mode of mPTP operation, which explains sustained mPTP functioning as Ca^{2+} transporting pathway in energized mitochondria.

mPTP properties in high conductance state observed in our work too differ from the properties of high conductance mPTP megachannel described in the literature. From the literature, it is known that mPTP transition from low- to high conductance states induced by very high calcium results in sustained mPTP functioning in the open state caused by Ca^{2+}-induced conformational changes to pore-forming membrane proteins [16]. Worth mention that, unlike works which described mPTP opening by very high [Ca^{2+}], in our work we studied the case when mPTP transition to high-conductance state was caused by membrane depolarization with protonophore CCCP. So, we suppose that channel studied in our work can differ in properties from the channel opened by high calcium and Ca^{2+}-induced conformational changes, which keep mPTP in permanently open state. Also, it needs to be considered that, based on the published data [34], membrane depolarization by CCCP could suppress ROS production required for mPTP activity, which in turn terminated mPTP functioning.

The role of ROS production in the regulation of mPTP activity under membrane depolarization requires more detailed study, especially, considering the relevance of such events for physiological conditions. Under physiological conditions, membrane depolarizations occur continuously, caused by numerous physiologically active agents. So, we suppose that mPTP transitions from sub- to high-conductance states caused by mitochondrial depolarization are frequent cellular events. We hypothesize that mPTP closure after membrane depolarization represents a sort of feedback mechanism aimed to preserve mitochondria in functionally active state under physiological states and conditions accompanied by elevated mPTP activity.

Conclusions. Based on the experiments, we came to the following conclusions:

1) Mitochondrial energization is required for prolonged mPTP operation as Ca^{2+} transporting system of mitochondria when mPTP is opened in low-conductance states;

2) Membrane depolarization increases mPTP activity, but reduces the timeframes of mPTP functioning and results in fast mPTP closure or inactivation;

3) Extramitochondrial Ca^{2+} exerts dual effect on mPTP activity: added at the onset of depolarization it increases mPTP activity, but added at larger time intervals, it promotes mPTP transition to inactive state in the course of Ca^{2+} release from mitochondria. So, membrane depolarization and extramitochondrial Ca^{2+} are the determinants, which strongly limit the timeframes of mPTP functioning in the open state and promote the transition of mPTP to inactive state during calcium release from mitochondria.

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ФУНКЦІОНАЛЬНА АКТИВНІСТЬ ЦИКЛОСПОРИНЧУТЛИВОЇ ПОРИ В ЕНЕРГІЗОВАНИХ І ДЕЕНЕРГІЗОВАНИХ МІТОХОНДРІЯХ ПЕЧІНКИ ЩУРІВ

О. В. Акопова✉*, Л. І. Колчинська, В. І. Носар*

Інститут фізіології ім. О. О. Богомольця
НАН України, Київ;
✉e-mail: ov_akopova@ukr.net

Вивчено відкривання циклоспоринчутливої пори (mPTP) в енергізованих і деенергізованих мітохондріях печінки щурів та оцінено вплив мітохондріальної деполяризації на її активність. Активність mPTP оцінювали спектрофотометрично за циклоспоринчутливим набуханням і циклоспоринчутливим виходом Ca^{2+}, який спостерігався після блокування Ca^{2+} уніпортеру рутенієвим червоним (RR) в енергізованих мітохондріях та після деполяризації мембрани протонофором CCCP. В енергізованих мітохондріях відкривання mPTP у станах низької провідності за концентрацій $Ca^{2+} \leq K_a$ вносило позитивний вклад у швидкість дихання, не впливаючи на $\Delta\Psi_m$. Проведено оцінку порогових концентрацій Ca^{2+}, вище котрих відкривання mPTP призводило до деполяризації. Оцінка активності mPTP за циклоспоринчутливим транспортом Ca^{2+} в умовах мітохондріальної деполяризації показала підвищення початкової швидкості (V_0) за зменшення константи швидкості (k) і часу напівперетворення ($t_{1/2}$), що вказувало на активацію mPTP порівняно з енергізованими мітохондріями. Попри це, в умовах деполяризації спостерігався неповний вихід Ca^{2+} через mPTP. Із застосуванням селективних блокаторів Ca^{2+} уніпортеру та mPTP, RR і циклоспорину А, знайдено парціальний вклад Ca^{2+} уніпортеру і mPTP в транспорт Ca^{2+}. «Титрування» транспортного процесу шляхом внесення RR в різні проміжки часу від скидання потенціалу показало, шо деполяризація різко скорочувала тривалість функціонування mPTP порівняно з енергізованими мітохондріями, що підтверджувалось кінетичними характеристиками циклоспоринчутливого транспорту Ca^{2+} після зняття $\Delta\Psi_m$. Добавлений із зовнішньої сторони мітохондріальної мембрани Ca^{2+} здійснював двоякий вплив на активність mPTP: спостерігалась активація в початковий момент часу з подальшим блокуючим ефектом. На підставі експериментів дійшли висновку, що енергізація мітохондрій необхідна для підтримання функціональної активності mPTP у станах субмаксимальної провідності, тоді як деполяризація мембрани сприяє переходу mPTP до неактивного стану в процесі вивільнення Ca^{2+} із мітохондрій.

Ключові слова: мітохондрії печінки щурів, кальцій, циклоспоринчутлива пора, мембранний потенціал.

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