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Involvement of palmitate/Ca^{2+}(Sr^{2+})-induced pore in the cycling of ions across the mitochondrial membrane

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ABSTRACT

The palmitate/Ca^{2+}-induced (Pal/Ca^{2+}) pore, which is formed due to the unique feature of long-chain saturated fatty acids to bind Ca^{2+} with high affinity, has been shown to play an important role in the physiology of mitochondria. The present study demonstrates that the efflux of Ca^{2+} from rat liver mitochondria induced by ruthenium red, an inhibitor of the energy-dependent Ca^{2+} influx, seems to be partly due to the opening of Pal/Ca^{2+} pores. Exogenous Pal stimulates the efflux. Measurements of pH showed that the Ca^{2+}-induced alkalization of the mitochondrial matrix increased in the presence of Pal. The influx of Ca^{2+} (Sr^{2+}) also induced an outflow of K^+ followed by the reuptake of the ion by mitochondria. The outflow was not affected by a K^+/H^+ exchange blocker, and the reuptake was prevented by an ATP-dependent K^+ channel inhibitor. It was also shown that the addition of Sr^{2+} to mitochondria under hypotonic conditions was accompanied by reversible cyclic changes in the membrane potential, the concentrations of Sr^{2+} and K^+ and the respiratory rate. The cyclic changes were effectively suppressed by the inhibitors of Ca^{2+}-dependent phospholipase A_2, and a new Sr^{2+} cycle could only be initiated after the previous cycle was finished, indicating a refractory period in the mitochondrial sensitivity to Sr^{2+}. All of the Ca^{2+}- and Sr^{2+}-induced effects were observed in the presence of cyclosporin A. This paper discusses a possible role of Pal/Ca^{2+} pores in the maintenance of cell ion homeostasis.

1. Introduction

Mitochondria play an important role in supporting Ca^{2+} homeostasis and signaling within the cell [1–3]. The system of mitochondrial Ca^{2+} transport, which has been studied since the middle of the past century [4], is well characterized from the functional point of view, and substantial progress has been achieved in revealing the identity of its components in the last few years. The identification of the mitochondrial Na^+/Ca^{2+} exchanger in 2010 [5] and the Ca^{2+} uniporter (MCU) in 2011 [6,7], both of which have been studied for a long time [8–12], filled in the major functional gaps in the mitochondrial Ca^{2+} transport. Additionally, other proteins were discovered that participate in the mitochondrial Ca^{2+} exchange: ryanodine receptor [13], leucine zipper EF hand-containing transmembrane protein 1 (Letm1) [14] and uncoupling proteins 2 and 3 (UCP2/3) [15]. Several reviews have been published recently to describe Ca^{2+} exchange in mitochondria and to connect the scheme of exchange to the functional data obtained on both isolated and intact organelles [3,11,12,16–18].

It is now clear that mitochondria serve as Ca^{2+} "dampers", quenching endoplasmic Ca^{2+} spikes in the vicinity of the reticulum by accumulating the ion in their matrix. The mitochondrial Ca^{2+} exchange system is therefore balanced for rapid Ca^{2+} uptake and slow Ca^{2+} release; as emphasized in [19], the maximal rate of Ca^{2+} influx via MCU is approximately 70-fold higher than the combined rate of all of the efflux pathways. Thus, mitochondria are under a constant potential threat of Ca^{2+} overload.

The overload of mitochondria with Ca^{2+} will eventually lead to the permeability transition (PT) in the inner mitochondrial membrane and, as suggested by Bernardi and his colleagues, PT can be a Ca^{2+} release mechanism, working in emergency situations [19,20]. However, PT is not selective; PT will lead to a rapid equilibration of all of the gradients of low-molecular solutes. Therefore, to function as an emergency Ca^{2+} release mechanism, while not leading to the loss of functional activity of the organelles, PT should be transient. In this respect, Cheng and his colleagues monitored mitochondrial PT by registering the superoxide flashes resulting from the burst acceleration of electron transport

Abbreviations: Pal, palmitic acid; Pal/Ca^{2+} pore, palmitate/Ca^{2+}-induced pore; RR, ruthenium red; MCU, mitochondrial Ca^{2+} uniporter; MPT, mitochondrial permeability transition; CsA, cyclosporin A; TPP^+, tetraphenylphosphonium; PLA_2, phospholipase A_2; TFP, trifluoperazine; $AACOCF_3$, arachidonyl trifluoromethyl ketone; P_i, inorganic phosphate; FFA, free fatty acids; $\Delta\psi$, mitochondrial transmembrane potential

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in the respiratory chain [21,22]. The transient character of those flashes confirmed that PT could occur in the form of a "flickering pore".

The flickering mode of mitochondrial PT – when PT occurs transiently and repeatedly – implies the induction of Ca^{2+} cycling across the membrane. In our earlier work, we suggested the activation of such a Ca^{2+} cycle in a specific case of mitochondrial PT — permeabilization of the mitochondrial membrane induced by long-chain saturated fatty acids and Ca^{2+}(or Sr^{2+}) [23]. The ability of palmitic acid (Pal) + Ca^{2+} to trigger a permeability transition in the mitochondrial membrane was shown after the discovery that saturated fatty acids (mainly palmitic and stearic) bind Ca^{2+} with high affinity [24]. It was established that the binding of Ca^{2+} to the fatty acid anions resulted in the formation of non-specific lipid pores; this effect was demonstrated on planar membranes (BLM), liposomes and mitochondria [24–27]. The mechanism of pore formation is supposedly based on the chemotropic phase transition in the lipid bilayer upon the formation of fatty acid/divalent cation complexes in the membrane [25,28]. One of the main features of lipid pores is their ability to close spontaneously with rapid restoration of membrane integrity [25,27], providing for the possibility of Ca^{2+} cycling across the mitochondrial membrane [23].

In this paper, we continue to investigate the phenomenon of Pal/Ca^{2+}- and Pal/Sr^{2+}-induced permeabilization of the mitochondrial membrane and show that the RR-induced efflux of Ca^{2+} from mitochondria described earlier seems to relate to the opening of the Pal/Ca^{2+}-induced pore (Pal/Ca^{2+} pore). The pore opening gives rise to the flux of ions down their concentration gradients: the efflux of Ca^{2+}(Sr^{+}) and K^{+} from mitochondria and the influx of H^{+} into the organelles.

We have found that under the experimental conditions used, the efflux of K^{+} is not inhibited by quinidine, a mitochondrial K^{+}/H^{+} exchange blocker, but the subsequent influx of the ion is blocked by glibenclamide. The addition of Sr^{2+} to mitochondria in a hypotonic medium has been shown to result in the CsA-insensitive cycling of ions and corresponding changes in $\Delta\Psi_m$, with the organelles becoming unresponsive to the subsequent Sr^{2+} additions until the PT cycle is complete. Evidence is obtained showing that PT-associated effects, e.g., cycling of Ca^{2+}(Sr^{2+}) and K^{+}, oscillations of membrane potential ($\Delta\Psi_m$) and respiration, are related to the activation of phospholipase A_2.

2. Materials and methods

2.1. Materials

$CaCl_2$ and tetraphenylphosphonium chloride were purchased from Merck (Germany); arachidonyl trifluoromethyl ketone ($AACOCF_3$) were purchased from Tocris (Great Britain); trifluoperazine (TFP), quinidine, glibenclamide and all of the common chemicals were purchased from Sigma-Aldrich (USA).

2.2. Isolation of rat liver mitochondria

Mitochondria were isolated from the liver of mature male Wister rats (220–250 g) using a standard differential centrifugation technique. The isolation medium contained 210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES/KOH (pH 7.4). The liver was cooled in the isolation medium, washed to remove blood, pressed through a plate with 1-mm holes and homogenized with a Teflon pestle in a Potter-type glass homogenizer (the tissue to medium w/v ratio was 1:8). The homogenate was centrifuged at 700 *g* (10 min), and mitochondria were sedimented for 15 min at 7000 *g*. The mitochondrial pellet was resuspended in a washing medium containing 210 mM mannitol, 70 mM sucrose, 0.1 mM EGTA and 10 mM HEPES/KOH (pH 7.4) and was centrifuged once more for 15 min at 7000 *g*. The pellet was resuspended in the washing medium (0.1 mL/g of liver) and stored on ice. All of the procedures were carried out at 0–4 °C. The resulting mitochondrial suspension contained 70–80 mg of protein per mL. The concentration of protein was determined by the Lowry method [29].

2.3. Estimation of the functional parameters of mitochondria

Mitochondrial membrane potential ($\Delta\Psi_m$) was estimated by the distribution of tetraphenylphosphonium (TPP^{+}) in the mitochondrial suspension and was measured with a TPP^{+}-sensitive electrode. TPP^{+} was added to the incubation medium at the concentration of 1 μM. The concentrations of Ca^{2+}(Sr^{2+}) and K^{+} ions in the incubation medium were determined with Ca^{2+}- and K^{+}-selective electrodes (Nico-Analyt, Russia). Changes in the medium pH were registered by a pH electrode — InLab Micro (Metler Toledo, Switzerland). Changes in the incubation medium pH and the concentrations of TPP^{+} and Ca^{2+} were recorded simultaneously in a 1-mL cell with constant stirring at 26 °C (thermostat controlled), using an original multichannel electrometrical system — Record 4 (Russia). The rate of oxygen consumption was measured polarographically with a Clark-type platinum electrode (Oxygraph-2k, Austria) at 26 °C under continuous stirring.

The composition of the incubation medium varied depending on the experimental requirements (see figure legends for details).

To test the effects of the various inhibitors, the inhibitors were added to the mitochondria 2 min before the addition of Ca^{2+}(Sr^{2+}). Stock solutions of the inhibitors were prepared in either ethanol (TFP, $AACOCF_3$) or DMSO (glibenclamide). The final concentration of the solvents in the incubation was <0.1 vol.%.

2.4. Data processing

Each experiment was repeated from 3 to 7 times. The data obtained were processed using Microsoft Excel software and characteristic curves, typical for each of the experiments, were presented in the figures. The curve smoothing (removing the noise from electrodes) was performed by the method of adaptive cubic spline filtering using the Filter package.

3. Results

3.1. Involvement of palmitic acid in the release of Ca^{2+} from mitochondria

Fig. 1 shows the RR-induced Ca^{2+} efflux from mitochondria after the organelles were loaded with Ca^{2+} (70 nmol·mg^{-1} of protein) in a P_i-containing medium, in the absence or presence of palmitate (Pal). As observed, Pal stimulates the Ca^{2+} efflux, and RR enhances this effect.

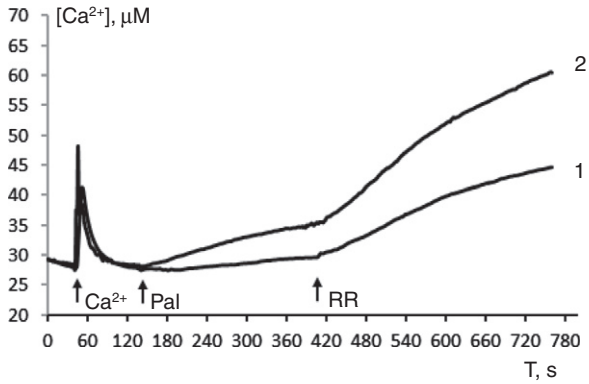

Fig. 1. Ruthenium red (RR)-induced Ca^{2+} release from rat liver mitochondria in the absence (1) or presence (2) of palmitic acid (Pal). Additions: 1) 70 μM $CaCl_2$ and 1 μM RR; 2) 70 μM $CaCl_2$, 30 μM Pal and 1 μM RR. The medium contained 210 mM mannitol, 70 mM sucrose, 1 mM NaH_2PO_4, 5 mM succinic acid, 1 mM rotenone, 10 μM EGTA, 10 mM Hepes-NaOH (pH = 7.4). The concentration of mitochondrial protein was 1 mg/mL.

This may indicate that Pal either affects one of the known Ca^{2+} efflux mechanisms or opens a new Ca^{2+} release pathway.

In our earlier work, we presented evidence that the Pal-induced Ca^{2+} release pathway was not associated with classic PT [23]. In the absence of P_i and in the presence of cyclosporin A (CsA), when classic PT was suppressed, the addition of Ca^{2+} (65 nmol·mg^{-1} of protein) without Pal led to the acidification of the external medium (Fig. 2), which was related to the Ca^{2+} uptake. The Pal-induced acidification was significantly lower under the same conditions, and pH_{out} was restored to almost the initial level, presumably because the mitochondria lost all of the accumulated Ca^{2+}. Therefore, in the presence of exogenous Pal, the efflux of Ca^{2+} from mitochondria, under those conditions, became equal to or exceeded its total influx.

3.2. Involvement of Pal/Sr^{2+} pore in the Sr^{2+}-induced changes of $\Delta\Psi_m$ and the efflux of ions from mitochondria

We showed previously that loading rat liver mitochondria with Sr^{2+} led to a high-amplitude CsA-insensitive swelling of the organelles. The swelling was partially suppressed by the preincubation of mitochondria with phospholipase A_2 inhibitors or albumin. We assume that Sr^{2+} activates mitochondrial phospholipase A_2, which is followed by an increase in the content of free fatty acids (including Pal) in the mitochondrial membrane, Pal/Sr^{2+}-induced PT and organelle swelling. In this paper, we continue to study Pal/Sr^{2+}-induced mitochondrial PT.

Fig. 3 shows data on the simultaneous measurements of $\Delta\Psi_m$, the external concentration of Sr^{2+} ($[Sr^{2+}]_{out}$) and the pH of the medium (pH_{out}) upon loading mitochondria with Sr^{2+} in the absence/presence of BSA (1 mg/mL). The addition of Sr^{2+} was followed by a cycle of fast uptake/fast release/slow reuptake of the ion, with the initial uptake phase being too fast to be resolved with inertial ion-selective electrodes (Fig. 3A). In the absence of albumin, the post-cycle baseline $(Sr^{2+})_{out}$ increased with every successive Sr^{2+} addition (Fig. 3A, line 1). The accumulation of Sr^{2+} in the mitochondrial matrix was accompanied by the acidification of the external medium (Fig. 3C, line 1) and the reduction of $\Delta\Psi_m$ (Fig. 3B, line 1). The pre-incubation of mitochondria with albumin enhanced their ability to accumulate Sr^{2+} ions; after every Sr^{2+} addition, all of the ions were taken up by the organelles, and the shifts of the other parameters (pH_{out} and $\Delta\Psi_m$) also diminished. The same effects were observed with the PLA_2 inhibitors $AACOCF_3$ (15 μM) and TFP (10 μM) (data not shown).

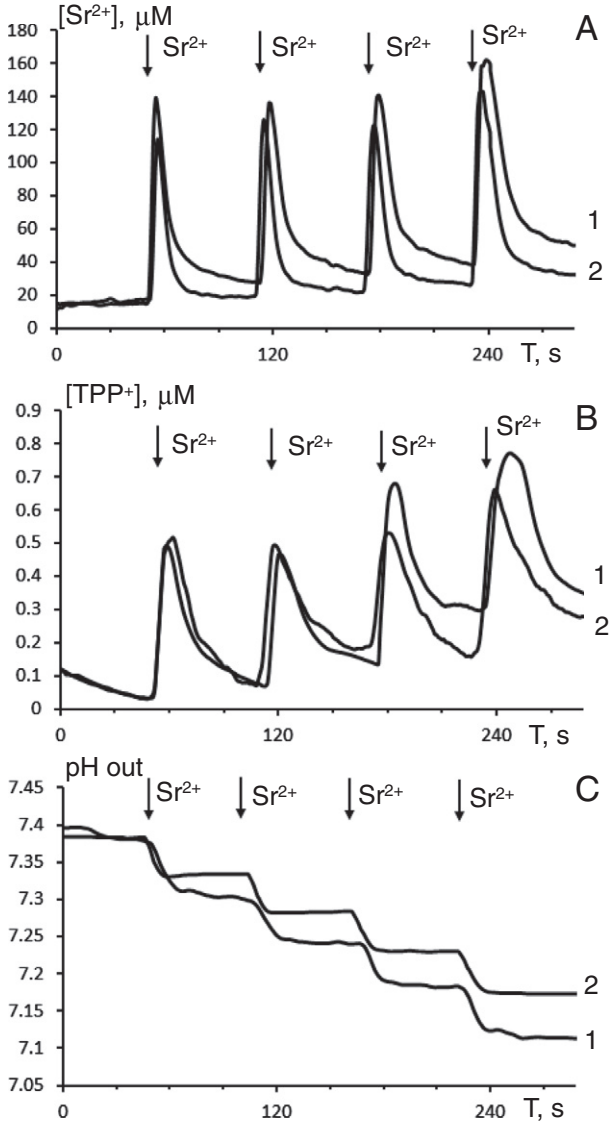

Fig. 3. Sr^{2+}-induced changes in external (Sr^{2+}) (A), (TPP^+) (B), and pH (C) in the suspension rat liver mitochondria in the absence (1) or presence (2) of 1 mg/mL bovine serum albumin. Each addition was 200 nmol $SrCl_2$/mg protein. The medium was the same as in Fig. 1 but supplemented with 1 μM CsA and 1 μM TPP^+.

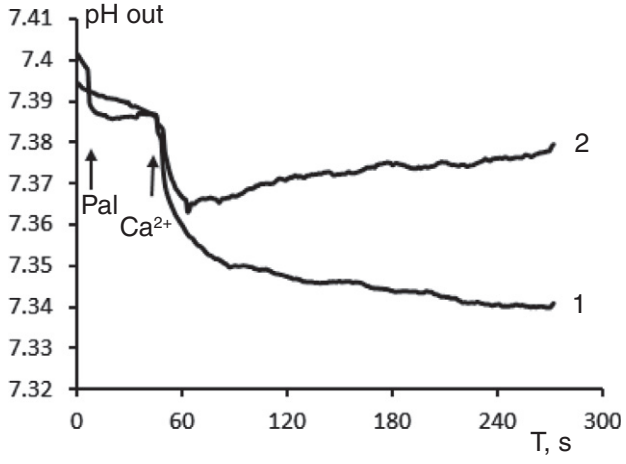

Fig. 2. Palmitic acid (Pal) prevents Ca^{2+}-induced medium acidification in the suspension of respiring rat liver mitochondria. Additions: 1) 65 μM $CaCl_2$; 2) 30 μM Pal and 65 μM $CaCl_2$. The incubation medium contained 210 mM mannitol, 70 mM sucrose, 1 mM KH_2PO_4, 5 mM succinic acid, 10 μM EGTA, 1 μM CsA, 1 μM rotenone and 3 mM Hepes-KOH (pH 7.4). The concentration of mitochondrial protein was 1 mg/mL.

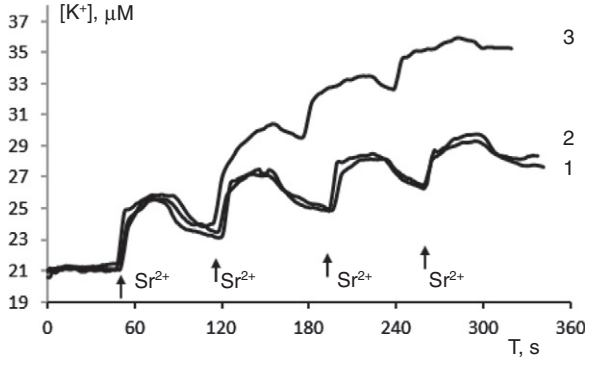

Fig. 4. The effect of 0.1 mM quinidine and 1 μM glibenclamide on the Sr^{2+}-induced potassium fluxes in rat liver mitochondria. Each addition was 200 nmol $SrCl_2$/mg protein; 1) control; 2) 0.1 mM quinidine; 3) 1 μM glibenclamide. The medium was the same as in Fig. 1 but supplemented with 1 μg/mL oligomycin.

The cycles of Sr^{2+} uptake/release/reuptake were accompanied not only by changes in $\Delta\Psi_m$ and pH_{out} but also by the cycling of K^+ (Fig. 4). The K^+ cycle seemed to follow the last two phases of the Sr^{2+} cycle; the efflux and the subsequent slow influx of the two ions occurred in parallel. To elucidate the pathways of K^+ influx and efflux, we conducted experiments with glibenclamide, an inhibitor of the mitochondrial ATP-dependent potassium channel ($mitoK_{ATP}$), and quinidine, a K^+/H^+ exchange blocker in mitochondria. The experiments showed that quinidine had no effect on K^+ efflux, whereas glibenclamide suppressed K^+ influx (Fig. 4). Apparently, the release of K^+ from mitochondria after the addition of Sr^{2+} was associated with Sr^{2+}-induced CsA-insensitive PT, with the subsequent slow uptake of K^+ by the organelles being mediated by $mitoK_{ATP}$.

3.3. Involvement of Pal/Sr^{2+} pores in the reversible Sr^{2+}-induced oscillations of ion fluxes, respiration rate and $\Delta\Psi_m$

The data obtained can explain the mechanism of Sr^{2+} and K^+ release from mitochondria in response to a pulse of Sr^{2+} in a hypotonic medium [32–35]. Under these conditions, oscillations of Sr^{2+} and K^+ fluxes across the mitochondrial membrane can be observed, and we believe that the mechanism of ion release is related to the opening of the Pal/Ca^{2+} pore in the membrane. Fig. 5 shows simultaneous records of the external concentrations of Sr^{2+}, K^+ and TPP^+ as well as the rate of mitochondrial O_2 consumption after a single addition of Sr^{2+} (47 nmol/mg protein) to rat liver mitochondria under hypotonic conditions. As observed from the figure, the addition of Sr^{2+} triggered

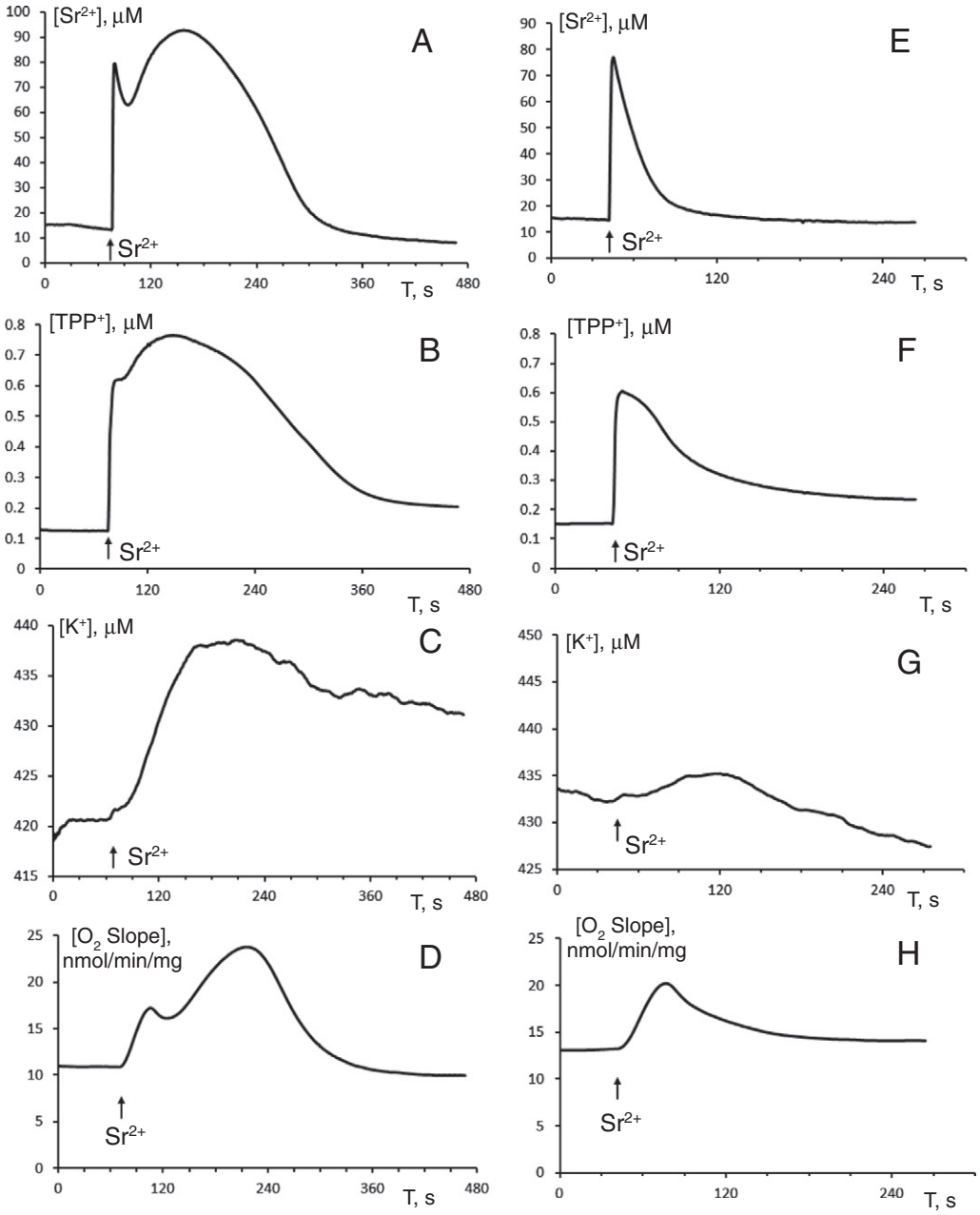

Fig. 5. Sr^{2+}-induced cyclic changes in the external concentrations of Sr^{2+}, TPP^+, K^+ and the mitochondrial respiration rate (V_2 state) in the absence (A, B, C, D) or presence (E, F, G, H) of 15 μM $AACOCF_3$ (phospholipase A_2 inhibitor). Addition: 47 nmol $SrCl_2$/mg of protein. The medium contained 20 mM sucrose, 1 mM KCl, 1 μM TPP^+, 1 μM CsA, 5 mM succinic acid/Tris (pH 7.3). The concentration of mitochondrial protein was 2 mg/mL.

spontaneous respiration rate oscillations (Fig. 5D) accompanied by the corresponding changes in $\Delta\Psi_m$ (Fig. 5B) and the flux of ions (Fig. 5A and C). The amplitudes of these changes depended on the amount of Sr^{2+} added to the organelles; in our experiments, the Sr^{2+} amount was equal to 37–54 nmol/mg of protein.

Similar oscillations were triggered by Sr^{2+} in heart mitochondria (data not shown). The Sr^{2+}-induced effects were not affected by CsA. It should be noted that these experiments were conducted in the absence of P_i. In the presence of P_i, oscillations disappeared, since their launch would require free Sr^{2+} in the medium.

It is known that hypotonia is accompanied by the activation of PLA_2 and phospholipid hydrolysis [36]. Therefore, the contribution of PLA_2-related mechanisms in membrane permeabilization should show an increase under hypotonic conditions. Accordingly, one could expect a stronger effect of PLA_2 inhibitors. Fig. 5E–H shows the effect of $AACOCF_3$, an inhibitor of mitochondrial PLA_2. As observed in the figure, the inhibitor effectively suppressed all of the Sr^{2+}-induced cyclic changes in mitochondria. In the presence of other PLA_2 inhibitors (aristolochic acid, TFP), the result was similar.

As shown in Fig. 6, the addition of Pal in the presence of the inhibitor resulted in the same oscillations that were observed in the control experiments. Furthermore, palmitoleic acid, which has a much lower affinity for Ca^{2+}, had no effect under the same conditions (data not shown). This result confirms our supposition that PLA_2 inhibitors and albumin prevent the accumulation of endogenous Pal.

3.4. The Pal/Sr^{2+} cycle and refractoriness of the inner mitochondrial membrane

After a cycle of Sr^{2+}-induced fluxes/changes is complete, the cycle can be initiated again by Sr^{2+} addition (Fig. 7A–C). After the second Sr^{2+} pulse, one can observe the same cyclic changes of $[Sr^{2+}]_{out}$ and $\Delta\Psi_m$ as in the first cycle, but the outer concentration of K^+ ($[K^+]_{out}$) is altered. After the initial release of K^+ during the first cycle, $[K^+]_{out}$ oscillates – slightly and slowly – against the general trend of gradual K^+ reuptake. Usually, the reuptake of K^+ is accelerated after the second Sr^{2+} pulse, when $\Delta\Psi_m$ is fully restored by the end of the second cycle.

As shown in Fig. 7D–F, the second cycle of Sr^{2+}-induced ion permeability changes could not be initiated until the first cycle was fully complete. Thus, to respond to the second stimulus, mitochondria require time to return to their initial state, i.e., they demonstrate a refractory period. As observed in the figure, the addition of another Sr^{2+} portion before the first Sr^{2+} cycle has been completed (i.e., if $\Delta\Psi_m$ has not been fully restored and not all of the previously added Sr^{2+} has entered the mitochondrial matrix) will not lead to reversible changes of ion flux and $\Delta\Psi_m$.

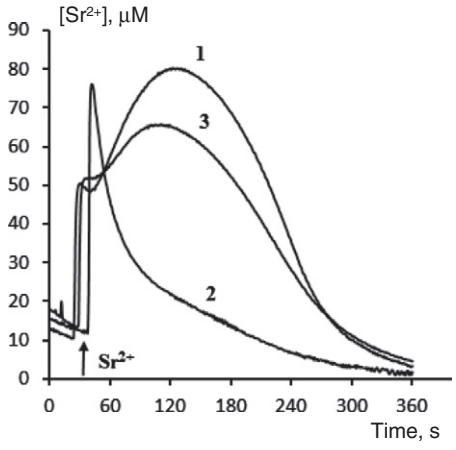

Fig. 6. Palmitic acid (Pal) re-induces the Sr^{2+}-induced permeability transition suppressed by $AACOCF_3$. The medium was the same as in Fig. 5. Addition: 41 nmol $SrCl_2$/mg of protein. 1. — Control; 2. — mitochondria preincubated with 15 μM $AACOCF_3$; 3. — mitochondria preincubated with 15 μM $AACOCF_3$ and 40 μM Pal.

4. Discussion

In this paper, we study the mode of mitochondrial function when the introduction of Ca^{2+} (or Sr^{2+}) into a suspension of organelles initiates the cycling of ions across the mitochondrial membrane. It is well known that RR, an inhibitor of the mitochondrial Ca^{2+} uniporter (MCU), induces the release of the Ca^{2+}ion from Ca^{2+}-loaded organelles [11,30,31]. This release indicates the existence of a Ca^{2+} efflux pathway, with the efflux compensated for by the MCU-mediated reuptake of the Ca^{2+} ion. Suppressing MCU with RR makes the efflux visible. We found that the RR-induced Ca^{2+} release was activated by Pal (Fig. 1).

The amount of Pal added to mitochondria exceeds the level of endogenous free Pal by two-fold [40,41]; therefore, the emergence of Pal in such amounts in the mitochondrial membrane seems quite possible under the conditions of PLA_2 activation by Ca^{2+}. Because of the RR-induced release of Ca^{2+} from respiration and because Ca^{2+}-loaded mitochondria are suppressed by PLA_2 inhibitors [37], we suppose that Pal may appear under these conditions and that the opening of Pal/Ca^{2+} pores is possible. It is known that the mitochondrial Ca^{2+}-dependent phospholipase (a β3 isoform of the Ca^{2+}-dependent cytoplasmatic phospholipases) shows both PLA_2 and PLA_1 activity (saturated fatty acids are mainly esterified in the A_1 position) [42,43]. Indeed, the accumulation of saturated FFA (Pal and stearic acid) in mitochondria incubated with divalent cations was confirmed earlier [37–39], along with the fact that long-chain saturated fatty acids ($C_{16:0}$–$C_{18:0}$) have a high affinity for Ca^{2+} [24] and, upon the formation of FFA/Ca^{2+} complexes, give rise to short-lived nonspecific CsA-insensitive lipid pores [24–27,40].

We hypothesize that the Ca^{2+} release pathway is initiated by saturated fatty acids and the formation of lipid pores in the inner mitochondrial membrane and by the mechanism of chemotropic phase transition in the lipid bilayer [25]. The mechanism is native because membranes always contain some free fatty acids (FFA) and because the addition of Ca^{2+} (Sr^{2+}) to mitochondria results in the activation of phospholipase A_2 (PLA_2) and the increase of FFA content. Therefore, the formation of lipid pores may, in principle, be initiated by the addition of Ca^{2+} alone. In this case, the addition of albumin, which binds FFA, or PLA_2 inhibitors should suppress the permeabilization of the mitochondrial membrane.

This supposition may be even better tested if Ca^{2+} is replaced with Sr^{2+}. Using Sr^{2+} – in combination with CsA – would guarantee that classic MPT is not involved. Like Ca^{2+}, Sr^{2+} is known to accumulate within energized mitochondria, to activate mitochondrial PLA_2 [39] and to cause permeabilization of artificial membranes upon binding to Pal.

The appearance of lipid pores implies a nonspecific change in membrane permeability. The nonspecific character of the membrane permeability changes can be observed in Figs. 2–4, which include data on the changes in mitochondrial H^+ and K^+ exchange. The accumulation of Ca^{2+} in the mitochondrial matrix is known to result in the acidification of the external medium, and this acidification decreases in the presence of exogenous Pal (Fig. 2), thus demonstrating an increase in the permeability of the membrane to H^+.

Another piece of evidence for the above-mentioned non-specificity is data on mitochondrial K^+ exchange. The Sr^{2+}-induced changes in membrane permeability are accompanied by changes in the K^+ flux (Fig. 4), and it seems that K^+ exits mitochondria following Sr^{2+}-induced nonspecific membrane permeabilization and enters mitochondria via mitoK_{ATP}. The first supposition is indirectly supported by the fact that K^+ release is not affected by quinidine, an inhibitor of the K^+/H^+ exchange system; the second conclusion is confirmed by the suppression of K^+ uptake with the inhibitor of mitoK_{ATP}, glibenclamide (Fig. 4).

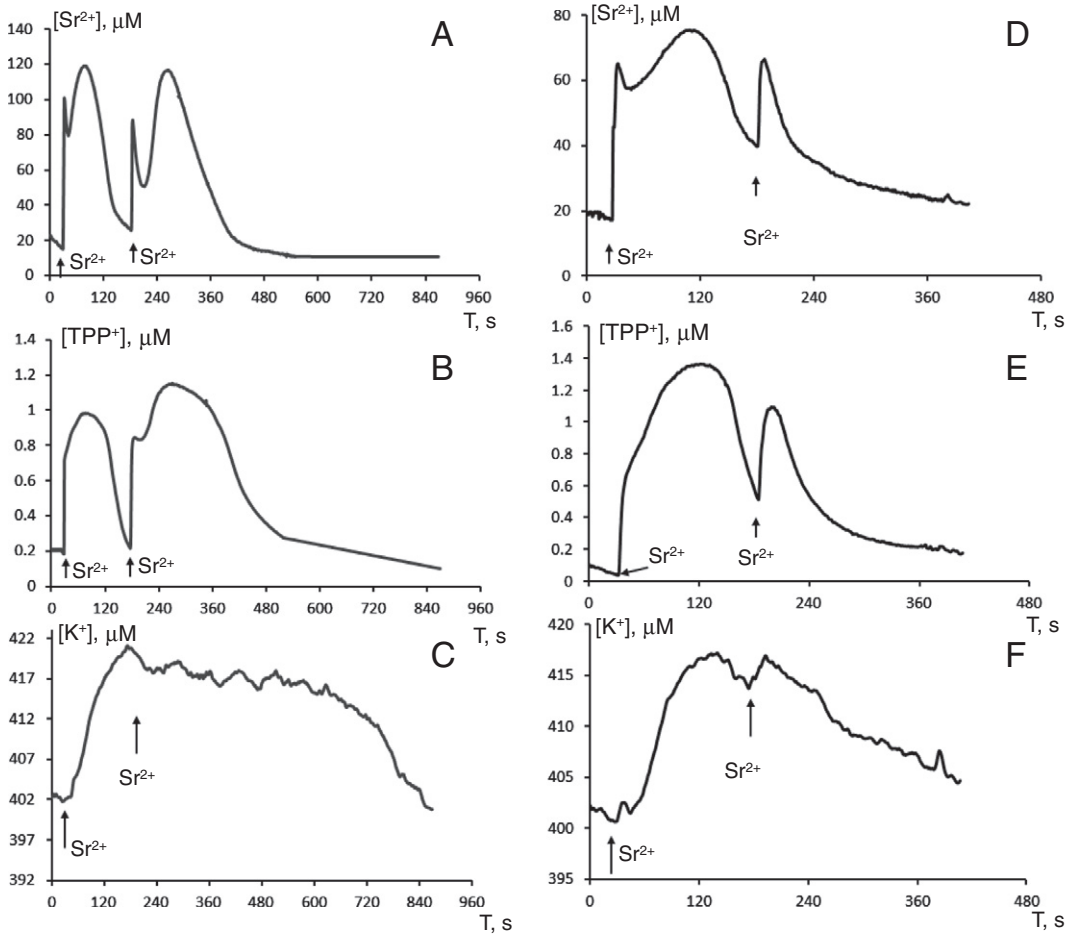

Fig. 7. The second Sr^{2+} pulse induces a new cycle of ion fluxes, and $\Delta\Psi_m$ changes only after all Sr^{2+} from the first addition is accumulated in the mitochondrial matrix and $\Delta\Psi_m$ is completely restored. A and D, changes in the concentration of Sr^{2+}; B and E, changes of $\Delta\Psi_m$; C and F, changes in the concentration of K^+. The 2nd addition of Sr^{2+} was made *after* (A–C) or *before* (D–F) the 1st Sr^{2+} portion was completely accumulated in the mitochondria. The medium was as in Fig. 5. Each addition was 40 nmol $SrCl_2$/mg of protein.

The lipid-pore mechanism discussed here has an important feature: the membrane permeabilization associated with the formation of lipid pores is transient [25,27]. The ability of lipid pores to rapidly heal – if their diameter does not exceed a certain value – is an intrinsic property [44]. Hence, the mechanism should inherently result in *cyclic* changes of membrane permeability. Membrane permeabilization will be followed by the restoration of membrane integrity, and the cycle will recur until there is a force driving the build-up of $\Delta\Psi_m$.

Depending on the strength of the stimuli that causes the formation of lipid pores, the frequency of their appearance, their diameter and, consequently, their lifetime will vary. Correspondingly, different functional modes of mitochondrial PT may exist. The periodical formation of single pores of a small diameter, resulting in local and transient membrane depolarization, should lead to partially uncoupled steady-state modes of mitochondrial functioning — when there are ion cycles initiated across the membrane but the mitochondria remain functionally active. Earlier, we wrote about the initiation of such a cycle, the Ca^{2+} cycle, by the addition of Pal and Ca^{2+} to respiring mitochondria in the absence of inorganic phosphate. Under those conditions, we observed a persistent fall in $\Delta\Psi_m$, which could still be removed with RR after 5–10 min of the organelles' operating in this depolarized state [23]. The measurement of pH changes in the suspension of mitochondria performed in the present study also indicates the activation of the Ca^{2+} cycle (Fig. 2).

Another interesting mode of mitochondrial PT that provides insight into its mechanism is the “pulse mode”, which is observed when PT is initiated with a single strong stimulus, e.g., a Sr^{2+} pulse. This model was described earlier by Holmuhamedov et al. [32–35]. The pulse results in the cycling of ion fluxes, which can be best observed under hypotonic conditions (Figs. 5–6). Here, after the initial fast uptake of Sr^{2+} by mitochondria (1st phase), the transmembrane ion gradients equilibrate (2nd phase) (Fig. 8). This strong PT is followed by the restoration

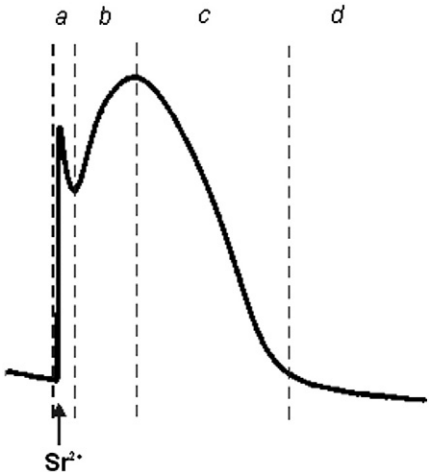

Fig. 8. Phases of the Sr^{2+} cycle: a) Fast initial uptake of Sr^{2+}; b) PT-associated Sr^{2+} release; c) slow reuptake of Sr^{2+} by mitochondria; d) the steady state of recovered $\Delta\Psi_m$ after the Sr^{2+} cycle is completed.

of membrane integrity and a relatively slow recharge of $\Delta\Psi_m$ and the reuptake of Sr^{2+} (3rd phase). The data obtained indicate that it is the Pal/Ca^{2+} pore, rather than the classic MPT pore, that is involved in the operation of Sr^{2+} cycle under the pulse mode of Sr^{2+} addition to mitochondria. Indeed, PLA_2 inhibitors and albumin prevent both the accumulation of endogenous FFA in mitochondria and the functioning of the Sr^{2+} cycle (Fig. 5); the addition of Pal in the presence of the PLA_2 inhibitor restarts the Sr^{2+} oscillations (Fig. 6). Palmitoleic acid did not exert such an effect (data not shown). At the same time, all of the experiments described above were carried out in the presence of CsA, and the MPT inhibitor did not affect Sr^{2+} cycling.

On the basis of the data obtained, it becomes clear that certain conditions should be met for such a Ca^{2+} cycle to operate in mitochondria. First, a high intramitochondrial concentration of free Ca^{2+} (Sr^{2+}) should be achieved, which is necessary for the activation of mitochondrial Ca^{2+}-dependent phospholipase and for the appearance of endogenous fatty acids. Without these factors, lipid pores will not be formed, and ions will not exit mitochondria. It was shown that a slow and continuous infusion of the same amount of Sr^{2+} ions into the suspension did not lead to reversible changes in ion flux [32], which was probably due to the precipitation of the ions in the mitochondrial matrix and a decrease in the concentration of free intramitochondrial Sr^{2+} under those conditions. Second, a high $\Delta\Psi_m$ is needed, which will occur only after the completion of the PT cycle. A high $\Delta\Psi_m$ seems to underlie the phenomenon of refractoriness, an interesting feature of the Sr^{2+} cycle reported earlier by Holmuhamedov's group [32]. In Fig. 7, during the 3rd phase of the Sr^{2+} cycle – when mitochondria slowly build $\Delta\Psi_m$ and when the cycle is not yet restored – an additional Sr^{2+} pulse will not lead to the repetition of the cycle. The cycle can only be repeated after the full restoration of $\Delta\Psi_m$. We relate this to the known fact that decreased $\Delta\Psi_m$ will result in lower rates of Ca^{2+} uptake, and the intramitochondrial concentration of free Ca^{2+} will not reach the level necessary for the formation of lipid pores and the subsequent efflux of ions. In this paper, we found that the phenomenon of refractoriness could be followed by not only measuring [Sr^{2+}] but also $\Delta\Psi_m$ and K^+ (Fig. 7). The mechanism of the phenomenon is still unknown and will require further studies.

The results presented in the paper confirm the involvement of FFA in the induction of the transmembrane Ca^{2+} (Sr^{2+}) cycle in the process of the accumulation of these divalent cations in the mitochondrial matrix. The fast MCU-mediated uptake of Ca^{2+} (Sr^{2+}) supposedly results in the activation of PLA_2 and the appearance of FFA, which are accumulated on the matrix side of the inner mitochondrial membrane in the form of Ca^{2+} (Sr^{2+}) complexes. In the case of palmitic and stearic acids, which are the major saturated fatty acids in mitochondria [41] and have the highest affinity for divalent cations [24], their accumulation will lead to the phase separation of their Ca^{2+} (Sr^{2+}) complexes and to the formation of lipid pores. This transient permeabilization of the mitochondrial membrane creates a pathway for the efflux of various ions, among them K^+, which reenters the mitochondria via mitoK_{ATP}.

However, there is a question whether such a cycle can function in the living cell. On the one hand, the formation of a small number of mitochondrial lipid pores lead to a situation where the launch of the futile Ca^{2+} and K^+ cycles will moderately reduce $\Delta\Psi_m$, protecting the cell from oxidative stress and regulating ion homeostasis and mitochondrial volume. On the other hand, a prolonged elevation of Ca^{2+} concentration in the cytoplasm, which is observed under various pathologies, will result in the activation of phospholipase, an increase in the level of free saturated fatty acids and to the formation of a large number of lipid pores – with eventual depolarization of the mitochondrial membrane and cell death. The possibility of this mechanism to be implemented in the cell was demonstrated in our earlier work, in a model of the glutamate-induced degradation of neurons. The degradation of neurons is characterized by the development of delayed Ca^{2+} (Sr^{2+}) deregulation and mitochondrial depolarization, which are not inhibited by CsA [45] but can be suppressed by the inhibitors of PLA_2 [46,47].

5. Conclusion

Mitochondrial permeability transition is considered an important pathway of Ca^{2+} release from the organelles. The data obtained allows us to suggest that the transient Pal/Ca^{2+}-induced pore can be an "emergency valve" system, preventing mitochondria from Ca^{2+} overload under physiological and pathological conditions and can support cell ion homeostasis. Studying palmitate/Ca^{2+}-induced PT is important for understanding the phenomena of ion oscillations in mitochondria.

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