J Bioenerg Biomembr (2007) 39:167–174
DOI 10.1007/s10863-007-9079-9

ORIGINAL PAPER

# Mitochondrial $Ca^{2+}$ cycle mediated by the palmitate-activated cyclosporin A-insensitive pore

**Galina D. Mironova · Konstantin N. Belosludtsev · Natalia V. Belosludtseva · Elena N. Gritsenko · Boris I. Khodorov · Nils-Erik L. Saris**

Received: 9 March 2007 / Accepted: 12 March 2007 / Published online: 25 May 2007


**Abstract** Earlier we found that in isolated rat liver mitochondria the reversible opening of the mitochondrial cyclosporin A-insensitive pore induced by low concentrations of palmitic acid (Pal) *plus* $Ca^{2+}$ results in the brief loss of $\Delta\psi$ [Mironova et al., J Bioenerg Biomembr (2004), 36:171–178]. Now we report that Pal and $Ca^{2+}$, increased to 30 and 70 nmol/mg protein respectively, induce a stable and prolonged (10 min) partial depolarization of the mitochondrial membrane, the release of $Ca^{2+}$ and the swelling of mitochondria. Inhibitors of the $Ca^{2+}$ uniporter, ruthenium red and $La^{3+}$, as well as EGTA added in 10 min after the Pal/$Ca^{2+}$-activated pore opening, prevent the release of $Ca^{2+}$ and repolarize the membrane to initial level. Similar effects can be observed in the absence of exogeneous Pal, upon mitochondria accumulating high [$Sr^{2+}$], which leads to the activation of phospholipase $A_2$ and appearance of endogenous fatty acids. The paper proposes a new model of the mitochondrial $Ca^{2+}$ cycle, in which $Ca^{2+}$ uptake is mediated by the $Ca^{2+}$ uniporter and $Ca^{2+}$ efflux occurs via a short-living Pal/$Ca^{2+}$-activated pore.

G. D. Mironova · K. N. Belosludtsev · N. V. Belosludtseva · E. N. Gritsenko ·
Institute of Theoretical and Experimental Biophysics RAS, Pushchino, Moscow Region 142290, Russia

G. D. Mironova (✉)
Pushchino State University, Pushchino, Moscow Region 142290, Russia
e-mail: mironova@iteb.ru

B. I. Khodorov
Institute of General Pathology and Pathophysiology RAMS, Moscow 125315, Russia

N.-E. L. Saris
Department of Applied Chemistry and Microbiology, POB 56 Biocenter 1, FIN-00014, University of Helsinki, Helsinki, Finland

**Keywords** Palmitic acid · Calcium · Strontium · Mitochondrial $Ca^{2+}$-dependent pore · $Ca^{2+}$-cycle

**Abbreviations** $\Delta\psi$: mitochondrial transmembrane potential · Pal: palmitic acid · PalCaP: palmitate/$Ca^{2+}$-activated pore · CsA: cyclosporin A · DNP: 2,4-dinitrophenol · $PLA_2$: phospholipase $A_2$ · PTP: permeability transition pore · RR: ruthenium red · BSA: bovine serum albumin · TFP: trifluoperazine

## Introduction

$Ca^{2+}$ transport in mitochondria is realized by separate systems of $Ca^{2+}$ influx and efflux, see the recent review (Saris and Carafoli, 2005). The study of $Ca^{2+}$ transport in mitochondria was started in the early 1960s by Saris (Saris, 1959; Saris, 1963) and others (De Luca and Engstrom, 1961; Vasington and Murphy, 1962). The uptake of the ion is considered to be realized electrophoretically by the $Ca^{2+}$ uniporter (Heaton and Nicholls, 1976). The physiological efflux mechanisms are mediated, depending on the tissue, by the $Ca^{2+}/nNa^+$ and $Ca^{2+}/nH^+$ exchangers, also called antiporters (Carafoli et al., 1974; Carafoli, 1979). When $\Delta\psi$ collapses, $Ca^{2+}$ efflux might occur by the reversal of the uniporter (Reed and Lardy, 1988). The operation of these systems in energized mitochondria provides the cycling of the cation, this contributing to the maintaining of a steady-state $Ca^{2+}$ concentration in the cytoplasm (Bernardi, 1999).

It is believed that aside from the specific ways of $Ca^{2+}$ efflux, which function in the energized mitochondria with intact membranes, there is a nonspecific way, namely the opening of the permeability transition pore, PTP (Bernardi and Petronilli, 1996; Bernardi, 1999; Saris and Carafoli, 2005). The factors that stimulate or inhibit $Ca^{2+}$ efflux via the

$Na^+$-dependent and $Na^+$-independent pathways will also affect PTP (Bernardi and Petronilli, 1996), this making it difficult to determine which mechanism is in operation under the experimental conditions used. Meanwhile, the opening of PTP is accompanied by the high-amplitude swelling of mitochondria and results in their damage (Saris, 1963; Zoratti and Szábo, 1995; Zoratti et al., 2005). It therefore seems unlikely that PTP would function as a system of $Ca^{2+}$ efflux under normal conditions.

As has been shown recently, there exists another pore, which differs from PTP and may be opened in mitochondria under certain conditions (Sultan and Sokolove, 2001; Mironova et al., 2004; Belosludtsev et al., 2005; Belosludtsev et al., 2006). The opening of this pore is induced by the formation of Pal/$Ca^{2+}$ complexes in the inner mitochondrial membrane (Mironova et al., 2001; Mironova et al., 2004; Belosludtsev et al., 2005). This pore (PalCaP) is insensitive to cyclosporin A (CsA) and closes spontaneously (Sultan and Sokolove, 2001; Agafonov et al., 2003), which leads to the restoration of $\Delta\psi$ (Mironova et al., 2004). Our data obtained on the model lipid membranes indicate that PalCaP has a lipid nature (Mironova et al., 2001; Agafonov et al., 2003; Agafonov et al., 2007).

This paper continues the study of PalCaP. We have found that the time needed for $\Delta\psi$ recovery after the opening of PalCaP in mitochondria depends on the concentrations of Pal and $Ca^{2+}$. At the Pal concentration of 30 nmol/mg protein and $Ca^{2+}$ concentration of 70 nmol/mg protein, $\Delta\psi$ decreases (but not collapses) within a few minutes. The addition of ruthenium red (RR), $La^{3+}$ or EGTA to the mitochondria that have not recovered $\Delta\psi$ for 10 min or more restores it almost to the initial level. The accumulation of high [$Sr^{2+}$] in the matrix also results in the depolarization of the mitochondrial membrane, efflux of $Sr^{2+}$ from mitochondria and swelling of organelles. RR recovers $\Delta\psi$ and reduces $Sr^{2+}$ release from mitochondria. The inhibition of phospholipase $A_2$ or addition of bovine serum albumine (BSA) decreases the effect of $Sr^{2+}$. In this paper we offer a new model of the mitochondrial $Ca^{2+}$ cycle, in which $Ca^{2+}$ uptake is mediated by the $Ca^{2+}$ uniporter and $Ca^{2+}$ efflux occurs through the PalCaP.

## Materials and methods

Mitochondria were isolated from the liver of Wistar rats (220–250 g) using a standard differential centrifugation technique (Belosludtsev et al., 2005). The isolation medium contained 210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM Hepes/KOH buffer (pH 7.4); the washing medium was of the same content, except that EDTA was replaced with 50 $\mu$M EGTA. The final suspension contained 90–100 mg of mitochondrial protein/ml. The concentration of mitochondrial protein was determined by Lowry's method using BSA as a standard (Lowry et al., 1951).

Mitochondrial swelling was followed by the absorbance change (at 540 nm) at 25°C using an Ocean Optics USB-2000 spectrometric fiber-optic system (Ocean Optics Inc, USA). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinic acid, 5 $\mu$M EGTA, 1 $\mu$M rotenone and 10 mM Hepes/KOH buffer (pH 7.4). The concentration of mitochondrial protein in the cuvette was about 0.4 mg/ml. In some experiments, the incubation medium was supplemented with 1 $\mu$M CsA.

Mitochondrial potential ($\Delta\psi$) was estimated from the distribution of tetraphenylphosphonium ($TPP^+$) across the inner mitochondrial membrane. In this case the incubation medium was supplemented with 1 $\mu$M $TPP^+$. The concentration of $TPP^+$ was measured with a $TPP^+$-selective electrode (Kamo et al., 1979). The concentration of $Ca^{2+}$ or $Sr^{2+}$ in the reaction medium was measured with an ion-selective electrode. The concentration of mitochondrial protein in the cuvette was about 1 mg/ml.

Oxygen consumption by isolated rat liver mitochondria was measured using a Clark-type oxygen electrode. The incubation medium contained 120 mM KCl, 5 mM $NaH_2PO_4$, 5 mM succinic acid, 5 $\mu$M EGTA, 1 $\mu$M rotenone, 1 $\mu$M CsA and 10 mM Hepes/KOH buffer (pH 7.4). The concentration of mitochondrial protein in the cuvette was about 1.3 mg/ml.

Sucrose, palmitic acid, CsA, Hepes, EGTA, EDTA, $LaCl_3$, succinic acid, rotenone, ruthenium red, Tris, BSA, trifluoperazine and $NaH_2PO_4$ were from Sigma-Aldrich (USA); mannitol, $CaCl_2$ and $SrCl_2$ were from Merck AG (Germany).

## Results

### Pal and $Ca^{2+}$ induce a stable partial depolarization of the mitochondrial membrane and the swelling of mitochondria

Earlier we found that the Pal/$Ca^{2+}$-induced pore in mitochondrial and artificial membranes is short-living (Agafonov et al., 2003; Mironova et al., 2004). In the absence of $P_i$, the addition of Pal (15 nmol/mg protein) and $Ca^{2+}$ (35 nmol/mg protein) to the suspension of rat liver mitochondria induced the swelling of organelles and the loss of $\Delta\psi$, which then fully recovered in 3 min (Mironova et al., 2004). The recovery of $\Delta\psi$ points out that the pore closes spontaneously. All the experiments were carried out in the presence of 1 $\mu$M CsA.

In the present study we estimated $\Delta\psi$ and $\Delta Ca^{2+}$ upon the PalCaP opening induced by Pal (30 nmol/mg protein) and $Ca^{2+}$ (70 nmol/mg protein) (Fig. 1A and B). The addition of Pal slightly decreased $\Delta\psi$ due to the well-known

uncoupling properties of Pal (Skulachev, 1998). After the addition of $Ca^{2+}$ $\Delta\psi$ significantly decreased and no membrane repolarization occurred during the recording time (15 min) (Fig. 1A, dashed line). $Ca^{2+}$ was rapidly (in 2 min) accumulated in mitochondria, this followed by the efflux of the ion (Fig. 1B, dashed line). Figure 1C shows that under these conditions, a large-amplitude mitochondrial swelling was observed. In the absence of Pal, the addition of $Ca^{2+}$ (70 nmol/mg protein) to the mitochondrial suspension resulted in the complete uptake of the cation by mitochondria in 3 min, during which $\Delta\psi$ first temporarily dropped but finally got completely restored (data not presented).

The inhibitors of $Ca^{2+}$ uniporter, ruthenium red and $La^{2+}$, remove the PalCaP-induced mitochondrial depolarization and stop the release of $Ca^{2+}$ from mitochondria

The addition of 1 $\mu$M RR at the 6th min after the PalCaP opening resulted in a substantial repolarization of mitochondria (Fig. 1A) and stopped further release of $Ca^{2+}$ (Fig. 1B). These experiments were carried out in the presence of 1 $\mu$M CsA.

In case of the PTP opening induced by the addition of 200 nmol $Ca^{2+}$/mg protein in the presence of 1 mM $P_i$ and absence of CsA, mitochondria rapidly lost the accumulated

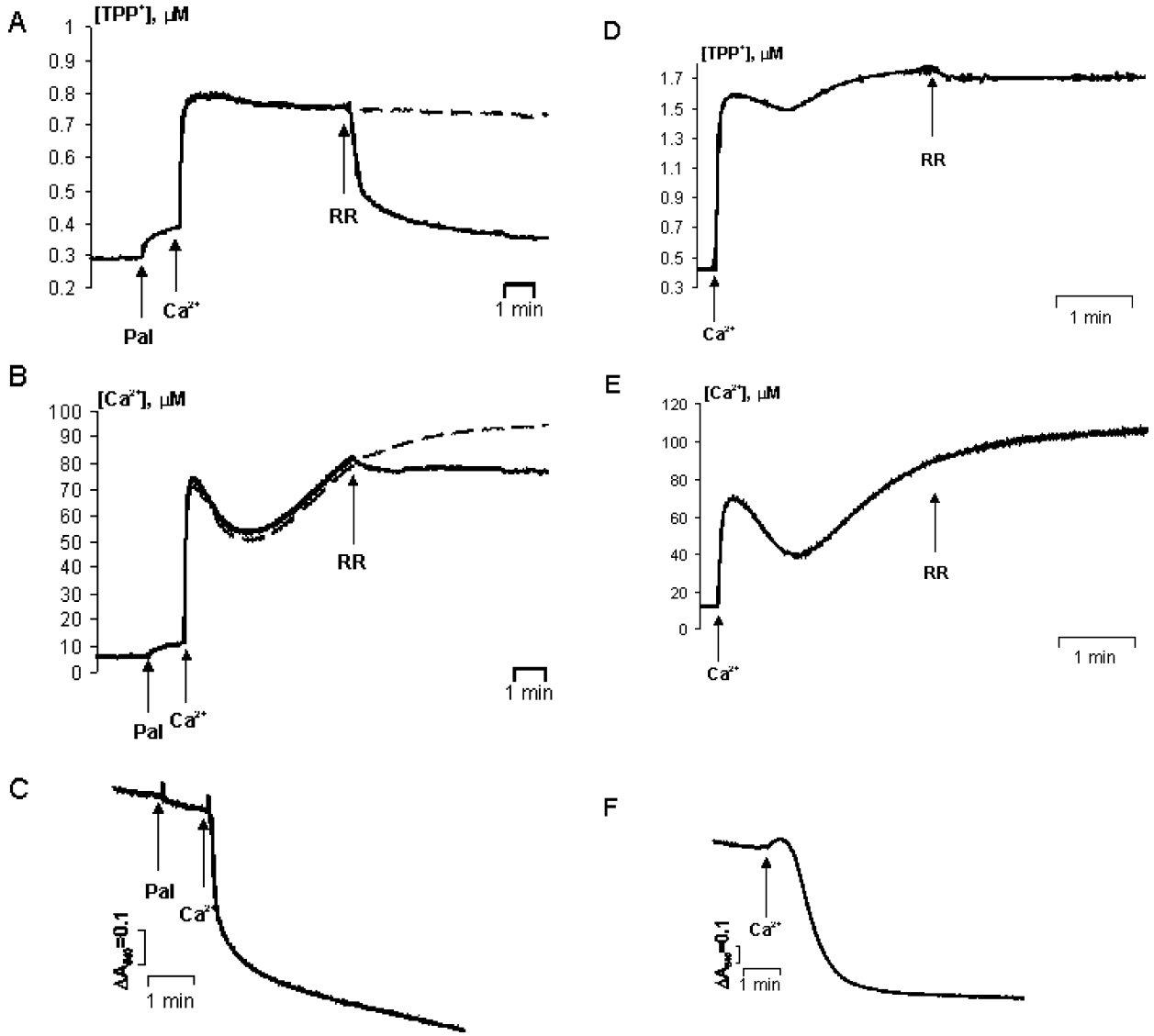


**Fig. 1** The effects of 1 $\mu$M ruthenium red (RR) on the changes in mitochondrial $\Delta\psi$ (A, D), [$Ca^{2+}$] (B, E) and swelling of mitochondria (C, F) that were induced by opening of either the palmitate/$Ca^{2+}$-activated pore (A–C) or PTP (D–F). Dashed line, control (without RR). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 5 $\mu$M EGTA, 1 $\mu$M rotenone, and 10 mM Hepes/KOH (pH 7.4). Additions: Pal (30 nmol/mg protein) and $Ca^{2+}$ (70 nmol/mg protein). The incubation medium was supplemented with 1 $\mu$M CsA (A–C); $Ca^{2+}$ (200 nmol/mg protein). The incubation medium was supplemented with 1mM $P_i$ (D–F). Typical traces are shown ($n = 17$)

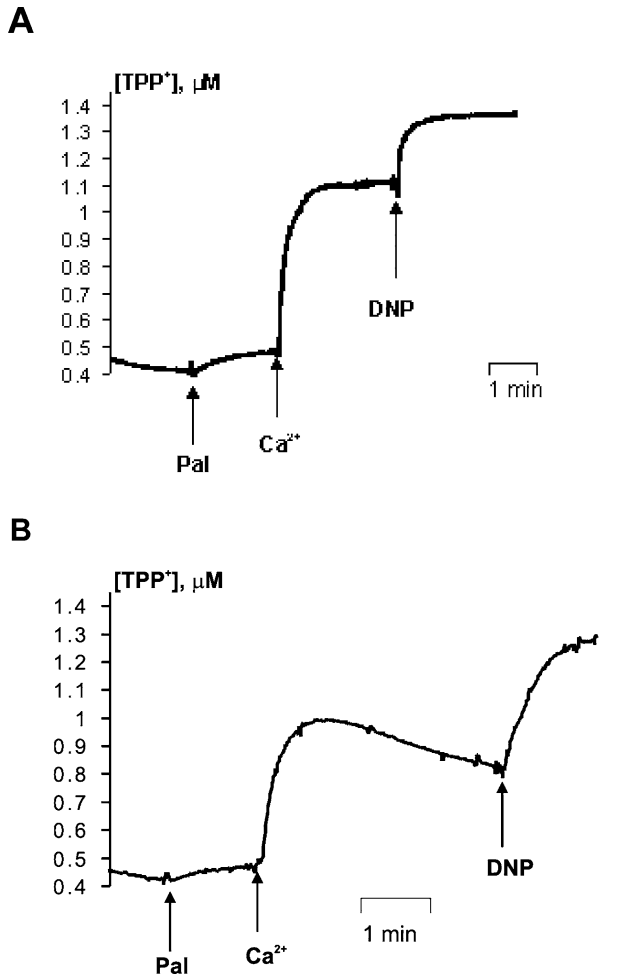


**Fig. 2** The changes in $\Delta\psi$ induced by different concentrations of Pal and $Ca^{2+}$. Additions: Pal (30 nmol/mg protein), $Ca^{2+}$ (70 nmol/mg protein) and DNP (50 $\mu$M) (A); Pal (20 nmol/mg protein), $Ca^{2+}$ (60 nmol/mg protein) and DNP (50 $\mu$M) (B). The incubation medium was as in Fig. 1A. Typical traces are shown ($n = 4 - 7$)

$Ca^{2+}$ and swelled (Fig. 1E and F), $\Delta\psi$ collapsed (Fig. 1D). However, the addition of RR did not lead to repolarization of the inner mitochondrial membrane (Fig. 1D and E). The amplitude of swelling (Fig. 1F) was approximately 1.5 times larger than in case of the PalCaP opening (Fig. 1C).

It is important to note that upon the PalCaP opening, $\Delta\psi$ decreases but does not collapse completely. This can be shown by adding DNP after the PalCaP opening, which removes any residual $\Delta\psi$ (Fig. 2).

The experiments shown in Fig. 1A and B were repeated using another inhibitor of $Ca^{2+}$ uniporter, $La^{3+}$ (Fig. 3A and B), which, in contrast to RR, acts competitively (Reed and Bygrave, 1974). The addition of 5 $\mu$M $La^{3+}$ also led to repolarization of the mitochondrial membrane and inhibited further release of $Ca^{2+}$. The temporary efflux of $Ca^{2+}$ caused by the addition of $La^{3+}$ (Fig. 3B) could be an artefact due to $La^{3+}$ giving a signal with the $Ca^{2+}$ electrode. The chelation of $Ca^{2+}$ by 1 mM EGTA had a similar effect: $\Delta\psi$ returned to the level that was prior to the addition of $Ca^{2+}$ (Fig. 3C and D).

## The accumulation of $Sr^{2+}$ results in the depolarization of mitochondria, activation of $Sr^{2+}$ release and mitochondrial swelling

It may be supposed that the PalCaP opening would also occur in the absence of exogeneous Pal, in response to the activation of phospholipase $A_2$ ($PLA_2$), when the level of endogeneous fatty acids in the mitochondrial membrane increases. It is well-known that high $Ca^{2+}$ concentrations activate $PLA_2$ (Saris, 1994). However, these $Ca^{2+}$ concentrations will also induce the PTP opening and degradation of mitochondria. Taking this into account, we used $Sr^{2+}$ in the next series of experiments. It is known that $Sr^{2+}$ can be transported into mitochondria by the same mechanism as $Ca^{2+}$, i.e. by the uniporter (Gunter and Gunter, 1994; Saris and Carafoli, 2005), and high $Sr^{2+}$ concentrations also activate $PLA_2$ (Saris, 1994). But in contrast to $Ca^{2+}$, $Sr^{2+}$ does not induce PTP opening and damage of mitochondria (Zoratti and Szabo, 2005). At the same time, $Sr^{2+}$ can promote opening of the Pal-activated pore (Sultan and Sokolove, 2001).

The addition of $Sr^{2+}$ (200 nmol/mg protein) to mitochondria in the presence of 1 mM $P_i$ first led to a fall of $\Delta\psi$, which was associated with the influx of $Sr^{2+}$ via the uniporter, and then $\Delta\psi$ returned to a certain stable level (Fig. 4A and B). After 3–4 $Sr^{2+}$ additions the stable level of $\Delta\psi$ began to decrease, and after the 7$^{th}$ addition $\Delta\psi$ did not longer recover (Fig. 4A). At the same time, the release of $Sr^{2+}$ and mitochondrial swelling were initiated (Fig. 4B and C). The shrinkage of mitochondria (an increase in $A_{540}$) induced by additions of $Sr^{2+}$ before their swelling is probably caused by the release of $K^+$, the osmotic cation, from the organelles (Sidash et al., 1994). CsA affected neither $Sr^{2+}$ transport nor swelling. This indicates that the decrease of $\Delta\psi$, $Sr^{2+}$ release and mitochondrial swelling were due to opening of a non-selective CsA-insensitive pore, probably, PalCaP. According to the data presented above, PalCaP can be closed by inhibiting the $Ca^{2+}$ ($Sr^{2+}$) influx. The addition of RR to the swollen mitochondria resulted in the $\Delta\psi$ recover (Fig. 5A) and reduced the efflux of $Sr^{2+}$ (Fig. 5B, cf. Fig. 4B).

## The $Sr^{2+}$-induced mitochondrial swelling is blocked by a phospholipase $A_2$ inhibitor and BSA

The mitochondrial phospholipase $A_2$ inhibitor trifluoperazine (TFP) significantly decreased the $Sr^{2+}$-activated mitochondrial swelling at the concentration of 10 $\mu$M (Fig. 6).

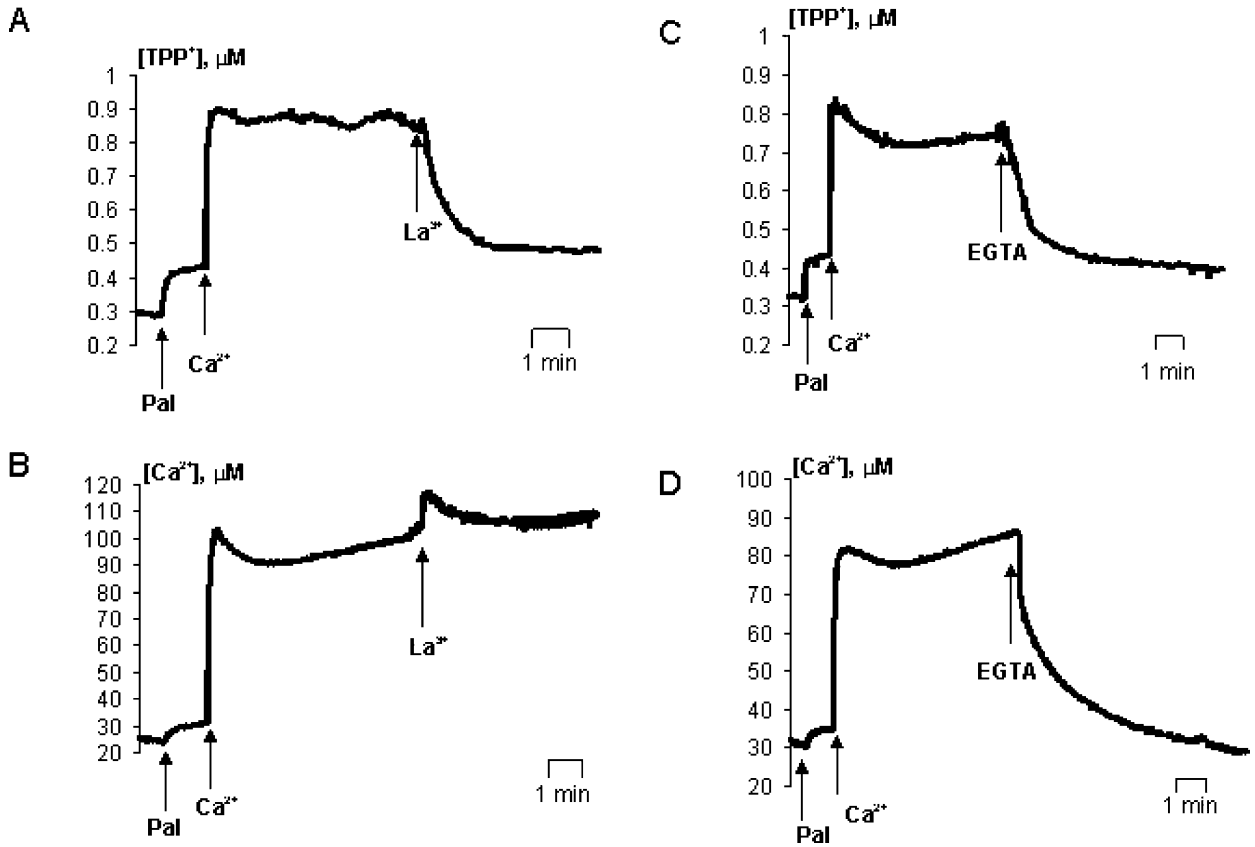


**Fig. 3** The effects of 5 $\mu$M $La^{3+}$ (A-B) and 1 mM EGTA (C-D) on the changes in $\Delta\psi$ and [$Ca^{2+}$] induced by the opening of PalCaP. The incubation medium and additions were as in Fig. 1A. Typical traces are shown ($n = 3$)

At the same time, 10 $\mu$M TFP did not affect the mitochondrial respiration and the rate of $Sr^{2+}$ influx (Table 1). Unfortunately, it was not possible to estimate the effect of TFP on the $Sr^{2+}$-activated $\Delta\psi$ decrease and $Sr^{2+}$ release from mitochondria since TFP interferes with TPP and $Ca^{2+}$ electrodes.

When mitochondria were preincubated with BSA to remove free fatty acids, mitochondrial depolarization and $Sr^{2+}$ efflux decreased as did the amplitude of $Sr^{2+}$-induced mitochondrial swelling (Fig. 7).

## Discussion

In this paper we continue to study properties of the mitochondrial Pal/$Ca^{2+}$-induced cyclosporin A-insensitive pore. As shown in Fig. 1A–C, the combination of Pal (30 nmol/mg protein) and $Ca^{2+}$ (70 nmol/mg protein) induces the opening of a CsA-insensitive pore, this followed by swelling of mitochondria, persistent depolarization of the inner membrane and the release of $Ca^{2+}$ from the organelles. At the same time, mitochondria seem to retain their functional intactness, since the addition of RR restores $\Delta\psi$.

We suppose that the prolonged depolarization of the membrane after the opening of PalCaP is due to operation of a $Ca^{2+}$ cycle which PalCaP is involved in. This pore may therefore be regarded as a nonspecific system of $Ca^{2+}$ efflux from mitochondria.

Different models for the mechanism of $Ca^{2+}$ cycling and $Ca^{2+}$ efflux from mitochondria can be considered. The $Ca^{2+}$ cycle in mitochondria can operate with the ion influx and efflux being mediated by the $Ca^{2+}$ uniporter and $Ca^{2+}$ antiporters (i.e. $Ca^{2+}/nNa^{+}$ or $Ca^{2+}/nH^{+}$) respectively (Carafoli, 1979; Bernardi, 1999; Saris and Carafoli, 2005). In our experiments, however, a $Na^{+}$-free medium was used. Moreover, these efflux mechanisms are not inhibited by RR and are not accompanied by swelling of mitochondria (Jurkowitz et al., 1983).

$Ca^{2+}$ can be released from mitochondria by reversal of the uniporter after the collapse of $\Delta\psi$ (Litsky and Pfeiffer, 1997). In this case, however, a selective RR-sensitive $Ca^{2+}$ efflux would be observed, and mitochondria would not undergo the swelling that we observed in our experiments on the opening of a large non-selective Pal/$Ca^{2+}$-induced pore (Fig. 1C).

Thus we suppose that the $Ca^{2+}$ cycle, operating in mitochondria under our experimental conditions, would

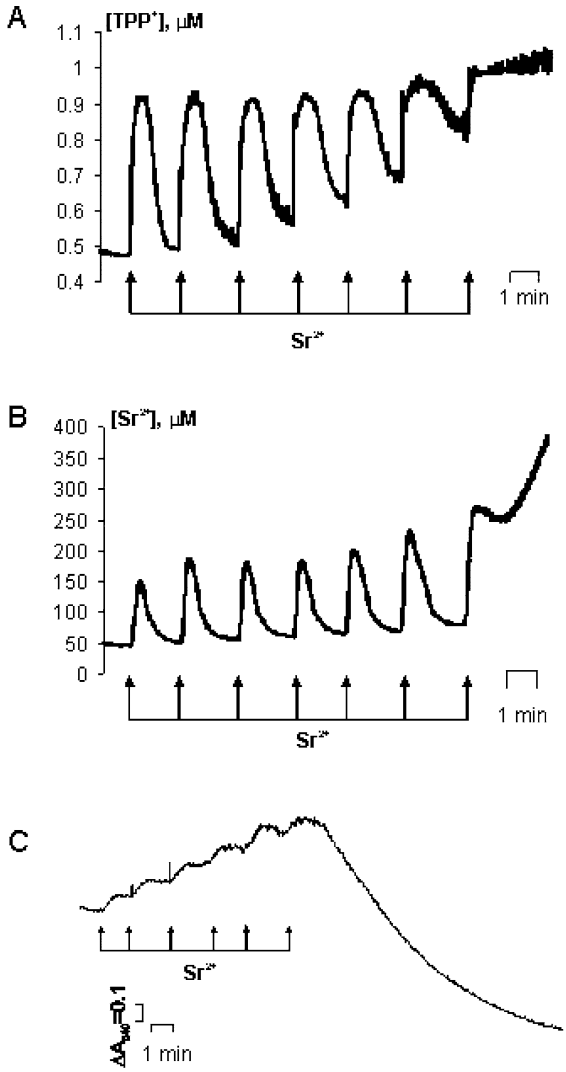


**Fig. 4** The changes in $\Delta\psi$ (A), $[Sr^{2+}]$ (B) and CsA-insensitive swelling of mitochondria (C) induced by the sequential additions of $Sr^{2+}$ (200 nmol/mg protein). The incubation medium was as in Fig. 1A but supplemented with 1 mM $P_i$. Typical traces are shown ($n = 4$)

include $Ca^{2+}$ influx via the uniporter and efflux via the open Pal/$Ca^{2+}$-activated pore.

The addition of Pal to mitochondria will result in the rapid distribution of its molecules between the matrix and cytosolic leaflets of the inner mitochondrial membrane (Hamilton, 1998). This will be accompanied by weak uncoupling (Skulachev, 1998). With $Ca^{2+}$ added to the system, mitochondria will take up this cation via the $Ca^{2+}$ uniporter, and therefore $Ca^{2+}$ will be accumulated in the mitochondrial matrix. Thus, the conditions, arising on the matrix side of the inner mitochondrial membrane, would favor the formation of Pal/$Ca^{2+}$ complexes. Once the concentration of these complexes in the membrane exceeds a certain critical value, the formation of lipid pores will begin. The mechanism of PalCaP opening may be explained in view of our recent data

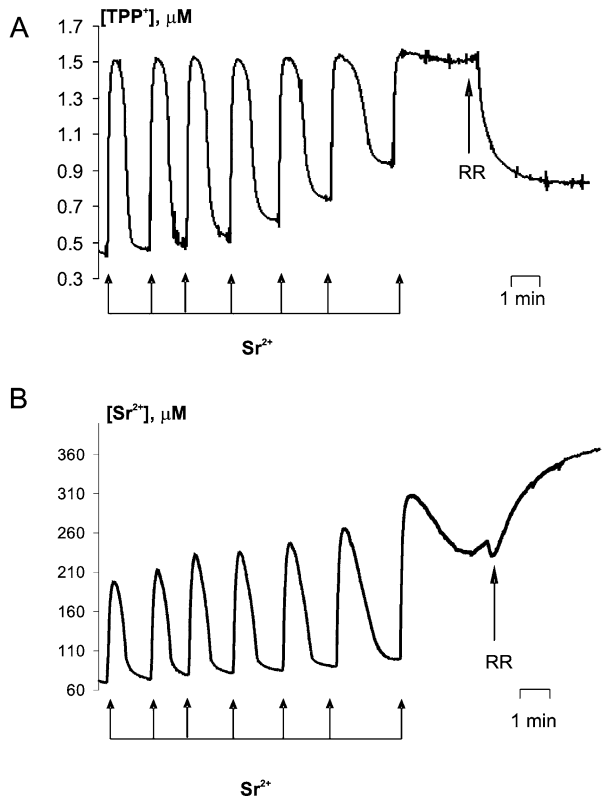


**Fig. 5** The effect of 1 $\mu$M ruthenium red (RR) on the changes in $\Delta\psi$ and $[Sr^{2+}]$ induced by the sequential additions of $Sr^{2+}$ (200 nmol/mg protein). The incubation medium was as in Fig. 4. Typical traces are shown ($n = 7$)

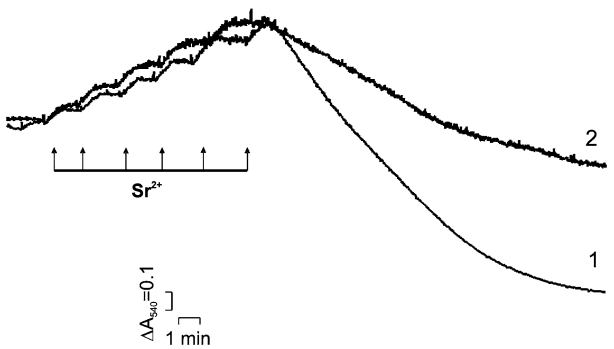


**Fig. 6** The effect of 10 $\mu$M trifluoperazine, a phospholipase inhibitor, on the CsA-insensitive swelling of mitochondria induced by the sequential additions of $Sr^{2+}$ (200 nmol/mg protein). The incubation medium was as in Fig. 4. Typical traces are shown ($n = 5$)

that binding of $Ca^{2+}$ to palmitic acid leads to its segregation into distinct solid membrane domains (Agafonov et al., 2007). This is in good agreement with the theory of lipid pore formation upon chemotropic phase transition (Antonov and Shevchenko, 1995). After the opening of PalCaP $\Delta\psi$ will collapse, this eliminating the motive force that drives the accumulation of Pal/$Ca^{2+}$ complexes on the matrix side of the membrane. However, since the membrane integrity

**Table 1** The effect of TFP on mitochondrial respiration[a]

| | Respiration rate (nmol $O_2 \cdot min^{-1} \cdot$ mg protein$^{-1}$) | | | |
|---|---|---|---|---|
| [TFP] ($\mu$M) | $V_4$ | $V_3$ | $V_{DNP}$ | $V_{Strontium}$ |
| 0 | 10.25 ± 1.18 | 35.07 ± 0.30 | 44.74 ± 1.03 | 22.45 ± 1.61 |
| 10 | 11.19 ± 1.79 | 39.18 ± 1.66 | 48.38 ± 2.62 | 22.11 ± 1.86 |

[a]The incubation medium was as described in "Materials and Methods". Phosphorylation was induced by the addition of 200 $\mu$M ADP, mitochondrial respiration was measured in the presence of 50 $\mu$M DNP and $Sr^{2+}$ (100 nmol/mg protein). Mean values ± SD are presented ($n = 4$).

will be restored in a short time (which is an intrinsic feature of the lipid-pore mechanism), $\Delta\psi$ will recover (Mironova et al., 2004). It seems that in spite of prolonged depolarization, some residual $\Delta\psi$ (50–70 mV) is preserved (Fig. 2),

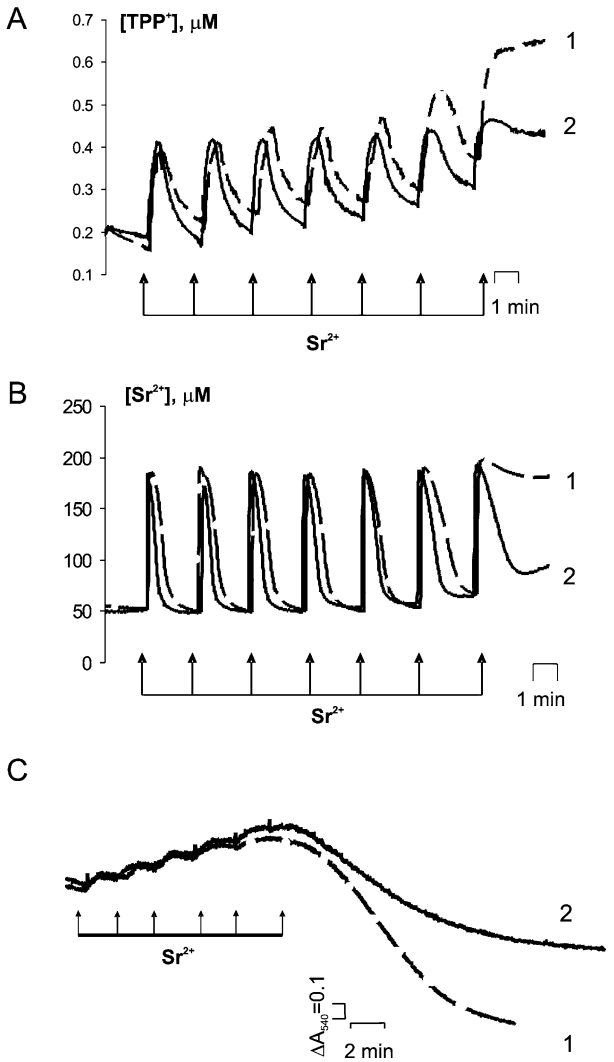


**Fig. 7** The effect of BSA (1 mg/ml) (trace 2) on the changes in $\Delta\psi$ (A), [$Sr^{2+}$] (B) and CsA-insensitive swelling of mitochondria (C) induced by the sequential additions of $Sr^{2+}$ (200 nmol/mg protein). Dashed line (trace 1), control (without BSA). The incubation medium was as in Fig. 4. Typical traces are shown ($n = 6$)

which would enable mitochondria to take up the earlier released $Ca^{2+}$ via the uniporter. Thus, the closure of pores will return the cycle to its beginning: mitochondria will start to accumulate $Ca^{2+}$, and the cycle will be repeated.

In view of this mechanism one can explain the effects of $Ca^{2+}$-uniporter inhibitors and EGTA. As mentioned above, the PalCaP opening would require the presence of $Ca^{2+}$ in the mitochondrial matrix (Sultan and Sokolove, 2001). The inhibition of $Ca^{2+}$ influx by RR or $La^{3+}$ will prevent the formation of new PalCaPs; this, in its turn, can result in inhibition of the $Ca^{2+}$ efflux through the PalCaP and fast recovery of $\Delta\psi$ (Figs. 1A and 3A). At the same time, EGTA would prevent $Ca^{2+}$ influx and PalCaP opening by chelating the $Ca^{2+}$ ions that are being released from mitochondria (Fig. 3C and D). Interestingly, saturated fatty acids were earlier found to stimulate the release of $Ca^{2+}$ from mitochondria (De Villiers and Lochner, 1986).

In the case of PTP, the picture is different. Since this large pore does not close after opening (Sultan and Sokolove, 2001), the inhibition of $Ca^{2+}$ influx by RR does not result in the recovery of $\Delta\psi$ (Fig. 1D–F).

The cycling of $Ca^{2+}$ in mitochondria can be initiated upon the activation of $PLA_2$, which results in the formation of endogenous Pal. As mentioned above, $Ca^{2+}$ and $Sr^{2+}$ are known activators of $PLA_2$ (Saris, 1994). Since high $Ca^{2+}$ concentrations can induce the PTP opening, we preferred to use $Sr^{2+}$. Figure 4 shows that the successive additions of $Sr^{2+}$ cause persistent depolarization, the release of $Sr^{2+}$ from mitochondria and their swelling.

There are substantial similarities between the PalCaP and the $Sr^{2+}$-activated pore. They are non-selective and CsA-insensitive, they close after opening, and their prolonged open state is associated with mitochondrial swelling. The absence of sensitivity to CsA suggests that they are not related to the PTP and may have a lipid nature. It should be noted that the $Sr^{2+}$-induced depolarization, like that observed upon the opening of PalCaP, can be suppressed by RR (Fig. 4A). In addition, TFP and BSA inhibit the $Sr^{2+}$-induced swelling and depolarization of mitochondria (Figs. 6 and 7), this indicating the $PLA_2$ activation and the appearance of endogenous fatty acids.

These results are in good agreement with the data published by Kholmukhamedov et al. around 1990 (Kholmukhamedov et al., 1988, 1991). They found that the addition of $Sr^{2+}$ to rat liver mitochondria induced opening of a non-selective CsA-insensitive reversible pore in the inner mitochondrial membrane. Thus, it can be supposed that this pore is similar to the PalCaP, with $Sr^{2+}$ replaced by $Ca^{+}$ and Pal formed by the $Sr^{2+}$-activated $PLA_2$. The authors suggested that opening of this pore activated a $Ca^{2+}$ cycle in mitochondria, indicating a kind of excitability of the mitochondrial inner membrane (Kholmukhamedov et al., 1991).

In conclusion, the data presented indicate that when the cellular $Ca^{2+}$ increases, leading to the $PLA_2$ activation and elevated levels of free fatty acids (including Pal) in the inner mitochondrial membrane, a $Ca^{2+}$ cycle may be induced in mitochondria, with $Ca^{2+}$ uptake via the calcium uniporter and efflux through the emerging PalCaPs.

**Acknowledgements** This work was supported by grants from the Russian Foundation for Basic Research (04-04-97281) and ISTC (3301) to G. Mironova, and by a grant from the Medical Society of Finland and the Magnus Ehrnrooth Foundation to N.-E. Saris.

## References

Agafonov A, Gritsenko E, Belosludtsev K, Kovalev A, Gateau-Roesch O, Saris N-E, Mironova GD (2003) Biochim Biophys Acta 1609:163–160

Agafonov AV, Gritsenko EN, Shlyapnikova EA, Belosludtseva NV, Kharakoz DP, Saris N-EL, Mironova GD (2007) J Biol Membr (in press)

Antonov V, Shevchenko E (1995) Vestn Ross Acad Med Nauk (in Russian) 10:48–55

Belosludtsev KN, Belosludtseva NV, Mironova GD (2005) Biochemistry (Moscow) 70:815–821

Belosludtsev K, Saris N-EL, Andersson LC, Belosludtseva N, Agafonov A, Sharma A, Moshkov DA, Mironova GD (2006) J Bioenerg Biomembr 38:113–120

Bernardi P (1999) Physiol Rev 79:1127–1155

Bernardi P, Petronilli V (1996) J Bioenerg Biomembr 28:131–138

Carafoli E (1979) FEBS Lett 104:1–5

Carafoli E, Tiozzo R, Lugli G, Crovetti F, Kratzing C (1974) J Mol Cell Cardiol 6:361–371

De Luca M, Engstrom G (1961) Proc Natl Acad Sci USA 47:1744–1747

De Villiers M, Lochner A (1986) Biochim et Biophys Acta 876:309–317

Gunter K, Gunter T (1994) J Bioenerg Biomembr 26:471–485

Hamilton JA (1998) J Lipid Res 39:467–481

Heaton GM, Nicholls DG (1976) Biochem J 156:635–646

Kholmukhamedov EL, Chukhlova EA, Zinchenko VP, Evtodienko YV (1988) Biol Membr (Russian) 5:866–871

Kholmukhamedov EL, Teplova VV, Chukhlova EA (1991) Biol Membr (in Russian) 8:612–620

Jurkowitz M, Geisbuhler T, Jung D, Brierley G (1983) Arch Biochem Biophys 223:120–128

Kamo N, Muratsugu M, Hongoh R, Kobatake Y (1979) J Membr Biol 49:105–121

Litsky M, Pfeiffer D (1997) Biochemistry 36:7071–7080

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951) J Biol Chem 193:265–275

Mironova GD, Gateau-Roesch O, Levrat C, Gritsenko E, Pavlov E, Lazareva AV, Limarenko E, Rey P, Louisot P, Saris N-EL (2001) J Bioenerg Biomembr 33:319–331

Mironova GD, Gritsenko E, Gateau-Roesch O, Levrat C, Agafonov A, Belosludtsev K, France-Prigent A, Muntean D, Dubois M, Ovize M (2004) J Bioenerg Biomembr 36:171–178

Reed KC, Bygrave FL (1974) Biochem J 140:143–155

Reed PW, Lardy H (1988) J Biol Chem 247:6970–6977

Saris N-E (1959) Finska Kemistsamfundets Meddelanden 68:65–72 (In Swedish)

Saris N-E (1963) Soc Sci Fenn: Comment Phys-Math 28(11):1–77 (Doctoral thesis)

Saris N-EL (1994) Magnesium Res 7:5–10

Saris N-EL, Carafoli E (2005) Biochemistry (Moscow) 70:187–194

Sidash SS, Evtodienko YuV, Kholmukhamedov EL, Teplova VV (1994) Biol Membr (Russian) 11:429–436

Skulachev VP (1998) Biochim Biophys Acta 1363:100–124

Sultan A, Sokolove P (2001) Arch Biochem Biophys 386:37–51

Vasington FD, Murphy JV (1962) J Biol Chem 237:2670–2672

Zoratti M, Szábo I (1995) Biochim Biophys Acta 1241:139–176

Zoratti M, Szábo I, De Marchi U (2005) Biochim Biophys Acta 1706:40–52