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ORIGINAL PAPER

On the mechanism of palmitic acid-induced apoptosis: the role of a pore induced by palmitic acid and Ca^{2+} in mitochondria

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Abstract Palmitic acid (Pal) is known to promote apoptosis (Sparagna G et al (2000) Am J Physiol Heart Circ Physiol 279: H2124–H2132) and its amount in blood and mitochondria increases under some pathological conditions. Yet, the mechanism of the proapoptotic action of Pal has not been elucidated. We present evidence for the involvement of the mitochondrial cyclosporin A-insensitive pore induced by Pal/Ca^{2+} complexes in the apoptotic process. Opening of this pore led to a fall of the mitochondrial membrane potential and the release of the proapoptotic signal cytochrome *c*. The addition of cytochrome *c* prevented these effects and recovered membrane potential, which is in contrast to the cyclosporin A-sensitive mitochondrial permeability transition pore. Oleic and linoleic acids prevented the Pal/Ca^{2+}-induced pore opening in the intact mitochondria, this directly and significantly correlating with the effect of these fatty acids on Pal-induced apoptosis in cells (Hardy S et al (2003) J Biol Chem 278: 31861–31870). The specific probe for cardiolipin, 10-*N*-nonyl acridine orange, inhibited formation of this pore.

Keywords Apoptosis · Calcium · Cyclosporin A · Cytochrome *c* · Palmitic acid · Permeability transition pore

Abbreviations $\Delta\psi$: mitochondrial inner membrane potential · CsA: cyclosporin A · Pal: palmitic acid · PalCaP: Pal/Ca^{2+}-activated pore · FFA: free fatty acids · PTP: permeability transition pore · NAO: 10-*N*-nonyl acridine orange · TPP^{+}: tetraphenylphosphonium · cyt *c*: cytochrome *c*

Introduction

One of signaling mechanisms proposed for the induction of apoptosis is the increase in the permeability of the mitochondrial membrane leading to the release of proapoptotic proteins such as cytochrome *c* (cyt *c*), Smac/Diablo and apoptosis-inducing factor (AIF) (Liu et al, 1996; Susin et al, 1999). The caspase cascade and cell death programme is thereby activated (Kroemer et al, 1998).

Free fatty acids (FFA) are natural modifiers of biological membranes, affecting a number of physiological functions (Dyatlovitskaya and Bezuglov, 1998). In mitochondria they affect the function of the uncoupling protein (Jezek et al, 1994), the adenine nucleotide ATP/ADP translocator (Skulachev, 1998), and the opening of the mitochondrial permeability transition pore (PTP) (Zoratti and Szabò, 1995; Broekemeier and Pfeiffer, 1995). It was found that saturated FFA, such as palmitic (Pal) and stearic acid, but not unsaturated ones may induce apoptosis (Sparagna et al, 2000; Kong and Rabkin, 2000). The mechanism of the proapoptotic action of Pal is still poorly understood. It was shown that Pal affected the synthesis of the mitochondrial acidic

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phospholipid cardiolipin, and this effect was correlated with the release of cyt *c* from mitochondria (Ostrander et al, 2001; Hardy et al, 2003). Also, Pal can increase the permeability of the inner mitochondrial membrane (Kong and Rabkin, 2000). Unsaturated FFA, such as oleic and linoleic, reversed the proapoptotic affect of Pal (Ostrander et al, 2001; Hardy et al, 2003).

We have suggested that the proapoptotic effect of Pal could be due to the opening of a cyclosporin A (CsA)-insensitive permeability transition pore, which has been recently found in mitochondria (Sultan and Sokolove, 2001a; Mironova et al, 2004; Belosludtsev et al, 2005). The opening of this pore was found to be related to the formation of Pal/Ca^{2+} complexes in the mitochondrial membrane (Mironova et al, 2004). We found that Pal and stearic acids have a high affinity for Ca^{2+} in comparison to other FFA and the formation of the Pal/Ca^{2+} complex in artificial membranes, bilayer lipid membranes (BLM) and liposomes, increased the non-selective permeability of these membranes (Mironova et al, 2001; Agafonov et al, 2003).

The properties of the Pal/Ca^{2+}-activated pore (PalCaP) differ from those of the PTP. PalCaP opens temporarily (Sultan and Sokolove, 2001a; Mironova et al, 2004; Belosludtsev et al, 2005), what can be well explained in the light of the theory of lipid pores (Antonov and Shevchenko, 1995). It was found P_i, while being necessary for opening of the PTP, was not needed for opening of the PalCaP (Sultan and Sokolove, 2001a). Also, Sr^{2+} was able to activate PalCaP (Agafonov et al, 2003), whereas ADP did not prevent its opening (Belosludtsev et al, 2005).

The ability of PalCaP to close spontaneously, promoting the recovery of mitochondrial membrane potential ($\Delta\psi$), makes Pal more attractive as an activator of apoptosis. It is known that during apoptosis, mitochondria maintain a substantial level of cellular ATP (Liu et al, 1996). However, the possible participation of the PalCaP in the process of apoptosis has not yet been investigated.

In this paper, we present data indicating that the opening of PalCaP causes the release of cyt *c* from mitochondria. Oleic and linoleic acids prevent PalCaP opening. The specific probe for cardiolipin, 10-*N*-nonyl acridine orange (NAO), inhibits the formation of this pore but does not influence significantly the mitochondrial respiration. These data suggest that cardiolipin is important for the regulation of the PalCaP opening.

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 μM EGTA. The final suspension contained 90–100 mg of mitochondrial protein/ml. The concentration of the mitochondrial protein was determined by the Lowry method (Lowry et al, 1951). When isolated for electron microscopy examination, mitochondria were washed in a larger volume of the isolation medium (to remove microsomes more effectively), the ratio of tissue weight/isolation media being 1:20 instead of 1:8.

Mitochondrial swelling was measured as a decrease in A_{540} in a stirred and thermostated cuvette (25°C) using an USB-2000 spectroscopy fiber-optic system (Ocean Optics Inc, USA). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 5 μM EGTA, 1 μM rotenone, and 10 mM Hepes/KOH buffer (pH 7.4). The concentration of the mitochondrial protein in the cuvette was about 0.5 mg/ml. In some experiments, the incubation medium was supplemented with 1 μM CsA.

Changes in $\Delta\psi$ were recorded by following the distribution of 1 μM tetraphenylphosphonium (TPP^+) across the inner mitochondrial membrane. The concentration of TPP^+ was measured with a TPP^+-sensitive electrode (Kamo et al, 1979). The concentration of the mitochondrial protein in the cuvette was 1 mg/ml.

Oxygen consumption by isolated rat liver mitochondria was measured using a Clark-type oxygen electrode.

Samples for western-blot analysis were mixed with Laemmli's loading buffer, boiled for 5 min and subjected to SDS/PAGE (12% gel) at 200 V followed by electroblotting on nitrocellulose membranes for 2 h at 80 V. Membranes were blocked for 1 h with 2% (w/v) fat-free milk in PBS at room temperature (22°C) and subsequently incubated overnight with anti-cyt *c* antibodies (1:2500). The membranes were rinsed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:10000). After the incubation with secondary antibodies, the membranes were rinsed, and the enhanced chemiluminescence of bound antibodies was detected according to the manufacturer's instructions.

For electron microscopy samples of mitochondria were fixed for 2 h in 2.5% glutaraldehyde dissolved in isolation medium (pH 7.4) and postfixed in 1% osmium tetroxide. Samples were embedded in Epon 812. Microscopic sections were cut on an LKB-III microtome and stained with lead and uranylacetate. Electron microscopy was performed with a Tesla BS-500 microscope.

Preparation of liposomes (large unilamellar vesicles) loaded with sulforhodamine B (SRB) and measurement of their permeabilization were made as described earlier (Agafonov et al, 2003).

All chemicals were purchased from Sigma-Aldrich, St Louis, MO, U.S.A.

Table 1 Mitochondrial respiration before and after PalCaP opening in the presence and the absence of 10 μM cyt c^a

	Respiration rate (nmol O_2 $*$ min^{-1} $*$ mg $protein^{-1}$)			
	−cyt *c*		+10 μM cyt *c*	
PalCaP state	V_2	V_{DNP}	V_2	V_{DNP}
Before PalCaP opening	8.66 ± 1.46	44.37 ± 1.64	9.54 ± 1.71	51.10 ± 1.86
PalCaP opening	36.65 ± 1.21	43.55 ± 7.00	31.68 ± 0.85	48.50 ± 5.52
After PalCaP closure	12.25 ± 2.75	36.47 ± 6.51	13.71 ± 2.08	39.03 ± 5.06

[a]The incubation medium composition is as described "Materials and Methods" but supplemented with 1 μM CsA. Concentration of Pal −20 nmol/mg prot, Ca^{2+} −60 nmol/mg prot, DNP −50 μM. Mean values ± SD are presented ($n = 4$).

Results

The opening of PalCaP induces a CsA-insensitive release of cyt *c* from mitochondria

As was established earlier, 15 μM Pal (30 nmol/mg protein) and 30 μM Ca^{2+} (60 nmol/mg protein) induced the opening of a CsA-insensitive PalCaP (Mironova et al, 2004; Belosludtsev et al, 2005). Under these conditions, cyt *c* is released from mitochondria in the CsA-insensitive way (Fig. 1). The amount of cyt *c* released is comparable to that released upon opening of the CsA-sensitive PTP, which was induced by the addition of 100 μM Ca^{2+} (200 nmol/mg protein) in the presence of 1 mM P_i.

Cyt *c* prevents the PalCaP-induced mitochondrial depolarization but does not affect the depolarization caused by the PTP opening

The Pal/Ca^{2+}-induced permeabilization of the mitochondrial and artificial membranes was found to be of short duration (Agafonov et al, 2003; Mironova et al, 2004). After depolarization, following the PalCaP induced by Pal (15 nmol/mg of protein) and Ca^{2+} (35 nmol/mg of protein), $\Delta\psi$ was recovered within 10 min (Mironova et al, 2004). The rate of respiration (V_2) significantly increased within the PalCaP opening and decreased after the pore closure. However, it was still higher than that before the pore opening (Table 1).

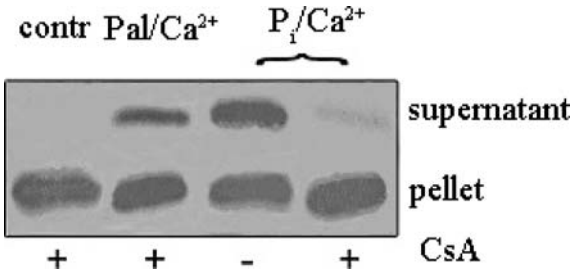

Fig. 1 The cyt *c* release from mitochondria induced by the Pal/Ca-activated pore opening (Pal (30 nmol/mg protein) and Ca^{2+} (60 nmol/mg protein)) and PTP opening (Ca^{2+} (200 nmol/mg protein) in the presence of 1 mM P_i) in the presence and the absence of 1 μM CsA

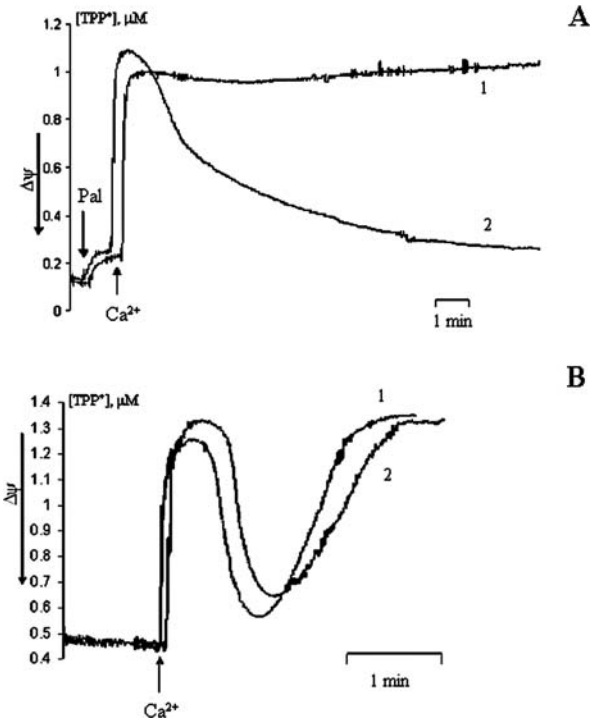

Fig. 2 Changes in $\Delta\psi$ on opening of the PalCaP (A) or PTP (B) in the absence (1) and the presence (2) of 10 μM cyt *c*. The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 5 μM EGTA, 1 μM rotenone, and 10 mM HEPES/KOH (pH 7.4). Additions: Pal (30 nmol/mg protein) and Ca^{2+} (70 nmol/mg protein), the medium was supplemented with 1 μM CsA (A); Ca^{2+} (200 nmol /mg protein), the medium was supplemented with 1 mM P_i. The typical curves are presented ($n = 5$)

After the addition of the same amount of Pal as in Fig. 1 (30 nmol/mg protein) and Ca^{2+} (70 nmol/mg protein), $\Delta\psi$ dropped and did not recover within 10 min (Fig. 2A). In those experiments, a small decrease in $\Delta\psi$ was observed upon the addition of Pal. This effect of Pal is related to its well-known uncoupling properties and is not associated with the mitochondrial swelling (Skulachev, 1998). The preincubation of mitochondria with 10 μM cyt *c* resulted in the recovery of $\Delta\psi$ approximately to the initial level within 5 min after the PalCaP opening. It should be mentioned that cyt *c* had no influence on the uncoupling effect of Pal. This indicates that

Fig. 3 The mitochondrial swelling induced by Pal (30 nmol/mg protein) and Ca^{2+} (60 nmol/mg protein) in the absence (1) and in the presence (2) of 10 μM cyt *c*. The incubation medium was the same as in Fig. 2A. The typical curves are presented ($n = 5$)

cyt *c* can not access the inter-membrane space of mitochondria if PalCaP does not open.

In case of PTP, the picture is different. Figure 2B shows that the PTP opening induced by 200 μM Ca^{2+} (200 nmol/mg protein) in the presence of 1 mM P_i caused a collapse of $\Delta\psi$ after a lag. Under these conditions, a release of cyt *c* from mitochondria was observed (Fig. 1). The addition of 10 μM or 20 μM cyt *c* (data not shown) to the mitochondrial suspension had little, if any, effect (Fig. 2B, trace 2).

Effect of cyt *c* on the mitochondrial swelling induced by the opening of PalCaP

The addition of 30 nmol Pal and 60 nmol Ca^{2+}/mg protein induced mitochondrial swelling, indicating the PalCaP opening. Typical curves are presented in Fig. 3. The rate of this swelling decreased only by 15–20% in the presence of 10 μM cyt *c*. There was no effect of cyt *c* on the swelling induced by the PTP opening (data not shown).

The electron microscopy examination confirmed that Pal/Ca^{2+} complexes induced swelling of mitochondria (Fig. 4B). The addition of 10 μM cyt *c* did not induce mitochondrial contraction, but observed in some organelles was an initiation of this process (Fig. 4C).

Oleic and linoleic acids prevent the PalCaP opening in mitochondria

As found earlier, the FFA that bind Ca^{2+} with high affinity are able to induce the mitochondrial swelling in the presence of Ca^{2+} (Mironova et al, 2004; Belosludtsev et al, 2005). Oleic (Fig. 5A, trace 2) and linoleic (Fig. 5B, trace 2) acids, which bind Ca^{2+} with low affinity, did not induce the opening of the CsA-insensitive Ca^{2+}-dependent pore. Moreover, these fatty acids prevented the CsA-insensitive swelling of mitochondria induced by Pal and Ca^{2+} (Fig. 5, traces 3). In the experiments with the release of sulforhodamine B from liposomes, Ca^{2+} had a little effect on vesicles containing linoleic acid — in comparison with its effect on the Pal-containing liposomes. At that same time, linoleic acid slightly prevented the Pal/Ca^{2+}-induced release of sulforhodamine B from liposomes (Table 2). The same results were obtained with oleic acid (data not shown).

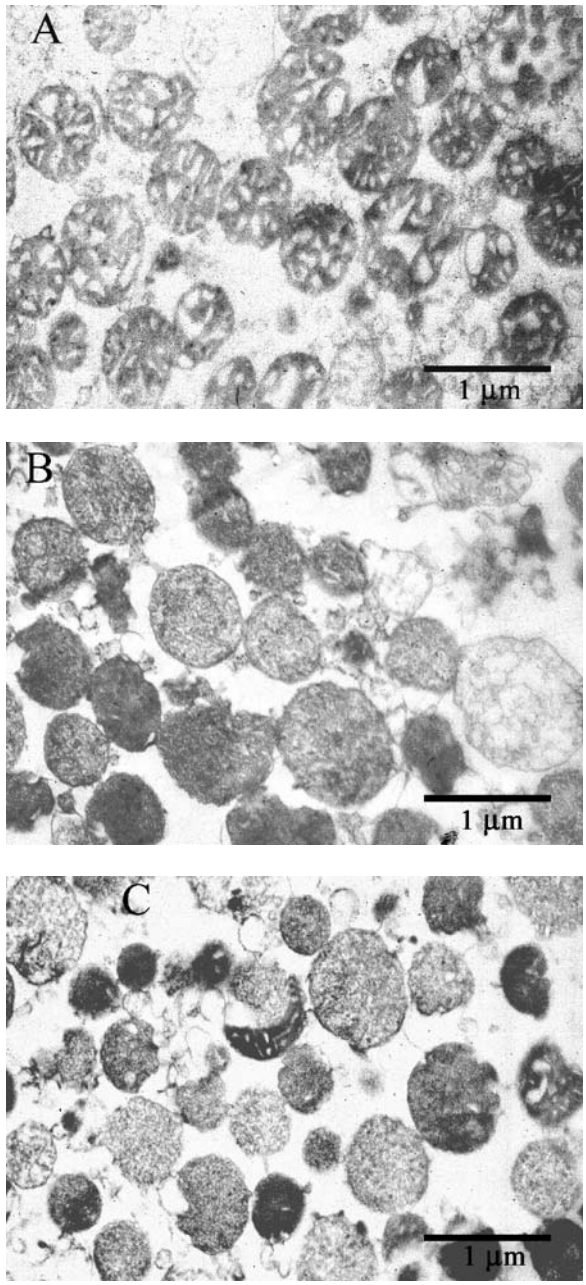

Fig. 4 Morphology of mitochondria following PalCaP opening in the absence (B) and presence (C) of 10 μM cyt *c*. As controls, mitochondria with no added Pal, Ca^{2+} and cyt *c* were also analyzed (A). Medium composition and additions are as in Fig. 3

Influence of NAO on the PalCaP opening and mitochondrial respiration

It was shown that the Pal-promoted induction of apoptotic cell death is accompanied by a decrease in the content

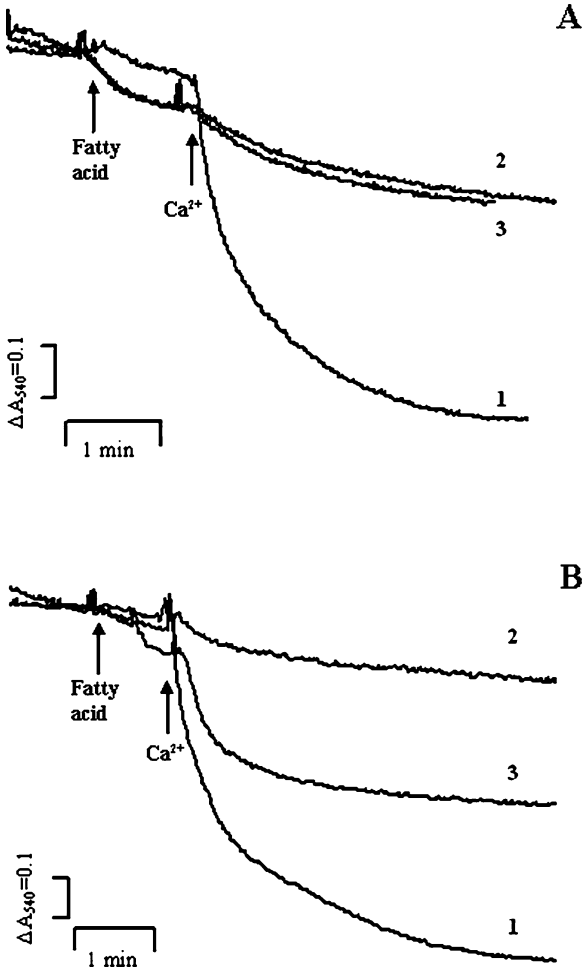

Fig. 5 The influence of oleic (A) and linoleic (B) acids on mitochondrial swelling, induced by Pal and Ca^{2+}. The incubation medium was the same as in Fig. 2A. Additions: (1) Pal (30 nmol/mg protein) and Ca^{2+} (60 nmol/mg protein); (2) oleic acid (30 nmol/mg protein) (A) or linoleic acid (30 nmol/mg protein) (B) and Ca^{2+} (60 nmol/mg protein); (3) Pal (30 nmol/mg protein) + oleic acid (30 nmol/mg protein) (A) or linoleic acid (30 nmol/mg protein) (B), Ca^{2+} (60 nmol/mg protein)

of cardiolipin (Hardy et al, 2003), an anionic phospholipid mainly found in the inner mitochondrial membrane, especially in the contact sites (Ardail et al, 1990). In order to study a possible role of cardiolipin in the induction of the PalCaP opening, we investigated the effect of the fluorescent probe NAO, which binds specifically

Table 2 The release of sulforhodamine B from liposomes induced by Pal and Ca^{2+}[a]

FFA	Sulforhodamine B release, % of total entrapped in liposomes
Pal	58.91 ± 5.69
Linoleic acid	15.36 ± 3.31
Pal+Linoleic acid	48.66 ± 0.83

[a] Medium contained 40 mM KCl, 5 μM EGTA and 10 mM Tris−HCl (pH 8.5). Concentration of Pal −30 μM, Linoleic acid −30 μM and Ca^{2+} −1 mM. Mean values ±SD are presented ($n = 4$).

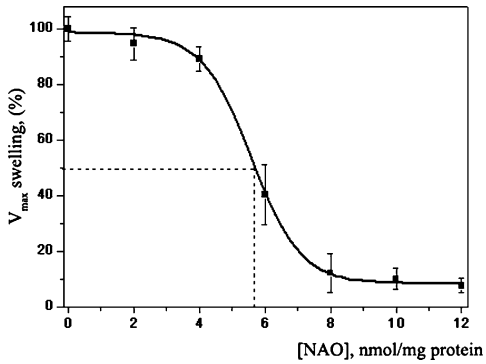

Fig. 6 The influence of NAO on the mitochondrial swelling induced by Pal (30 nmol/mg protein) and Ca^{2+} (60 nmol/mg protein). The incubation medium was the same as in Fig. 2A. Mean values ±SD are presented ($n = 6$)

to cardiolipin (Petit et al, 1992), on the PalCaP-induced swelling of mitochondria. It was found that NAO inhibited the Pal/Ca^{2+}-activated swelling of mitochondria in a dose-dependent manner (Fig. 6). A half-maximal inhibition of the swelling rate occurred in the presence of 5.69 nmol NAO/mg protein.

We tested the effect of NAO on the respiration of mitochondria in various energetic states (Table 3). NAO (10 nmol/mg protein) influenced nor the basic succinate-dependent respiration of mitochondria (state 2), nor the uncoupled respiration induced by DNP; and it had practically no effect on the Ca^{2+}-stimulated respiration. However, NAO inhibited the mitochondrial respiration by 30% in the state 3.

Table 3 The influence of NAO on mitochondrial respiration

	Respiration rate (nmol $O_2 * min^{-1} * mg\ protein^{-1}$)			
NAO, (nmol/mg prot)	V_2	V_3	V_{DNP}	$V_{Calcium}$
0	9.39 ± 2.66	22.82 ± 1.31	44.37 ± 1.64	43.42 ± 4.73
10	10.28 ± 2.70	15.34 ± 1.64	45.76 ± 2.20	37.64 ± 3.83

[a] Medium composition is as Fig. 2A but supplemented with 1 mM P_i. Phosphorylation was induced by the addition of 200 μM of ADP, the mitochondrial respiration was measured in the presence of 50 μM DNP and Ca^{2+} (200 nmol/mg protein). Mean values ± SD are presented ($n = 5$).

Discussion

This study shows that the opening of PalCaP may be one of mechanisms for the induction of apoptosis. It was found that saturated fatty acids (mainly Pal) can induce apoptosis (DeVeries et al, 1997). A metabolic role for mitochondria in the Pal-induced apoptosis in cell cultures has been suggested (Kong and Rabkin, 2000; Sparanga et al, 2000). In this report, we present data indicating that the proapoptotic effect of Pal is correlated with the opening of the PalCaP in mitochondria.

We found that the opening of the PalCaP leads to the release of cyt *c* from mitochondria in amounts comparable to those released after the opening of the PTP (Fig. 1). Since opening of PalCaP is temporary (Sultan and Sokolove, 2001a; Agafonov et al, 2003; Mironova et al, 2004), the presence of cyt *c* in the medium at the moment of pore opening should prevent, at least partially, its release from the inter-membrane space of mitochondria. This would be the cause of a slight stimulation of the mitochondrial respiration after the PalCaP closure (Table 1). It should be noted that a slight stimulation of the mitochondrial respiration was also observed without any external cyt *c*. However, the nature of these stimulations appears to be different. In the presence of the external cyt *c*, it will be stimulation of the coupled mitochondrial respiration, since cyt *c* recovers $\Delta\psi$ (Fig. 2A). In the absence of the external cyt *c*, slightly stimulated will be the uncoupled mitochondrial respiration.

In contrast to PalCaP, PTP does not close spontaneously after the induction of swelling and the collapse of $\Delta\psi$. Therefore, the addition of cyt *c* had little, if any, effect in this system (Fig. 2B). Probably, recovering mitochondria after PTP opening would require a more complex intervention, e.g. a combined action of several biochemical agents (Zoratti et al, 2005).

The swelling of mitochondria after the opening of PalCaP did not decrease with time, and cyt *c* only slightly affected the swelling (Fig. 3). The initiation of the contraction process in the presence of cyt *c* was observed only in some organelles (Fig. 4). Thus, the effect of external cyt *c* is due to its influence on $\Delta\psi$, which mitochondria can maintain even in the swollen state. It seems that after mitochondrial repolarization, a considerable time is needed to restore the original smaller volume of mitochondria.

The release of cyt *c* due to the PalCaP opening is a necessary but not a sufficient proof of our suggestion that the opening of PalCaP may contribute to the Pal-induced apoptosis. Cyt *c* has been reported to be released under conditions when the mitochondrial volume was increased without any pore opening (Gogvadze et al, 2004).

In order to obtain more evidence of the relevance of PalCaP to apoptosis, we studied opening of this pore in such conditions when the Pal-activated apoptosis is prevented on the cellular level. It is known that unsaturated FFA (oleic and linoleic acids) prevent the proapoptotic action of Pal (Hardy et al, 2003). Figure 5 shows that oleic and linoleic acids inhibit the PalCaP opening in mitochondria; but themselves, they do not induce a Ca^{2+}-dependent permeabilization of the mitochondrial membrane. Sokolove's group showed that unsaturated FFA induced the opening of a Ca^{2+}-dependent pore, sensitive to CsA (Sultan and Sokolove, 2001b). It should be noted that this effect of unsaturated FFA may be a consequence of them being oxidized. In our experiments, fresh unsaturated FFA did not cause mitochondrial swelling in the presence of Ca^{2+}, this being independent of CsA. But after exposure to air for several hours, unsaturated FFA began to induce the Ca^{2+}-dependent CsA-sensitive swelling of mitochondria (data not shown).

Earlier, we have supposed that PalCaP has a lipid nature (Agafonov et al, 2003; Mironova et al, 2004; Belosludtsev et al, 2005). It seems that the formation of this lipid pore is related to a Ca^{2+}-induced segregation of Pal into solid membrane domains (unpublished data). Hence, it may be supposed that the addition of unsaturated FFA hinders the segregation of Pal/Ca^{2+} complexes in the membrane and thus, prevents the PalCaP opening. However, the little inhibitory effect of linoleic acid (~17%) on PalCaP opening in liposomes (Table 2) does not fully confirm this assumption.

It has been shown that a possible cause of the proapoptotic action of Pal is that this fatty acid decreases the level of mitochondrial cardiolipin, which is necessary for cyt *c* retainment in the membrane (Choi and Swanson, 1995), and increases the levels of saturated cardiolipin precursors (Ostrander et al, 2001; Hardy et al, 2003). Supplementation of unsaturated FFA induced the channeling of Pal to inert triglyceride stores, restored the cardiolipin level and blocked the Pal-activated apoptosis thereby decreasing the lipotoxicity of Pal (Hardy et al, 2003; Listenberger et al, 2003). Thus, the inhibitory effect of unsaturated FFA on PalCaP opening possibly reflects a sum of these processes taking place in the mitochondrial membrane (some influence on the segregation of Pal/Ca^{2+} complexes and strong influence on the cardiolipin level).

Generally, the role of mitochondrial cardiolipin in the apoptosis can not be overestimated. Hence, to examine the participation of PalCaP in apoptosis we have studied a possible role of this phospholipid in the induction of PalCaP opening. We found that NAO, an agent that specifically binds to cardiolipin, is a powerful inhibitor of PalCaP (Fig. 6). This effect of NAO can not be explained simply by its possible influence on the mitochondrial respiration: used at a concentration which completely inhibits the pore opening (10 nmol/mg protein), NAO had no effect on the

rate of succinate-dependent respiration at different states of mitochondria (V_2, V_{DNP}, and $V_{Calcium}$) and inhibited the phosphorylation rate by 30% (Table 3). The latter effect of NAO is probably due to the involvement of cardiolipin in the regulation of the adenine nucleotide translocation (Beyer and Nuscher, 1996). It is known also that cardiolipin is important for the functioning of other mitochondrial membrane proteins (Schlame et al, 2000). Therefore, the mechanism of the influence NAO on PalCaP opening still needs to be clarified.

Thus, our data support the suggestion (Kong and Rabkin, 2000; Sparanga et al, 2000; Hardy et al, 2003) that the proapoptotic action of Pal is due to its effects on the mitochondrial functions. Both PalCaP and cardiolipin may be involved in this process. The decrease in the level of cardiolipin or changes in the cardiolipin state ("inactivation" by NAO or oxidation by ROS (Petrosillo et al, 2004)) would be a first step in the cyt *c* release from mitochondria, raising the concentration of cyt *c* in the inter-membrane space. The release of cyt *c* is then promoted by the mitochondrial swelling induced by the opening of PalCaP.

It is still unclear why decreasing the cardiolipin level influences the PalCaP opening. Probably, the restriction of cardiolipin synthesis in the presence of Pal leads to an additional increase in the amounts of Pal: we have found that cardiolipin and phosphatidyletahanolamine can be the sources of Pal in some cells (Mironova et al, 2004). According to our earlier data, the content of free and esterified Pal is higher in mitochondria of the TNF-sensitive cells (WEHI-164 line), comparatively to that in mitochondria of the TNF-insensitive cells (C_6 line), in which apoptosis is harder to be induced. So, PalCaP may also be of relevance to the TNF-activated apoptosis.

Concluding remarks

This study shows that opening of the PalCaP can be related to the induction of apoptosis. In our experiments close to endogenous amounts of Pal were used (Wojtczak, 1969). The amounts of Ca^{2+} needed to open this pore were also close to physiological and significantly smaller than those needed for opening the PTP. PalCaP closes spontaneously, without a permanent loss of mitochondrial functions. The opening of PalCaP leads to the release of cyt *c* from mitochondria. All this allows us to consider PalCaP as one of the possibly factors of the apoptotic cell death. In contrast, the irreversible opening of the PTP directs cell to the necrotic pathway (Crompton et al, 2003). Therefore, the role of PTP in the induction of apoptosis should be reconsidered (Nicholls and Chalmers, 2004), and the attention should be focused on another mitochondrial Ca^{2+}-dependent pore, that activated by the natural inductor of apoptosis, palmitic acid.

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