Biochimica et Biophysica Acta 1838 (2014) 2600–2606

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

# $Ca^{2+}$-dependent permeabilization of mitochondria and liposomes by palmitic and oleic acids: A comparative study

Konstantin N. Belosludtsev [a,*], Natalia V. Belosludtseva [a,b], Alexey V. Agafonov [a,b], Maxim E. Astashev [a,c], Alexey S. Kazakov [d], Nils-Erik L. Saris [e], Galina D. Mironova [a,b]

[a] Institute of Theoretical and Experimental Biophysics RAS, 142290 Pushchino, Moscow Region, Russia
[b] Pushchino State Institute of Natural Sciences, 142290 Pushchino, Moscow Region, Russia
[c] Institute of Cell biophysics RAS, Institutskaya 3, Pushchino, Moscow region 142290, Russia
[d] Institute for biological instrumentation RAS, Institutskaya 7, Pushchino, Moscow region 142290, Russia
[e] University of Helsinki, Helsinki, POB 56 Biocenter 1, FIN-00014, Finland

ARTICLE INFO

*Article history:*
Received 12 May 2014
Received in revised form 11 June 2014
Accepted 22 June 2014
Available online 2 July 2014

*Keywords:*
Palmitic acid
Oleic acid
Calcium
Mitochondria
Liposomes
Membrane permeabilization

ABSTRACT

In the present work, we examine and compare the effects of saturated (palmitic) and unsaturated (oleic) fatty acids in relation to their ability to cause the $Ca^{2+}$-dependent membrane permeabilization. The results obtained can be summarized as follows. (1) Oleic acid (OA) permeabilizes liposomal membranes at much higher concentrations of $Ca^{2+}$ than palmitic acid (PA): 1 mM versus 100 μM respectively. (2) The OA/$Ca^{2+}$-induced permeabilization of liposomes is not accompanied by changes in the phase state of lipid bilayer, in contrast to what is observed with PA and $Ca^{2+}$. (3) The addition of $Ca^{2+}$ to the PA-containing vesicles does not change their size; in the case of OA, it leads to the appearance of larger and smaller vesicles, with larger vesicles dominating. This can be interpreted as a result of fusion and fission of liposomes. (4) Like PA, OA is able to induce a $Ca^{2+}$-dependent high-amplitude swelling of mitochondria, yet it requires higher concentrations of $Ca^{2+}$ (30 and 100 μM for PA and OA respectively). (5) In contrast to PA, OA is unable to cause the $Ca^{2+}$-dependent high-amplitude swelling of mitoplasts, suggesting that the cause of OA/$Ca^{2+}$-induced permeability transition in mitochondria may be the fusion of the inner and outer mitochondrial membranes. (6) The presence of OA enhances PA/$Ca^{2+}$-induced permeabilization of liposomes and mitochondria. The paper discusses possible mechanisms of PA/$Ca^{2+}$- and OA/$Ca^{2+}$-induced membrane permeabilization, the probability of these mechanisms to be realized in the cell, and their possible physiological role.

© 2014 Elsevier B.V. All rights reserved.

## 1. Introduction

Free fatty acids (FFAs) have long been known as modulators of membrane permeability and the related membrane functions. Many general, nonspecific effects of FFAs on membranes are based on their ability to transport cations across the lipid bilayer and – when the FFA content is high enough – on their contribution to the structure and physical properties of the membrane. In the latter case, of primary importance is the index of saturation of a FFA molecule. The index determines how the molecule is shaped and how it will affect the phase state of the membrane [1–3].

The studies of the past decades revealed differences in the effects of saturated and unsaturated FFAs on the cell. One of these differences relates to the involvement of FFAs in the cell death, which is underlain by their ability to cause a $Ca^{2+}$-dependent transition in the permeability of the mitochondrial membrane [4–8].

In our earlier works, we discovered and investigated in details a specific case of mitochondrial $Ca^{2+}$-dependent permeability transition (PT): the cyclosporin A (CsA)-insensitive transition induced by long-chain saturated FFA, such as palmitic acid (PA) [9–12]. We showed that this type of PT was based on the high affinity of long-chain saturated FFAs to $Ca^{2+}$ at alkalescent pH, and we argued that the energization of mitochondria in the presence of long-chain saturated FFA and $Ca^{2+}$ should have led to the accumulation and phase separation of FFA/$Ca^{2+}$ complexes on the matrix side of the inner mitochondrial membrane [10,13,14]. A key point of the mechanism is that PA/$Ca^{2+}$ complexes will separate into a solid phase, resulting in the reduction of the area of the corresponding monolayer. This will create an interlayer disbalance of lateral pressure/tension forces in the membrane, which will be eventually resolved through the formation of short-living lipid pores [14,15].

Unsaturated long-chain FFAs are hardly able to permeabilize membranes by the mechanism described above. First of all, their affinity to $Ca^{2+}$ was shown to be an order of magnitude lower than that of saturated FFAs [13]. Second, the point of their liquid crystalline-to-gel transition lies below 0 °C, and binding of $Ca^{2+}$ to the anions of unsaturated FFA will not shift the point above physiological or even room

*Abbreviations:* PA, palmitic acid; OA, oleic acid; FFA, free fatty acid; CsA, cyclosporin A; PT, permeability transition; SRB, sulforhodamine B; DPPC, dipalmitoylphosphatidylcholine

* Corresponding author. Tel.: +7 4967739476; fax: +7 4967330553.
*E-mail address:* bekonik@gmail.com (K.N. Belosludtsev).

http://dx.doi.org/10.1016/j.bbamem.2014.06.017
0005-2736/© 2014 Elsevier B.V. All rights reserved.

temperatures. Hence, there will be no liquid-to-solid transition and the corresponding shrinking of the monolayer, which – as pointed out above – is the essential factor of the "phase-transitional" mechanism [14,15].

Indeed, the experiments conducted on liposomes and mitochondria showed that long-chain unsaturated FFAs did not induce membrane permeabilization under the conditions when saturated ones did [10,14,15]. Under different conditions, however, unsaturated FFAs can affect membrane permeability. For example, palmitoleic acid was reported to induce a CsA-insensitive swelling of mitochondria at relatively high concentrations of $Ca^{2+}$ in the medium [12].

We hypothesized earlier that palmitate/$Ca^{2+}$-induced pore might be a natural mechanism involved in the control of mitochondrial ion exchange under the conditions of $Ca^{2+}$ overload [16]. This hypothesis is based on the assumption that the accumulation of $Ca^{2+}$ in the mitochondrial matrix would result in the activation of $Ca^{2+}$-dependent phospholipase $A_2$ and, correspondingly, in the elevation of the FFAs content in the mitochondrial membrane [16,17]. The two major fatty acids of the membrane FFA pool are palmitic and oleic acids (PA and OA) [9]. Hence, to verify our hypothesis, we should first answer the question if these two fatty acids can be involved in the $Ca^{2+}$-induced PT.

Thus, the objectives of the present work were (1) to examine the ability of OA to cause $Ca^{2+}$-dependent membrane permeabilization — in comparison to the analogous ability of PA demonstrated in our earlier works; (2) to understand and compare mechanisms by which PA and OA permeabilize membranes upon binding of $Ca^{2+}$ ions; and (3) to evaluate the possibility of these mechanisms to work in mitochondria.

## 2. Materials and methods

### 2.1. Materials

Medium components, all inorganic chemicals, fatty acids, digitonin, sulforhodamine B, lecithin were purchased from Sigma-Aldrich; DPPC and cardiolipin were purchased from Avanti Polar Lipids.

### 2.2. Isolation of rat liver mitochondria

Mitochondria were isolated from the livers of Wistar rats (220–250 g) using a standard differential centrifugation technique [10]. 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 mitochondrial protein was determined by the Lowry method [18].

### 2.3. Obtaining mitoplasts

Mitoplasts were obtained from the suspension of rat liver mitochondria according to a conventional protocol [19,20]. One milliliter of the mitochondrial suspension (90–100 mg of protein/ml) was added to 1.0 ml of a medium containing 210 mM mannitol, 70 mM sucrose, 50 μM EGTA, and 10 mM Hepes/KOH buffer (pH 7.4) and 2% digitonin, this is followed by gentle stirring in an ice bath for 15 min. The suspension was diluted with 10 ml of the same medium without digitonin and centrifuged for 10 min at 12,000 g. The pellet was resuspended in 5 ml of the digitonin-free medium and centrifuged again under the same conditions. The mitoplast pellet was washed twice in 5 ml of the digitonin-free medium, and the final pellet was resuspended in the medium free of both digitonin and EGTA. The degree of purity of the resulting mitoplast suspension was evaluated by the activity of monoamino oxidase (MAO). MAO activity was judged by the oxidation of benzylamine to benzaldehyde, which was monitored by the increase in absorbance at 250 nm for 5 min [21]. Mitochondrial or mitoplast fractions (0.5 mg/ml) were incubated at 37 °C in a buffer containing 50 mM $K_2HPO_4$ (pH 7.4), 0.5% Triton X-100 and 1 mM benzylamine. The activity of MAO in the suspension of mitoplasts did not exceed 20% of that in the suspension of mitochondria.

### 2.4. Measuring mitochondrial and mitoplast swelling

Mitochondrial and mitoplast swelling was measured as a decrease in absorbance at 540 nm ($A_{540}$) in a stirred cuvette at the room temperature (about 22 °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, 6.5 μM EGTA, 1 μM rotenone 1 μM CsA, and 10 mM Hepes/KOH buffer (pH 7.4). The concentration of the mitochondrial protein in the cuvette was about 0.4 mg/ml. The rate of swelling ($V_{max} = \Delta A_{540}$/min per mg protein) was calculated as the change in absorbance within the first 30 s from the beginning of the high-amplitude swelling. The swelling rate is expressed as a percentage of the mean swelling rate recorded in a series of experiments with palmitic acid.

### 2.5. Preparation of liposomes

Liposomes (large unilamellar vesicles, LUV) were obtained by a conventional extrusion technique [14]. Dry egg phosphatidylcholine (lecithin; 0.75 mg) was hydrated for several hours with periodical stirring in 0.75 ml of a buffer containing 10 mM Tris–HCl (pH 8.5), 40 mM KCl and 50 μM EGTA. After five cycles of freezing/thawing at −20/+30 °C, the suspension of multilamellar liposomes was pressed 11 times through a 0.1 μm polycarbonate membrane using an Avanti microextruder (Avanti Polar Lipids, Birmingham, AL). All the operations (excluding the freezing/thawing procedure) were carried out at the room temperature.

### 2.6. Preparation of liposomes loaded with sulforhodamine B

Liposomes loaded with sulforhodamine B (SRB) were prepared from egg phosphatidylcholine by a procedure similar to that described above except that (1) the buffer for lipid hydration contained 50 mM SRB instead of 40 mM KCl and (2) after extrusion, liposomes were applied on a Sephadex G-50 column to remove the external SRB. The buffer for gel filtration contained 10 mM Tris–HCl (pH 8.5), 50 μM EGTA and 40 mM KCl.

The release of SRB was evaluated by the increase of its fluorescence as described earlier [14]. The medium contained 10 mM Tris–HCl (pH 8.5), 50 μM EGTA and 40 mM KCl. Fluorescence was measured using a USB-2000 spectroscopy fiber-optic system (Ocean Optics Inc., USA) (excitation wavelength, 565 nm; emission wavelength, 586 nm).

### 2.7. Determination of vesicle size

The size of vesicles in the suspension of liposomes was measured by dynamic light scattering (DLS) at 25 °C using a Zetasizer Nano ZS device (Malvern Instruments Ltd.). The back-scattered light from a 4 mW He/Ne laser (632.8 nm) was collected at an angle of 173°. Liposomes were prepared as described above. The concentration of lipid in the samples was 50 μM. The medium contained 10 mM Tris–HCl buffer (pH 8.5), 50 μM EGTA and 40 mM KCl. The acquisition time for a single autocorrelation function was 100 s, and the resulting autocorrelation function was averaged for 10 measurements. The volume-weighted size distributions were calculated using the following parameters: refractive index of the solution, 1.330; refractive index of particles (lecithin), 1.459; and solution viscosity, 0.8882 cp.

### 2.8. Acoustic measurements

The parameters of the main phase transition in the membrane of dipalmitoylphosphatidylcholine (DPPC) liposomes were measured

with a temperature-scanning differential ultrasonic fixed-length interferometer [22,23]. The parameters measured were the relative changes in sound velocity and absorption caused by the dispersed lipid of the concentration $c$: $[U] = (u-u_0)/u_0c$ and $[A] = (\alpha\lambda-\alpha_0\lambda_0)/c$. Scan rates on cooling runs were ~0.3 K/min. Liposomes were obtained as described above except that (1) DPPC was used instead of egg phosphatidylcholine, (2) the concentration of DPPC in samples was higher (4.07 mM) and (3) all operations were carried out at 45 °C. The medium contained 10 mM Tris–HCl buffer (pH 8.5), 50 μM EGTA and 40 mM KCl.

### 2.9. Statistical analysis

The data were analyzed using the GraphPad Prism 5 and Excel software and presented as means ± SD of 4–8 experiments. Statistical differences between data points were determined by a two-tailed $t$ test.

## 3. Results

### 3.1. Does $Ca^{2+}$ induce permeabilization of OA-containing liposomes and if it does, how is it different from the PA/$Ca^{2+}$-induced membrane permeabilization?

We demonstrated earlier that the addition of $Ca^{2+}$ to the PA-containing SRB-loaded azolectin liposomes in alkalescent media would result in the partial release of SRB at the moment of the addition. The study of the effect led us to the conclusion that the cause of the dye release was the formation of lipid pores in the liposomal membranes. In this work, we have conducted analogous experiments on lecithin liposomes, comparing the effect of PA and OA.

Fig. 1 shows the results of the experiment, in which a 15 μM fatty acid (PA or OA) and then $CaCl_2$ were added to the SRB-loaded liposomes. As can be seen in the figure, 1 mM $Ca^{2+}$ induced release of the dye in the presence of both fatty acids. In the presence of PA, though, the effect was stronger (44% versus 28% in the case of OA). The difference substantially increased at lower concentrations of $Ca^{2+}$. When $Ca^{2+}$ was added at the concentration of 0.1 mM, it caused a 25% release of SRB from the PA-containing liposomes; with the OA-containing vesicles, the effect was close to zero.

The difference in the effect of PA and OA is well correlated to their affinity to $Ca^{2+}$ in alkalescent media [13]. Taking this into account, one can conclude that the formation of OA/$Ca^{2+}$ complexes does result in membrane permeabilization, with the effect being comparable with that of PA. One cannot say, however, how similar are the mechanisms of the two effects. To answer this question, we conducted further experiments, examining changes in the phase state of liposomal membranes and the size of vesicles.

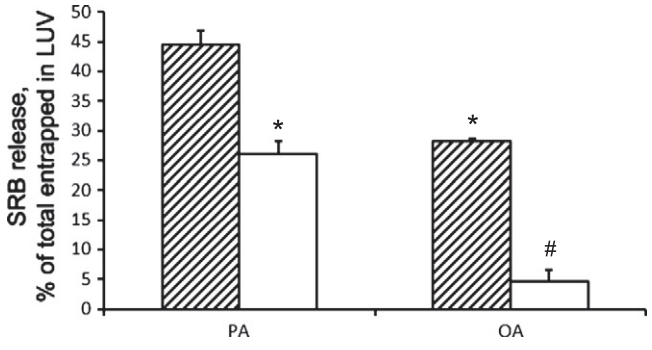


**Fig. 1.** $Ca^{2+}$-induced release of SRB from PA- and OA-containing liposomes. Additions: 15 μM PA or OA and 1 mM or 0.1 mM $Ca^{2+}$. The medium contained 10 mM Tris–HCl (pH 8.5), 50 μM EGTA and 40 mM KCl. Shaded bars, 1 mM $Ca^{2+}$; white bars, 0.1 mM $Ca^{2+}$. Mean values ± SD are represented (n = 4). *Differences between the control (SRB release induced by 15 μM PA and 1 mM $Ca^{2+}$) and experiment are significant at $p < 0.05$; #the difference with respect to the experiment with 15 μm OA and 1 mM $Ca^{2+}$ is significant at $p < 0.05$.

### 3.2. How do palmitic and oleic acids affect the phase state of liposomal membranes, and how does this state change upon the addition of $Ca^{2+}$?

To examine changes in the phase state of lipid bilayer induced by fatty acids and $Ca^{2+}$, we used the method of ultrasound interferometry [22,23]. Fig. 2 shows the temperature dependence of specific sound velocity (Fig. 2A, C) and specific sound absorption (Fig. 2B, D) for the suspension of DPPC liposomes and the changes of these parameters induced by PA, OA and $Ca^{2+}$. A sharp increase or decrease in the sound velocity or a peak in the sound absorption indicates a change of the phase state of membrane lipids. It can be seen that PA and OA differently affect the point of the DPPC main phase transition: PA shifts it in the region of higher temperatures (Fig. 2A, B; dashed line), whereas OA decreases it (Fig. 2C, D; dashed line). These results agree with the data of literature on the effect of saturated and unsaturated fatty acids on lipid phase transitions [3,24,25].

The effect of $Ca^{2+}$ on the phase state of PA- and OA-containing liposomes is also different for the two acids. The addition of $Ca^{2+}$ to the PA-containing DPPC liposomes eliminates the changes caused by the fatty acid: specific sound velocity and absorption shift to values close to those observed for pure phospholipid (Fig. 2A, B; dotted line). The addition of $Ca^{2+}$ to the OA-containing liposomes does not cause such an effect: the shapes of temperature curves for specific sound velocity and absorption remain the same as they were in the presence of OA (Fig. 2C, D; dotted line).

### 3.3. Does the addition of $Ca^{2+}$ to PA- and OA-containing liposomes change the size of vesicles?

Earlier we showed, using the method of correlation fluorescent spectroscopy, that the addition of $Ca^{2+}$ to the PA-containing azolectin liposomes did not change the size and number of vesicles in the suspension [14]. It was a proof that $Ca^{2+}$ did not cause disintegration of liposomes. In the present work, we confirmed those results with the method of dynamic light scattering (Fig. 3). The addition of 30 μM PA and then 1 mM $CaCl_2$ to lecithin vesicles did not change their size (Fig. 3A): the average hydrodynamic diameter of liposomes was 142 nm at the beginning, 136 nm after the addition of PA, and 142 after the subsequent addition of $Ca^{2+}$. OA did not affect the size of vesicles as well (149 nm) (Fig. 3B). At the same time, the addition of 1 mM $CaCl_2$ to the OA-containing liposomes led, on the one hand, to an increase in the size of most vesicles (to 230 nm) and, on the other hand, to the appearance of a population of smaller vesicles (72 nm). This can be interpreted as the results of fusion and fission of liposomes.

Thus, the mechanisms by which PA and OA permeabilize membranes upon binding $Ca^{2+}$, seem to be different. Apparently, the mechanism of PA/$Ca^{2+}$-induced membrane permeabilization is based on the phase transition in the lipid matrix, whereas OA/$Ca^{2+}$-induced loss of membrane integrity is caused by destabilization of the bilayer, which can also result in the fusion and fission of vesicles. Can these two different mechanisms be realized in the mitochondrial membrane?

### 3.4. Can oleic acid, like palmitic one, induce $Ca^{2+}$-dependent CsA-insensitive PT in mitochondria?

As shown in Fig. 4, 15 μM PA in the presence of 30 μM $CaCl_2$ is able to induce a high-amplitude swelling of rat liver mitochondria, which is not inhibited by CsA. At the same time, the rate of the mitochondrial swelling induced by 15 μM OA and 30 μM $CaCl_2$ is substantially lower. The increase of $Ca^{2+}$ concentration to 100 μM accelerates the OA-dependent swelling of mitochondria in the presence of CsA, having practically no effect on the PA-dependent mitochondrial swelling. The addition of RR inhibits the swelling caused by 15 μM OA and 100 μM $Ca^{2+}$. $Ca^{2+}$ alone does not result in the mitochondrial swelling under those conditions.

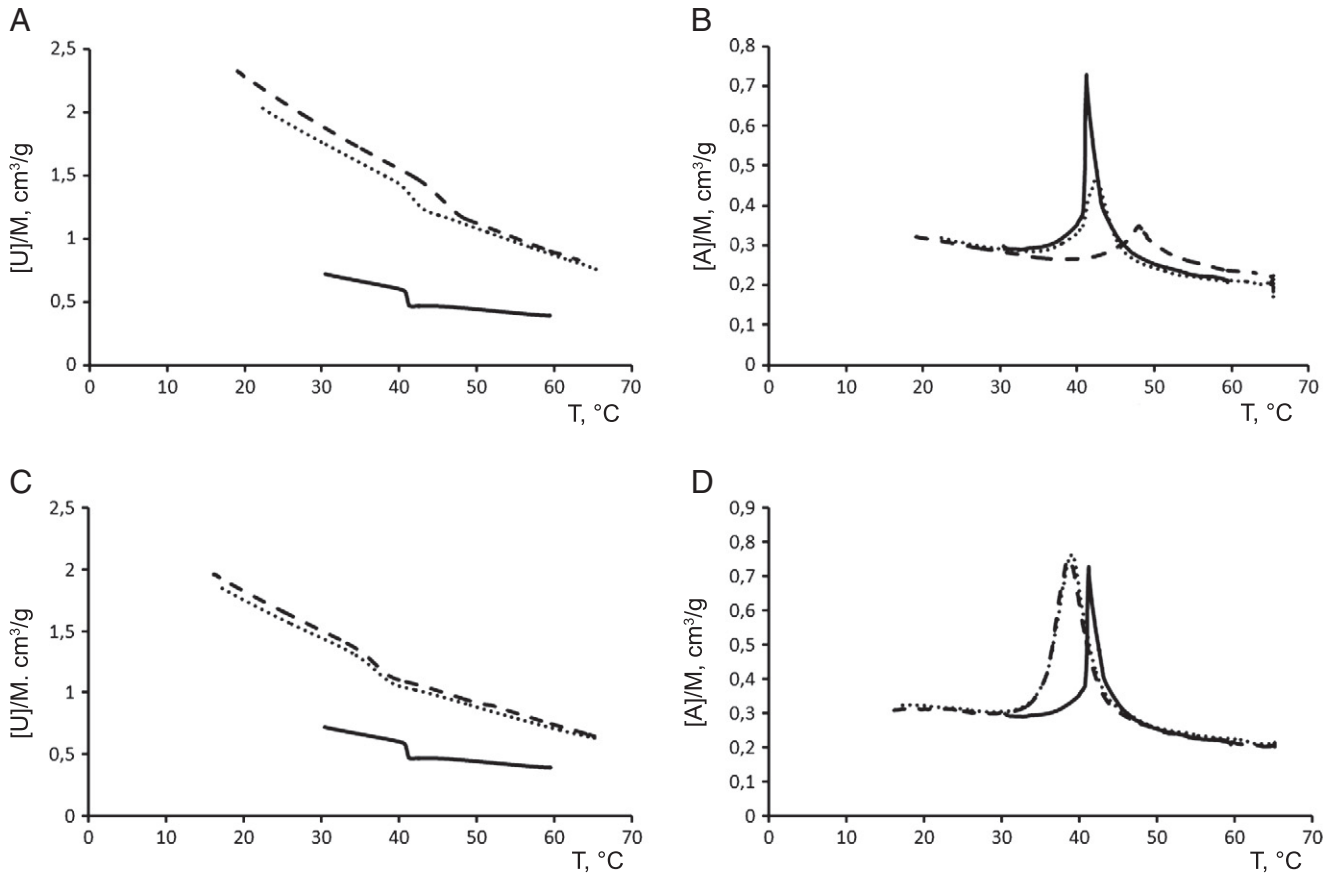


**Fig. 2.** Effect of PA and $Ca^{2+}$ (A,B) and OA and $Ca^{2+}$ (C,D) on the phase state of DPPC liposomes. The figure shows temperature curves of the specific sound velocity (A, C) and sound absorption (B, D) in the cell with DPPC liposomes. The difference between curves along the ordinate (A, C) is due to the presence of ethanol, which was introduced into the suspension with fatty acids (at the concentration used, ethanol enhances the sound velocity but does not affect the character of phase transition). Solid line, pure 4 mM DPPC; dashed line, DPPC + 1 mM PA or OA; dotted line, DPPC + PA or OA + 0.5 mM $CaCl_2$. The medium was as in Fig. 1.

Thus, both acids can cause a PT in the mitochondrial membrane, which would require the organelles to accumulate $Ca^{2+}$ in their matrix. Hence, in both cases, PT should be underlain by the accumulation of FFA/$Ca^{2+}$ complexes on the matrix side of the inner mitochondrial membrane. In case of PA, accumulation of these complexes probably leads to the formation of lipid pores in the inner membrane by the phase-transitional mechanism. Perhaps, OA/$Ca^{2+}$-induced PT is also based on the formation of lipid pores, yet taking into account the fusogenic effects of OA, we thought that a possibility of OA causing a fusion of the inner and outer mitochondrial membranes should have been considered too. Correspondingly, we conducted experiments with mitoplasts.

### *3.5. Are palmitic and oleic acids able to cause PT in mitoplasts?*

As seen in Fig. 5, PA and OA cardinally differ in their ability to induce $Ca^{2+}$-dependent swelling of mitoplasts. The effect of PA is comparable to that observed for mitochondria, whereas the OA/$Ca^{2+}$-induced swelling is greatly suppressed. Therefore, the presence of the outer membrane seems to be an important factor for OA/$Ca^{2+}$-induced PT to occur. If so, the mechanism of OA/$Ca^{2+}$-induced PT may, indeed, be based on the fusion of the inner and outer mitochondrial membranes.

Thus, our experiments indicate that the nature of PA/$Ca^{2+}$- and OA/$Ca^{2+}$-induced mitochondrial PT is different. We have made suppositions on the mechanisms, yet plausible as they seem to us, these suppositions are based on the data obtained on artificial membranes. Therefore, we conducted further experiments, trying to check how these mechanisms would work under more native conditions, closer to those in the cell. We have addressed two issues: the effect of cardiolipin, the presence of which in the membrane is a characteristic feature of mitochondria; and the effect of $Ca^{2+}$ on the membranes containing a mixture of PA and OA.

### *3.6. Would the presence of cardiolipin in the membrane affect PA/$Ca^{2+}$- and OA/$Ca^{2+}$-induced permeabilization?*

Fig. 6 shows the results of our experiments performed on liposomes formed from a mixture of lecithin and cardiolipin (3:1, mol/mol). As can be seen, the effectiveness of the OA/$Ca^{2+}$-induced SRB release in the presence of cardiolipin decreased considerably. At the same time, cardiolipin stimulated the release of the dye from the PA-containing liposomes. Therefore, cardiolipin may lower the possibility of the OA/$Ca^{2+}$-induced PT in mitochondria, but it may also stimulate the formation of lipid pores induced by the binding of $Ca^{2+}$ to PA.

### *3.7. How would $Ca^{2+}$ permeabilize membranes containing a mixture of PA and OA?*

The FFAs pool of cellular membranes is a mixture of saturated and unsaturated acids, among which PA and OA dominate. Examination of the combined effect of these two acids on the permeability of membranes would, therefore, show if the mechanisms specific for saturated and unsaturated fatty acids are able to work under more native cellular conditions.

Fig. 7A shows the results of the experiment, in which $Ca^{2+}$ was added to liposomes, containing a mixture of PA and OA. As seen in the figure, a small increase in the concentration of OA (against the equivalent decrease in the concentration of PA so that the total concentration of the

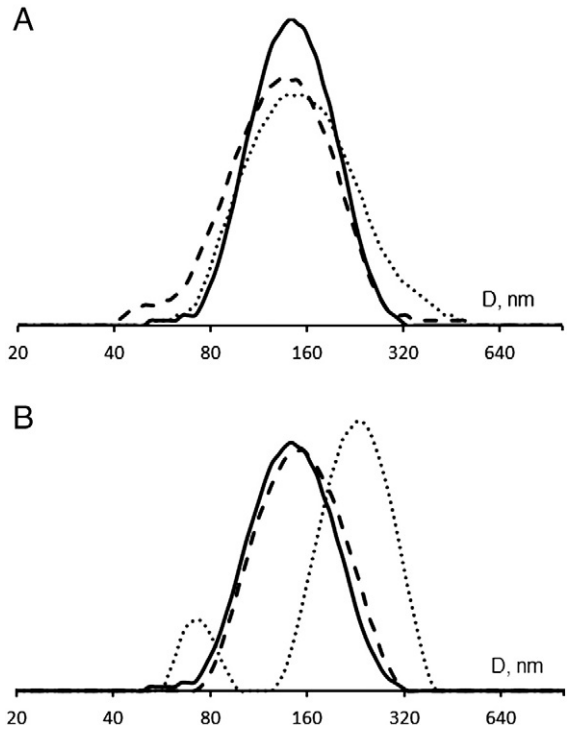


**Fig. 3.** Changes in the size of vesicles observed upon addition of PA and $Ca^{2+}$ (A) and OA and $Ca^{2+}$ (B) to the suspension of lecithin liposomes. Solid line, liposomes; dashed line, liposomes + 30 μM PA or OA; dotted line, liposomes + 30 μM PA or OA + 1 mM $CaCl_2$. The medium was as in Fig. 1.

two acids was the same) results in the stimulation of $Ca^{2+}$-induced permeabilization of lecithin liposomes. The dependence of the effect on the PA/OA ratio is bell-shaped, with the maximum shifted towards PA. Experiments with mitochondria revealed an analogous picture (Fig. 7B).

## 4. Discussion

In this work, we conducted a comparative study of $Ca^{2+}$-induced permeabilization of the PA- and OA-containing membranes. The experiments performed on liposomes show that both acids are able to cause a loss of membrane integrity upon binding of $Ca^{2+}$ ions, but the mechanism of these permeability changes seems to be different for saturated and unsaturated FFAs.

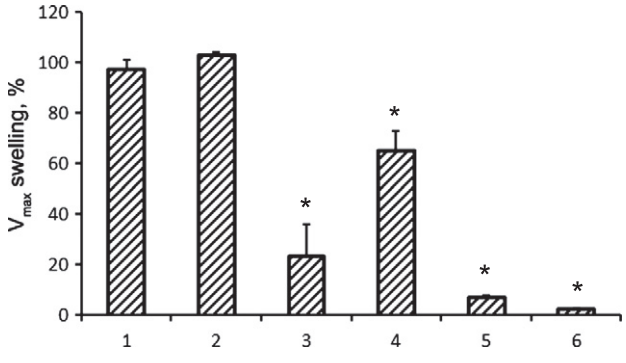


**Fig. 4.** The rate of CsA-insensitive swelling of rat liver mitochondria induced by 15 μM PA and 30 μM $CaCl_2$ (1); 15 μM PA and 100 μM $CaCl_2$ (2); 15 μM OA and 30 μM $CaCl_2$ (3); 15 μM OA and 100 μM $CaCl_2$ (4); 15 μM OA and 100 μM $CaCl_2$ in the presence of 1 μM RR (5); 100 μM $CaCl_2$ (6). The rate of mitochondrial swelling induced by 15 μM PA and 30 μM $CaCl_2$ is taken as 100%. The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5 mM succinate, 6.5 μM EGTA, 1 μM rotenone, 1 μM CsA, and 10 mM Hepes/KOH buffer (pH 7.4). Mean values ± SD are represented (n = 5–8). *Differences between the control (mitochondrial swelling induced by 15 μM PA and 30 μM $Ca^{2+}$) and experiments are significant at $p < 0.05$.

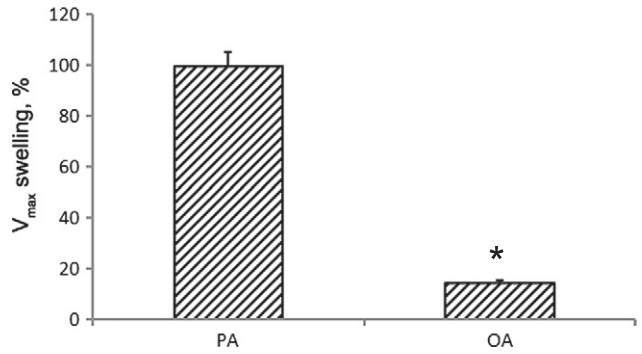


**Fig. 5.** The rate of CsA-insensitive swelling of mitoplasts induced by 15 μM PA and 30 μM $CaCl_2$ and 15 μM OA and 100 μM $CaCl_2$. The rate of swelling of mitoplasts induced by 15 μM PA and 30 μM $CaCl_2$ is taken as 100%. The incubation medium was as in Fig. 4. Mean values ± SD are represented (n = 4). *Differences between PA/$Ca^{2+}$-induced swelling of mitoplasts and OA/$Ca^{2+}$-induced swelling of mitoplasts are significant at $p < 0.05$.

As follows from our previous studies [10,14,15], as well as the data presented here (Figs. 1,2,4), in the core of PA/$Ca^{2+}$-induced membrane permeabilization lies separation of PA/$Ca^{2+}$ complexes into solid membrane domains, which is accompanied by the reduction of the area of the corresponding lipid monolayer. If PA/$Ca^{2+}$ complexes only form at one side of the membrane, the reduced monolayer becomes expanded in relation to the coupled monolayer, which becomes compressed. The increased lateral pressure in the compressed monolayer will force the expanded monolayer to break, and the exposed hydrophobic membrane interior will by immediately filled with lipids from the compressed monolayer. As a result, the two monolayers will fuse with the formation of a hydrophilic lipid pore [15,26].

Apparently, binding of $Ca^{2+}$ to OA anions does not lead to phase separation of OA/$Ca^{2+}$ complexes (Fig. 2C, D), and even if it should do, it would not result in the appearance of solid domains, i.e., in the liquid-to-solid phase transition. Correspondingly, there would not be formation of lipid pores by the mechanism described above. Yet in the experiments with liposomes, we observed a loss of membrane integrity upon binding of $Ca^{2+}$ to OA-containing vesicles (Fig. 1). We also saw that the size of vesicles changed: most of them became larger, but there also appeared a population of smaller vesicles (Fig. 3B). This can be interpreted as the results of fusion and fission of liposomes respectively.

The fact that binding of $Ca^{2+}$ to negatively charged lipid vesicles can result to their fusion is not new [27–30], and oleic acid, in particular, was shown to possess fusogenic properties [29]. It is also known that interacting with negatively charged lipids, $Ca^{2+}$ may cause a

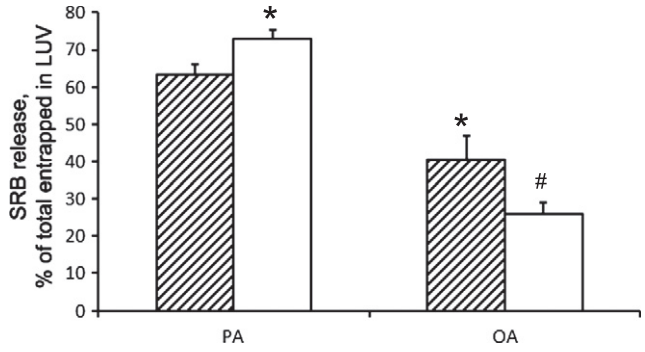


**Fig. 6.** $Ca^{2+}$-induced release of SRB from PA- and OA-containing liposomes. Additions: 30 μM PA or OA and 1 mM $Ca^{2+}$. The medium was as in Fig. 1. Shaded bars, liposomes formed from lecithin; white bars, liposomes formed from lecithin and cardiolipin (3:1, mol/mol). Mean values ± SD are represented (n = 5). *Differences between the control (PA/$Ca^{2+}$-induced SRB release from lecithin LUV) and experiment are significant at $p < 0.05$; #the difference with respect to the experiment with OA/$Ca^{2+}$-induced SRB release from lecithin LUV is significant at $p < 0.05$.

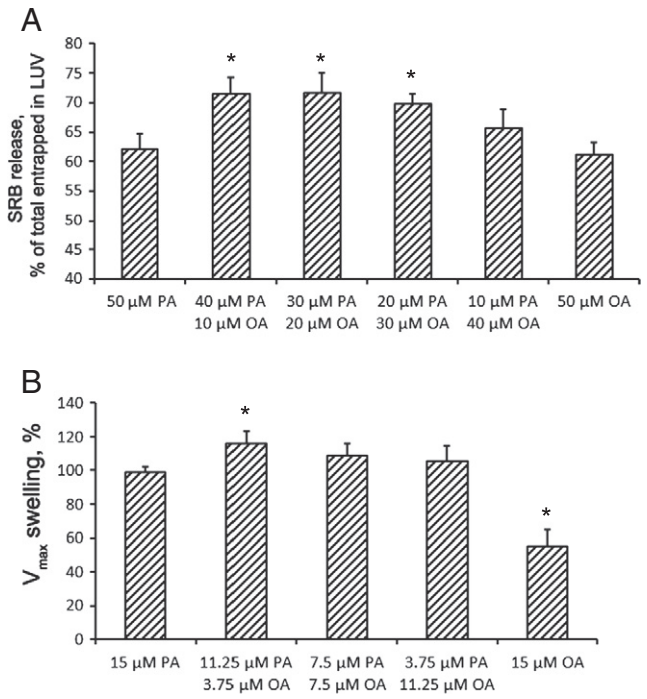


**Fig. 7.** The influence of PA and OA mixed at different ratios and $Ca^{2+}$ on the permeabilization of liposomes (A) and mitochondria (B). A) $Ca^{2+}$-induced release of SRB from liposomes containing both PA and OA mixed at different ratios. Additions: 0–50 μM PA or OA and 1 mM $Ca^{2+}$. The medium was as in Fig. 1. Mean values ± SD are represented (n = 4). *Differences between the control (PA/$Ca^{2+}$-induced SRB release from lecithin LUV) and experiment are significant at $p < 0.05$. B) CsA-insensitive swelling of mitochondria induced by $Ca^{2+}$ and a mixture of PA and OA. Additions: 0–15 μM PA or OA and 100 μM $Ca^{2+}$. The rate of mitochondrial swelling induced by 15 μM PA and 100 μM $CaCl_2$ is taken as 100%. The medium was as in Fig. 4. Mean values ± SD are represented (n = 4) *Differences between the control (mitochondrial swelling induced by 15 μM PA and 30 μM $Ca^{2+}$) and experiments are significant at $p < 0.05$.

polymorphic phase transition in the membrane with the formation of non-bilayer lipid phases [30]. All these effects imply that lipid bilayer gets destabilized; it becomes strained and thermodynamically unstable. That is what we suppose to occur when $Ca^{2+}$ binds to OA incorporated in the membrane. Our assumption is that binding of $Ca^{2+}$ may reduce the average cross-section area in the region of the OA head as compared to that in the tail-end region. If so, the OA/$Ca^{2+}$-rich zones of the OA-containing monolayer would tend to adopt an outwardly concave shape. The monolayer containing OA/$Ca^{2+}$ complexes can become rippled, with the ripple slopes being outwardly concave. As a consequence, the monolayer may break, since ripples add to the surface that needs to be covered by lipids. This would result to the formation of lipid pores and could account for the release of SRB that we observed in the experiments with liposomes. Another consequence of the OA-containing monolayer adopting the outwardly concave shape may be membrane fusion. If two rippled monolayers come to a close contact, they may merge, since the ridges of ripples are very stressed zones, while merging will eliminate the stress. Merging of the outward monolayers of two vesicles will lead to their subsequent fusion [31], and that is what we probably saw in the light-scattering experiments.

The tendency of the OA/$Ca^{2+}$-rich zones to become outwardly concave can also be realized through bending of the entire membrane. In this case, the corresponding zones of the coupled monolayer will become outwardly convex, and these membrane regions might protrude, forming bulges. Protrusion of the membrane can become the first step in the process of vesicle budding. The population of small vesicles, emerging upon the addition of $Ca^{2+}$ to the OA-containing liposomes, may have been formed this way.

Thus, the alterations induced by $Ca^{2+}$ in the PA-containing lipid bilayer are primarily associated with the change of the phase state (Figs. 1,2), and the major effect is the formation of lipid pores at the moment of phase transition [14,15]. The alterations observed upon binding of $Ca^{2+}$ to the OA-containing membrane can be generally characterized as destabilization and straining of the bilayer [3,30,32], with the possible outcomes being the loss of membrane integrity and membrane fusion (which manifests itself as fusion of liposomes and vesicle budding).

On the basis of these suppositions, we can explain the results of the experiments when $Ca^{2+}$ was added to the membranes containing both PA and OA at different ratios (Fig. 7). In those experiments, we observed a kind of synergy between PA and OA, with the highest effect achieved when PA was dominating and OA was presented in a small amount. This indicates that OA/$Ca^{2+}$-induced permeabilization is slightly, if at all, affected by PA, whereas PA/$Ca^{2+}$-induced formation of lipid pores does benefit from the presence of OA in the membrane. One can suppose that the straining of lipid bilayer caused by OA facilitates breakage of the expanded monolayer, which is the initial step of pore-formation process in the phase-transitional mechanism of membrane permeabilization [16]. Perhaps, the stimulation of PA/$Ca^{2+}$-induced SRB release by cardiolipin has the same nature (Fig. 6).

The situation with mitochondria is, no doubts, more complex. Permeabilization of the mitochondrial membrane occurs after accumulation of $Ca^{2+}$ in the mitochondrial matrix (which is proved by the inhibitory effect of RR), and the cause of the FFA/$Ca^{2+}$-induced PT should be the formation of the FFA/$Ca^{2+}$ complexes on the matrix side of the inner mitochondrial membrane. The results of our earlier studies [9,10] and the data presented here (Fig. 4) show that there are no reasons to reject the supposition of the PA/$Ca^{2+}$-induced PT in mitochondria to occur by the phase-transitional mechanism. There are all the conditions for this mechanism to work in the mitochondrial membrane, and the presence of unsaturated fatty acids or cardiolipin would not be a hindrance (Figs. 6,7). The results of this study show that their presence would rather be a stimulating factor.

A more intriguing question is about the mechanism of the OA/$Ca^{2+}$-induced mitochondrial PT. Our experiments with mitoplasts show that the mechanism of OA/$Ca^{2+}$-induced PT is, probably, not based on the formation of lipid pores in the inner mitochondrial membrane (Fig. 5). The need for the outer membrane indicates that OA/$Ca^{2+}$-induced PT may result from the fusion of the inner and outer mitochondria membranes.

The fusion of the inner and outer mitochondria membranes can occur in the region of contact sites by the mechanism described above for vesicle fission (budding). The appearance of the OA/$Ca^{2+}$-rich zones on the matrix side of the inner mitochondrial membrane can lead to the bending of the corresponding membrane regions and their protrusion towards the outer membrane. Upon a close contact of the outer membrane and the expanded, outwardly convex regions of the outer leaflet of the inner membrane, the two membranes may fuse, forming a local toroidal intermembrane anastomose (fusion pore).

We believe that, theoretically, both mechanisms (formation of lipid pores in the inner membrane or fusion of the inner and outer membranes) can be realized in the cell, but the probability of their realization is different. The appearance of lipid pores – due to the binding of $Ca^{2+}$ to PA and phase separation of PA/$Ca^{2+}$ complexes – should be a more probable and frequent event. The PA/$Ca^{2+}$-induced membrane permeabilization occurs at lower intramitochondrial concentrations of $Ca^{2+}$ than the OA/$Ca^{2+}$-induced PT, and there are always some FFAs in the membranes, of which PA is the most abundant. It is possible, therefore, that the PA/$Ca^{2+}$-induced mitochondrial PT will be triggered under physiological conditions. An important feature of lipid pores is their spontaneous and rapid closure; their lifetime is on the millisecond scale [26]. Correspondingly, the lipid pore-based PT should be transient, and this mechanism can lead to the modes of transmembrane ion cycling — when PT will play the role of an "emergency valve". Its opening would result in the mild uncoupling and lowering of transmembrane ion gradients.

In contrast to that of lipid pores, the lifetime of intermembrane anastomoses (fusion pores) may be longer, since their structure should be maintained until the causes resulted in their formation exist. That is, it should be maintained until the dissociation of OA/$Ca^{2+}$ complexes, which will probably occur after the collapse of $\Delta\Psi_m$, release of $Ca^{2+}$ and matrix acidification. Therefore, the fusion pore-based PT should result in a deeper and longer membrane depolarization — and we do observe such kind of depolarization under pathological conditions, i.e., glutamate overload of neurons [33]. Under those conditions, the concentration of $Ca^{2+}$ in the cell (and, correspondingly, in the mitochondrial matrix) increases significantly, which is accompanied by the activation of $PLA_2$ and accumulation of FFAs in the mitochondrial membrane. We showed earlier that the addition of $PLA_2$ inhibitors prevented mitochondrial depolarization and delayed $Ca^{2+}$ disregulation upon the hyperstimulation of brain neurons with glutamate [34]. This implies involvement of FFAs in the mechanisms of mitochondrial PT on the late stages of neuronal degradation. Perhaps, this deep and prolonged PT does result from the fusion of the inner and outer mitochondrial membranes.

## Acknowledgments

This study was supported by the Russian Foundation for Basic Research (project No. 12-04-00430a) to K.N. Belosludtsev, by the Government of RF (project No. 14.Z50.31.0028) and by a grant from Magnus Ehrnrooth Foundation to Prof. N.-E. Saris.

## References

[1] M.I. Di Paola, M. Lorusso, Interaction of free fatty acids with mitochondria: coupling, uncoupling and permeability transition, Biochim. Biophys. Acta 1757 (2006) 1330–1337, http://dx.doi.org/10.1016/j.bbabio.2006.03.024.
[2] P. Bernardi, Mitochondrial transport of cations: channels, exchangers, and permeability transition, Physiol. Rev. 79 (4) (1999) 1127–1155 (http://physrev.physiology.org/content/79/4/1127 ).
[3] T. Inoue, S. Yanagihara, Y. Misono, M. Suzuki, Effect of fatty acids on phase behavior of hydrated dipalmitoylphosphatidylcholine bilayer: saturated versus unsaturated fatty acids, Chem. Phys. Lipids 109 (2001) 117–133, http://dx.doi.org/10.1016/S0009-3084(00)00170-5.
[4] D. Penzo, C. Tagliapietra, R. Colonna, V. Petronilli, P. Bernardi, Effects of fatty acids on mitochondria: implications for cell death, Biochim. Biophys. Acta 1555 (2002) 160–165, http://dx.doi.org/10.1016/S0005-2728(02)00272-4.
[5] S. Hardy, W. El-Assaas, E. Przybytkowsky, E. Joly, M. Prentki, Y. Langelier, Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role of cardiolipin, J. Biol. Chem. 278 (2003) 31861–31870, http://dx.doi.org/10.1074/jbc.M300190200.
[6] G.C. Sparagna, D.L. Hickson-Bick, L.M. Buja, J.B. McMillin, A metabolic role for mitochondria in palmitate-induced cardiac myocyte apoptosis, Am. J. Physiol. 279 (2000) H2124–H2132, http://dx.doi.org/10.1152/ajpheart.00726.2001.
[7] H.G. Cheon, Y.S. Cho, Protection of palmitic acid-mediated lipotoxicity by arachidonic acid via channeling of palmitic acid into triglycerides in C2C12, J. Biomed. Sci. 21 (13) (2014) 1–10, http://dx.doi.org/10.1186/1423-0127-21-13.
[8] L. Yuzefovych, G. Wilson, L. Rachek, Different effects of oleate vs. palmitate on mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells: role of oxidative stress, Am. J. Physiol. Endocrinol. Metab. 299 (6) (2010) E1096–E1105.
[9] G.D. Mironova, E. Gritsenko, O. Gateau-Roesch, C. Levrat, A. Agafonov, K. Belosludtsev, A.F. Prigent, D. Muntean, M. Dubois, M. Ovize, Formation of palmitic acid/$Ca^{2+}$ complexes in the mitochondrial membrane: a possible role in the cyclosporin-insensitive permeability transition, J. Bioenerg. Biomembr. 36 (2) (2004) 171–178, http://dx.doi.org/10.1023/B:JOBB.0000023620.42653.b7.
[10] K.N. Belosludtsev, N.V. Belosludtseva, G.D. Mironova, Possible mechanism for formation and regulation of the palmitate-induced cyclosporin A-insensitive mitochondrial pore, Biochemistry (Mosc) 70 (2005) 815–821, http://dx.doi.org/10.1007/s10541-005-0189-x.
[11] A. Sultan, P.M. Sokolove, Palmitic acid opens a novel cyclosporin A-insensitive pore in the inner mitochondrial membrane, Arch. Biochem. Biophys. 386 (1) (2001) 37–51, http://dx.doi.org/10.1006/abbi.2000.2194.
[12] A. Sultan, P.M. Sokolove, Free fatty acid effects on mitochondrial permeability: an overview, Arch. Biochem. Biophys. 386 (1) (2001) 52–61, http://dx.doi.org/10.1006/abbi.2000.2195.
[13] G.D. Mironova, O. Gateau-Roesch, C. Levrat, E. Gritsenko, E. Pavlov, A.V. Lazareva, E. Limarenko, C. Rey, P. Louisot, N.-E.L. Saris, Palmitic and stearic acids bind $Ca^{2+}$ with high affinity and form nonspecific channels in black-lipid membranes. Possible relation to $Ca^{2+}$-activated mitochondrial pores, J. Bioenerg. Biomembr. 33 (4) (2001) 319–331.
[14] A. Agafonov, E. Gritsenko, K. Belosludtsev, A. Kovalev, O. Gateau-Roesch, N.-E.L. Saris, G.D. Mironova, A permeability transition in liposomes induced by the formation of $Ca^{2+}$/palmitic acid complexes, Biochim. Biophys. Acta 1609 (2) (2003) 153–160, http://dx.doi.org/10.1016/S0005-2736(02)00666-1.
[15] A.V. Agafonov, E.N. Gritsenko, E.A. Shlyapnikova, D.P. Kharakoz, N.V. Belosludtseva, E.I. Lezhnev, N.-E.L. Saris, G.D. Mironova, $Ca^{2+}$-induced phase separation in the membrane of palmitate-containing liposomes and its possible relation to membrane permeabilization, J. Membr. Biol. 215 (1) (2007) 57–68, http://dx.doi.org/10.1007/s00232-007-9005-4.
[16] G.D. Mironova, K.N. Belosludtsev, N.V. Belosludtseva, E.N. Gritsenko, B.I. Khodorov, N.-E.L. Saris, Mitochondrial $Ca^{2+}$ cycle mediated by the palmitate-activated cyclosporin A-insensitive pore, J. Bioenerg. Biomembr. 39 (2) (2007) 167–174, http://dx.doi.org/10.1007/s10863-007-9079-9.
[17] K.M. Broekemeier, P.C. Schmid, H.H. Schmid, D.R. Pfeiffer, Effects of phospholipase $A_2$ inhibitors on ruthenium red-induced $Ca^{2+}$ release from mitochondria, J. Biol. Chem. 260 (1) (1985) 105–113 (WOS:A1985TZ26100014).
[18] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1) (1951) 265–275 (http://www.jbc.org/content/193/1/265.citation ).
[19] C. Schnaitman, J.W. Greenawalt, Enzymatic properties of the inner and outer membranes of rat liver mitochondria, J. Cell Biol. 38 (1) (1968) 158–175, http://dx.doi.org/10.1083/jcb.38.1.158.
[20] J.A. Ronchi, A.E. Vercesi, R.F. Castilho, Reactive oxygen species and permeability transition pore in rat liver and kidney mitoplasts, J. Bioenerg. Biomembr. 43 (6) (2011) 709–715, http://dx.doi.org/10.1007/s10863-011-9384-1.
[21] C.W. Tabor, H. Tabor, S.M. Rosenral, Purification of amine oxidase from beef plasma, J. Biol. Chem. 208 (2) (1954) 645–662 (http://www.jbc.org/content/208/2/645.citation ).
[22] D.P. Kharakoz, M.S. Panchelyuga, E.I. Tiktopulo, E.A. Shlyapnikova, Critical temperatures and a critical chain length in saturated diacylphosphatidylcholines: calorimetric, ultrasonic and Monte Carlo simulation study of chain-melting/ordering in aqueous lipid dispersions, Chem. Phys. Lipids 150 (2007) 217–228, http://dx.doi.org/10.1016/j.chemphyslip.2007.08.004.
[23] M.E. Astashev, K.N. Belosludtsev, D.P. Kharakoz, Method for digital measurement of phase–frequency characteristics for a fixed length ultrasonic spectrometer, Acoust. Phys. 60 (2014) 335–341, http://dx.doi.org/10.1134/S1063771014030038.
[24] R.D. Koynova, B.G. Tenchov, P.J. Quinn, P. Laggner, Structure and phase behavior of hydrated mixtures of L-dipalmitoylphosphatidylcholine and palmitic acid. Correlations between structural rearrangements, specific volume changes and endothermic events, Chem. Phys. Lipids 48 (1988) 205–214, http://dx.doi.org/10.1016/0009-3084(88)90091-6.
[25] A. Ortiz, J.C. Gomez-Fernandez, A differential scanning calorimetry study of the interaction of free fatty acids with phospholipid membranes, Chem. Phys. Lipids 45 (1987) 75–91, http://dx.doi.org/10.1016/0009-3084(87)90041-7.
[26] V.F. Antonov, E.V. Shevchenko, Lipid pores and stability of cell membranes, Vestn. Ross. Akad. Med. Nauk (10) (1995) 48–55.
[27] D. Papahadjopoulos, S. Nir, N. Düzgünes, Molecular mechanisms of calcium-induced membrane fusion, J. Bioenerg. Biomembr. 22 (1990) 157–179, http://dx.doi.org/10.1007/BF00762944.
[28] K. Jacobson, D. Papahadjopoulos, Phase transitions and phase separations in phospholipid membranes induced by changes in temperature, pH, and concentration of bivalent cations, Biochemistry 14 (1975) 152–161, http://dx.doi.org/10.1021/bi00672a026.
[29] J. Wilschut, J. Scholma, S.J. Eastman, M.J. Hope, P.R. Cullis, $Ca^{2+}$-induced fusion of phospholipid vesicles containing free fatty acids: modulation by transmembrane pH gradients, Biochemistry 31 (1992) 2629–2636, http://dx.doi.org/10.1021/bi00125a001.
[30] A. Ortiz, J.A. Killian, A.J. Verkleij, J. Wilschut, Membrane fusion and the lamellar-to-inverted-hexagonal phase transition in cardiolipin vesicle systems induced by divalent cations, Biophys. J. 77 (4) (1999) 2003–2014, http://dx.doi.org/10.1016/S0006-3495(99)77041-4.
[31] L.V. Chernomordik, M.M. Kozlov, Mechanics of membrane fusion, Nat. Struct. Mol. Biol. 15 (2008) 675–683, http://dx.doi.org/10.1038/nsmb.1455.
[32] G.V. Richieri, A. Anel, A.M. Kleinfeld, Interactions of long-chain fatty acids and albumin: determination of free fatty acid levels using the fluorescent probe ADIFAB, Biochemistry 32 (29) (1993) 7574–7580, http://dx.doi.org/10.1021/bi00080a032.
[33] B. Khodorov, Glutamate-induced deregulation of calcium homeostasis and mitochondrial dysfunction in mammalian central neurons, Prog. Biophys. Mol. Biol. 86 (2) (2004) 279–351, http://dx.doi.org/10.1016/j.pbiomolbio.2003.10.002.
[34] G.D. Mironova, K.N. Belosludtsev, A.M. Surin, A.S. Trudovishnikov, N.V. Belosludtseva, V.G. Pinelis, I.A. Krasilnikova, B.I. Khodorov, Mitochondrial lipid pore in the mechanism of glutamate-induced calcium deregulation of brain neurons, Biochem. (Mosc.) Suppl. A Membr. Cell Biol. 6 (1) (2012) 45–55, http://dx.doi.org/10.1134/S1990747811060080.