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A permeability transition in liposomes induced by the formation of Ca^{2+}/palmitic acid complexes

Alexey Agafonov[a], Elena Gritsenko[a], Konstantin Belosludtsev[a], Alexandr Kovalev[b], Odile Gateau-Roesch[c], Nils-Erik L. Saris[d,*], Galina D. Mironova[a,*]

[a] *Laboratory Mitochondrial Transport, Institute of Theoretical and Experimental Biophysics RAS, Pushchino, Moscow Region 1422290, Russia*

[b] *Institute of Cell Biophisics RAS, Pushchino, Moscow Region 1422290, Russia*

[c] *Department of Biochemistry, INSERM 189, Lyon-Sud Medical School, P.O. Box 12, FR-69921 Oullins Cedex, France*

[d] *Department of Applied Chemistry and Microbiology, P.O. Box 56, FIN-00014 University of Helsinki, Helsinki, Finland*

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Abstract

Formation of palmitic acid/Ca^{2+} (PA/Ca^{2+}) complexes was suggested to play a key role in the non-classical permeability transition in mitochondria (NCPT), which seems to be involved in the PA-induced apoptosis of cardiomyocytes. Our previous studies of complexation of free fatty acids (FFA) with Ca^{2+} showed that long-chain (C:16–C:22) saturated FFA had an affinity to Ca^{2+}, which was much higher than that of other FFA and lipids. The formation of FFA/Ca^{2+} complexes in the black-lipid membrane (BLM) was demonstrated to induce a nonspecific ion permeability of the membrane. In the present work, we have found that binding of Ca^{2+} to PA incorporated into the membrane of sulforhodamine B (SRB)-loaded liposomes results in an instant release of a part of SRB, with the quantity of SRB released depending on the concentration of PA and Ca^{2+}. The pH-optimum of this phenomenon, similar to that of PA/Ca^{2+} complexation, is in the alkaline range. The same picture of SRB release has been revealed for stearic, but not for linoleic acid. Along with Ca^{2+}, some other bivalent cations (Ba^{2+}, Sr^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}) also induce SRB release upon binding to PA-containing liposomes, while Mg^{2+} turns out to be relatively ineffective. As revealed by fluorescence correlation spectroscopy, the apparent size of liposomes does not alter after the addition of PA, Ca^{2+} or their combination. So it has been supposed that the cause of SRB release from liposomes is the formation of lipid pores. The effect of FFA/Ca^{2+}-induced permeabilization of liposomal membranes has several analogies with NCPT, suggesting that both these phenomena are of similar nature.

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Keywords: Fatty acid; Calcium; Liposome; Sulforhodamine B; Permeability transition; Lipid pore

1. Introduction

Palmitic acid (PA) has recently been found to be a physiological activator of programmed cell death (apoptosis) [1,2]. The proapoptotic action of PA is of interest in pathology, since in myocardial infarction, the content of free fatty acids (FFA), including PA, increases substantially both in the plasma and in the affected tissue [3–5]. As was shown, PA promoted an increase in the permeability of the inner mitochondrial membrane, followed by the release of cytochrome *c* [1]. The nature of this permeability, however, has not been finally established. On the one hand, the increase in permeability was shown to be sensitive to cyclosporin A, indicating opening of the classical mitochondrial permeability transition (MPT) pore [1]. But on

Abbreviations: BLM, black-lipid membrane; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FFA, free fatty acids; LUV, large unilamellar vesicles; MPT, mitochondrial permeability transition; NCPT, non-classical permeability transition; PA, palmitic acid; *N*-Rh-PE, Lissamine™ rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine DHPE); SRB, sulforhodamine B; TX-100, Triton X-100

* Corresponding authors. G.D. Mironova is to be contacted at Tel.: +7-95-923-74-67; fax: +7-96-779-05-53. N.-E.L. Saris, Department of Applied Chemistry and Microbiology, P.O. Box 56, FIN-00014 University of Helsinki, Helsinki, Finland. Fax: +358-9-191-59301.

E-mail addresses: saris@helsinki.fi (N.-E.L. Saris), mironova@iteb.ru (G.D. Mironova).

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the other hand, FFA (mainly long-chain saturated ones) have recently been found to promote a non-classical permeability transition (NCPT), which has been identified as the opening of a novel cyclosporin-insensitive pore [6,7].

In order to understand the nature of FFA-induced increase in permeability of mitochondrial membrane, one should first distinguish between the effects of FFA on proteins and the processes that occur in the lipid matrix. Indeed, underlying opening of the classical MPT pore can be the binding of FFA to adenine nucleotide translocator [8], which is believed to be the core of the protein pore-forming megachannel [9,10]. But no evidence on participation of this megachannel in the NCPT was found, leaving the possibility of NCPT to be a phenomenon, which is intrinsic to the lipid bilayer. Taking into account that concentrations of FFA needed to trigger NCPT are rather high ($\sim 10^{-5}$–10^{-4} M [6,7]), this hypothesis should be subjected to a serious examination.

Earlier, we showed that long-chain saturated FFA (C:16–C:22) bound Ca^{2+} with an affinity, which was two orders of magnitude higher than that of other FFA and phospholipids [11]. In addition, we found that in the presence of Ca^{2+}, PA or stearic acid (SA) but not unsaturated FFA induced an increase in the nonspecific conductance of the black-lipid membrane (BLM) [11]. The aim of the present work was to study possible effects of FFA/Ca^{2+} complexes on the permeability of the liposomal membrane to the rather large ($\sim$ 1.5 nm in diameter) molecules of sulforhodamine B (SRB). As a result, we have found that in the presence of PA, the addition of Ca^{2+} or some other bivalent cations leads to the permeabilization of liposomes for SRB molecules, the effect depending on the concentration of PA and Ca^{2+}. Moreover, some features of this phenomenon are similar to those of the mitochondrial NCPT.

2. Materials and methods

2.1. Preparation of large unilamellar vesicles

Large unilamellar vesicles (LUV) loaded with SRB (Molecular Probes, Inc.) were prepared by a conventional extrusion technique. Dry azolectin (5 mg, a mixture of soybean phospholipids with phosphatidylcholine predominance, Sigma) was hydrated in 0.5 ml of a buffer for several hours, with periodical stirring on a vortex mixer. The buffer contained 10 mM Tris–HCl (pH 8.5), 50 μM EGTA and 50 mM SRB. After five cycles of freezing/thawing at $-10/+30$ °C, the suspension of multilamellar liposomes was pressed for 11 times through a 0.1-μm polycarbone membrane using an "Avanti" microextruder (Avantilipids, Inc.). The resulting SRB-loaded LUV were separated from free SRB on a Sephadex G-50 column (10×1 cm) equilibrated with a buffer, containing 10 mM Tris–HCl (pH 8.5), 50 μM EGTA and 40 mM KCl. All operations on LUV preparation (excluding freezing/thawing procedure) were carried out at a room temperature. SRB-loaded LUV were stored at +4 °C and were used for 2–3 days.

2.2. Measurement of permeabilization of SRB-loaded liposomes

The release of SRB from LUV was detected by the increase in fluorescence due to the dissociation of SRB excimers after the dilution of dye in the external medium. SRB fluorescence was measured at 25 °C using a "Kontron" spectrofluorimeter (excitation wavelength, 565 nm; emission wavelength, 586 nm). The fluorescence of SRB was found to be stable for hours and had a linear dependence on the SRB concentration in the range 10^{-8}–10^{-6} M.

In most of our experiments, SRB-loaded LUV were added to 2 ml of buffer (10 mM Tris–HCl, 50 μM EGTA and 40 mM KCl, pH 8.5) and their fluorescence was measured before and after various additions. Each experiment was concluded with the final estimation of a maximal fluorescence observed upon the total release of SRB from LUV, the latter being achieved by the addition of 0.1% Triton X-100 (TX-100). The quantity of LUV to be added to a sample was adjusted so that the maximal fluorescence was always at the same level. The molar phospholipid concentration in samples measured by modified Bartlett technique [12,13] was 10 μM.

FFA were added to samples as ethanol solutions, with the final concentration of ethanol never exceeding 1%. At this concentration, ethanol did not cause any release of SRB from LUV.

The release of SRB from LUV upon the effect of an acting factor (i.e. a chemical or a combination of chemicals) was calculated as a percentage of total SRB entrapped in LUV:

$$R = \frac{k_F F_F - F_B}{0.83 \cdot F_T - F_B} 100\%,$$

where R, release of SRB from LUV induced by an acting factor; F_B, base fluorescence level observed after the addition of LUV; F_F, fluorescence after the effect of an acting factor; F_T, fluorescence upon the total release of SRB from LUV after the addition of TX-100; k_F, correction coefficient for F_F- it was used when the fluorescence of SRB was affected by the acting factor itself (in most cases, k_F was equal to 1); 0.83, a value of correction coefficient for TX-100, which was found to increase SRB fluorescence.

In order to simplify calculations, we adopted that the fluorescence of SRB entrapped in LUV was low and could be neglected. We think it is acceptable in our case because we do not draw any conclusions that would require a very accurate estimation of SRB concentrations.

2.3. Fluorescence correlation spectroscopy measurements

LUV (0.1 μm in diameter) from azolectin with 5% (mol/mol) *N*-Rh-PE were obtained by the extrusion technique as described above, except that no SRB was loaded and the buffer used contained 10 mM Tris–HCl (pH 8.5), 50 μM EGTA and 40 mM KCl. Measurements were made with a "Confocor" instrument (Zeiss, Jena and Evotec, Hamburg, Germany). It consists of an inverted microscope with an objective lens (C-Apochromat 40 × /1.2 W Korr), a He/Ne continued wave laser (1.5 mW) at 543.5 nm as excitation source (1376, Uniphase, Manteca CA, USA), an avalanche photodiode (SPCM-AQ-131, EG&G, Canada) in the single photon counting mode and a digital correlator (ALV 5000/E, ALV GmbH, Germany). Fluorescence from the samples was observed through He/Ne 543 nm No. 015 filter slider (Carl Zeiss, Germany). The detection pinhole had a diameter of 45 μm.

Samples (250 μl) were measured in LAB-TEK chamber slides with eight chambers and a ~ 150-μm-thick cover slide on the bottom (Nunc, Denmark). The focus of the lens was placed inside the solution to be analyzed and ~ 200 μm above the inner surface of the cover slide. For each sample equilibrated to room temperature for ~ 4 min, the particles number presented in confocal volume and its diffusion time through the volume were determined by averaging five runs of 20 s each. The correlation functions were fitted with the FCS Access Fit software package [14], assuming the absence of the triplet fraction, in accordance with the following equation:

$$G(t) = 1 + \frac{1}{N}\frac{1}{N(1+t/\tau)\sqrt{1+t/(K^2\tau)}},$$

where $G(t)$, autocorrelation function; t, delay time; N, number of particles presented in the confocal volume; τ, diffusion time required for a fluorescing particle to pass through the confocal volume $V_{\mathrm{conf}}=\pi^{1.5}\,Kr_0^3$ [15].

Confocal volume formed by the highly focused laser light is defined by the distances from the centre to the edge

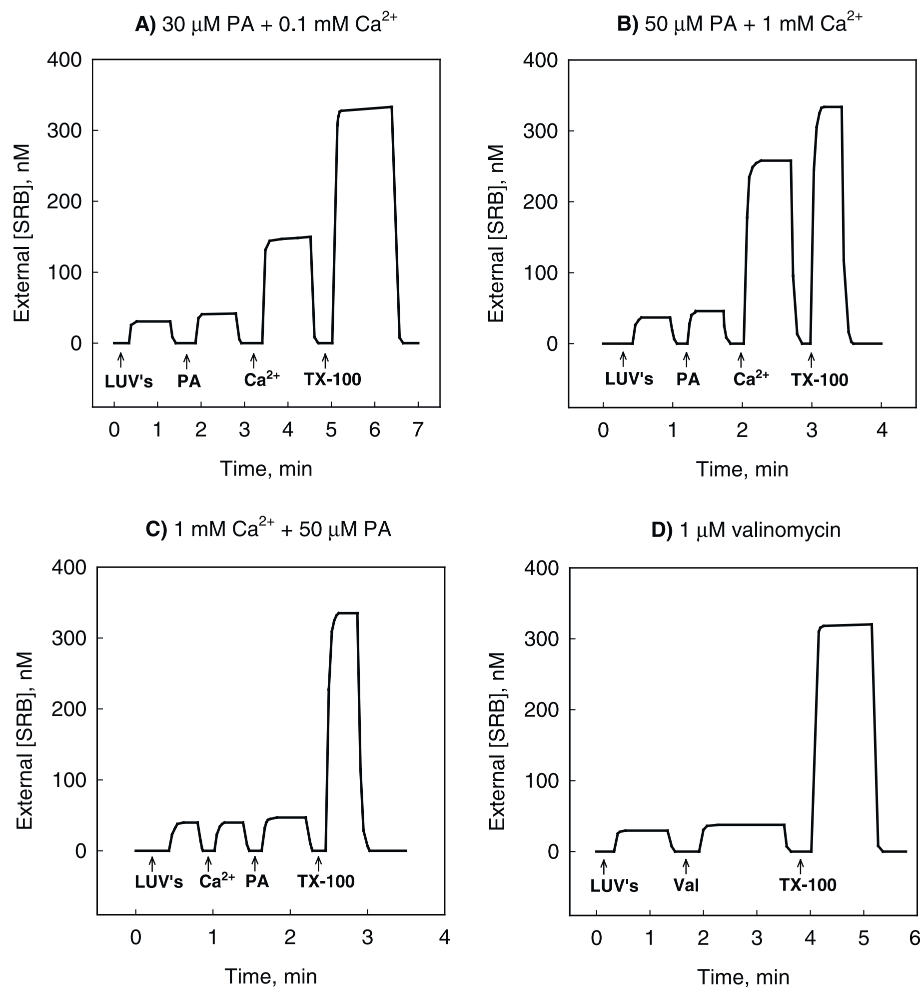

Fig. 1. Fluorescence changes in the suspension of SRB-loaded LUV upon the addition of PA and $CaCl_2$. Drops to the zero fluorescence level just reflect the opening of the cuvette chamber. Medium composition: 10 mM Tris–HCl buffer (pH 8.5), 50 mM KCl, 50 μM EGTA. Common additions: LUV (10 μM azolectin), 0.1% TX-100. (A) Addition of 30 μM PA and 0.1 mM $CaCl_2$ in the direct order (first PA, then $CaCl_2$); (B) addition of 50 μM PA and 1 mM $CaCl_2$ in the direct order; (C) addition of 50 μM PA and 1 mM $CaCl_2$ in the inverse order (first $CaCl_2$, then PA); (D) addition of 1 μM valinomycin.

of the confocal volume in the radial (r_0) and axial (Kr_0) directions. The parameter K and r_0 were calibrated prior to each experiment using rhodamine 6G, which has a known diffusion coefficient, D, of 2.8×10^{-10} $m^2 \cdot s^{-1}$ [16]. Parameter r_0 was estimated using the following equation: $r_0 = \sqrt{4D\tau}$ [17]. The concentration of fluorescing particles was calculated according to the formula:

$$C = \frac{N}{N_A V_{conf}}$$

where N_A is Avogadro's number.

3. Results

3.1. Formation of PA/Ca^{2+} complexes in the liposomal membrane results in the instant release of SRB from LUV, the effect depending on the concentration of PA and Ca^{2+}

In our previous study, we showed that the addition of 0.5% (w/w) PA or SA to the total brain lipid/cardiolipin mixture used to make BLM increased ion membrane permeability only in the presence of 0.1–1 mM Ca^{2+} [11]. Similar results were obtained with the liposomal membranes (Fig. 1). No efflux of SRB from LUV was observed in the presence of PA or Ca^{2+} alone. However, the combination of PA and Ca^{2+} resulted in the increase in SRB fluorescence, indicating the release of SRB from LUV (Fig. 1A and B). Fluorescence increased to a stable higher level immediately after the addition of Ca^{2+}. The higher were the concentrations of PA and Ca^{2+}, the more pronounced was the observed effect (Fig. 1B). The order in which PA and Ca^{2+} were added to liposomes was crucial for the effect to develop. The increase in fluorescence took place only when PA was added prior to Ca^{2+} (Fig. 1A and B). The addition of these agents in the inverse order did not change membrane permeability, even at high concentrations of PA and Ca^{2+} (Fig. 1C). The interpretation of this experiment is quite clear-cut. The cause of SRB release would be the formation of PA/Ca^{2+} complexes, but only in the case when these complexes are formed in the membrane (Fig. 1A and B). When added to liposomes, PA is known to be rapidly incorporated into the lipid bilayer [18–20]. The subsequent addition of Ca^{2+} would result in its binding to PA at the lipid/water interface and it is this event that seems to be responsible for the SRB release. However, if Ca^{2+} is added prior to PA, PA/Ca^{2+} complexes will be formed in the water phase, with no influence on the integrity of liposomal membranes.

As PA/Ca^{2+} complexes are able to increase the non-specific ion membrane permeability, one may suppose the SRB release to be due to an osmotic rupture of LUV. The rupture could result from the influx of the external K^+ (40 mM) into vesicles leading to an increase in the internal osmotic pressure. However, the addition of valinomicin does not induce SRB release, indicating that LUV are osmotically stable (Fig. 1D). So increasing the internal osmotic pressure cannot be a sufficient cause for SRB release. For LUV to be permeabilized, the formation of PA/Ca^{2+} complexes in the membrane is necessary.

Dependence of SRB release from LUV on the concentration of PA and Ca^{2+} is shown in Fig. 2. The concentration curves have a form, which is typical for the adsorption/binding processes, once again confirming the conclusion that SRB release is triggered by the formation of PA/Ca^{2+} complexes. The degree of permeabilization depends upon the PA (Fig. 2A) and Ca^{2+} (Fig. 2B) concentrations, with almost total SRB release observed in the presence of 50 μM PA and 1 mM Ca^{2+}. In case of 30 μM PA and 100 μM Ca^{2+}, the release is about 30% of the maximal level.

3.2. Dependence of PA/Ca^{2+}-induced SRB release from LUV on pH and ionic strength

The results presented above suggest that the PA/Ca^{2+}-induced SRB release from LUV would depend on the ability of PA and Ca^{2+} to form a complex. As we found

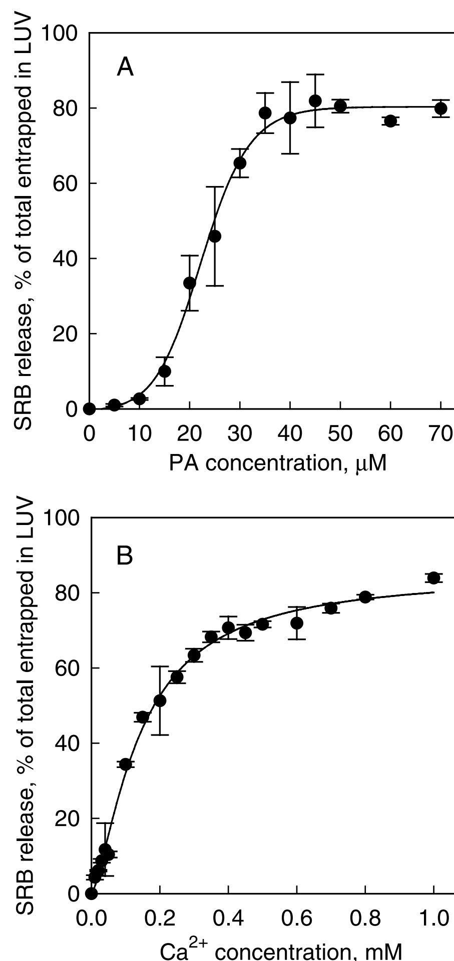

Fig. 2. Dependence of SRB release from LUV on the concentration of PA (A) and Ca^{2+} (B). Medium composition, common additions and the addition order are as in Fig. 1A. (A) Concentration of Ca^{2+} is 1 mM; (B) concentration of PA is 50 μM.

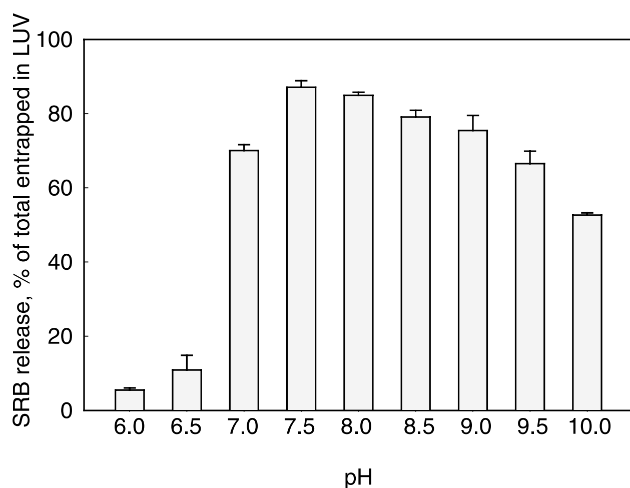

Fig. 3. pH-dependence of SRB release from LUV after the formation of PA/Ca^{2+} complexes in the liposomal membrane. Medium composition, common additions and the addition order are as in Fig. 1A. Concentration of PA—50 μM, $CaCl_2$—1 mM.

before, binding of Ca^{2+} to PA was clearly pH-dependent [11]. The maximal binding was observed at neutral to alkaline pH, while hardly any binding was found at pH 6.5. A similar pH-dependence was revealed for the PA/Ca^{2+}-induced permeabilization of LUV (Fig. 3).

Another factor that should influence formation of PA/Ca^{2+} complexes is the ionic strength. Earlier, we showed that raising the Na^+ concentration decreased binding of Ca^{2+} to PA [11]. The PA/Ca^{2+}-induced SRB release was also shown to be dependent on Na^+ concentration (Table 1). Both these facts (the dependence of SRB release on pH and Na^+ concentration) support the hypothesis that the permeabilization of the liposomal membrane is associated with the formation of a complex between PA and Ca^{2+}.

3.3. Ability of other fatty acids and bivalent cations to cause the permeabilization of liposomal membrane to SRB

The phenomenon of membrane permeabilization described in the present work is not specific for PA and Ca^{2+}. A number of bivalent cations (Table 2) and SA (Table 3) exert a similar effect on the liposomal membranes. However, linoleic acid, which has a low affinity to Ca^{2+} [11], turns out to be quite ineffective (Table 3). It is also noteworthy that in the case of Mg^{2+}, the membrane permeabilization is thrice as low comparatively to that induced by Ca^{2+} (Table 2).

Table 1
Release of SRB from LUV upon addition of 50 μM PA and 1 mM $CaCl_2$ at different ionic strength levels[a]

NaCl (mM)	SRB release, percentage of total entrapped in LUV
0	74.27 ± 2.22
50	60.33 ± 2.42
100	51.69 ± 1.30

[a] Medium composition, common additions and the addition order are as in Fig. 1A.

Table 2
The ability of bivalent cations to induce the release of SRB from LUV in the absence or presence of 50 μM PA[a]

Me^{2+} (1 mM)	SRB release, percentage of total entrapped in LUV	
	in the absence of PA	in the presence of PA
Ca^{2+}	0.65 ± 0.29	74.27 ± 2.22
Ba^{2+}	0.45 ± 0.10	74.10 ± 3.65
Sr^{2+}	0.00 ± 0.33	47.63 ± 5.18
Mn^{2+}	0.82 ± 0.29	71.97 ± 2.13
Ni^{2+}	1.15 ± 0.36	60.72 ± 3.00
Co^{2+}	0.93 ± 0.26	52.01 ± 2.37
Mg^{2+}	0.00 ± 0.00	22.45 ± 3.99

[a] Medium composition, common additions and the addition order are as in Fig. 1A.

3.4. Binding of CA^{2+} to PA incorporated in the liposomal membrane does not lead to the disintegration of LUV

Speaking above on the phenomenon of PA/Ca^{2+}-dependent SRB release from LUV, we consider it as a "permeabilization of the liposomal membrane". But what, in fact, happens with LUV after the addition of PA and Ca^{2+}? Maybe this is not permeabilization of the membrane but just a complete disintegration of LUV? To test whether LUV are disintegrating in the presence of PA and Ca^{2+}, we have performed an experiment using the fluorescence correlation spectroscopy approach. *N*-Rh-PE-containing LUV were treated with PA, Ca^{2+} or their combinations and then the number and radius of fluorescing particles in the confocal volume were evaluated. The results are given in Table 4. First, with 0.5 mM LUV, we have calculated the radius of a liposome to be 51 ± 2 nm, this corresponds well to the radius of pores in the polycarbone membrane used to prepare LUV. Then we lowered LUV concentration to the value of 10 μM (the concentration that was in our experiments with SRB-loaded LUV). Although this led to an increase of the error in radius determination, it also reduced the total fluorescence emitted by LUV, and hence, raised the sensitivity in detecting small fluorescing particles, should

Table 3
Release of SRB from LUV upon binding of Ca^{2+} to the different FFA, incorporated into the lipid bilayer[a]

FFA (50 μM)	SRB release, percentage of total entrapped in LUV	
	without $CaCl_2$	+1 mM $CaCl_2$
Palmitic	2.95 ± 0.39	79.10 ± 1.79
Stearic	2.02 ± 0.51	42.41 ± 3.01
Linoleic	8.64 ± 1.09	10.02 ± 1.35

[a] Medium composition, common additions and the addition order are as in Fig. 1A.

Table 4
Radius and concentration of fluorescing particles in the suspension of Rh-PE-labeled LUV

Experiment	Particle radius (nm)	Particle concentration (nM)
0.5 mM LUV	50.74 ± 1.96	19.530 ± 2.147
10 μM LUV	61.11 ± 10.16	0.384 ± 0.014
10 μM LUV + 50 μM PA	45.32 ± 8.82	0.676 ± 0.096
10 μM LUV + 1 mM Ca^{2+}	53.10 ± 3.69	0.396 ± 0.037
10 μM LUV + 50 μM PA + 1 mM Ca^{2+}	45.26 ± 10.76	0.386 ± 0.073
10 μM LUV + 1 mM Ca^{2+} + 50 μM PA	51.51 ± 8.47	0.335 ± 0.019
10 μM LUV + 0.1% TX-100	4.08 ± 0.24	504.385 ± 14.279

they appear after an experimental treatment. However, no essential changes in both the radius and the number of fluorescing particles were seen upon the addition of PA, Ca^{2+} and their combinations. And what is more important, no small particles were revealed. Therefore, in our experiments, LUV are not disintegrating but keep themselves as entities. The disintegration of LUV can be demonstrated with 0.1% TX-100 (Table 4). When TX-100 is added to LUV, the concentration of fluorescing particles increases by three orders of magnitude and the particle radius is reduced to 4 nm—a value, typical for micelles.

4. Discussion

The ability of FFA to induce changes in the permeability of the inner mitochondrial membrane has been known for many years but until recently, it has never been attributed to the complexation of FFA with Ca^{2+}. However, the fact that long-chain (C:16–C:22) saturated FFA bind Ca^{2+} with high affinity [11] makes one reconsider the possible role of FFA/Ca^{2+} complexes in the cell. It seems that the mechanism of some physiological processes can be based on the formation of FFA/Ca^{2+} complexes: among such processes are the Ca^{2+} sequestration by sarcoplasmic reticulum [21–24] and the NCPT in mitochondria [6,7].

Considering the role of FFA/Ca^{2+} complexes in the NCPT, one should investigate how these complexes influence the membrane permeability. Earlier, we demonstrated that formation of PA/Ca^{2+} or SA/Ca^{2+} complexes in the BLM resulted in the appearance of a nonspecific ion permeability of the membrane. The effect was much more pronounced when BLM was formed from the mitochondrial lipids, rather than from the total brain lipid extract [11]. In the present study, we observed PA/Ca^{2+}-induced membrane permeabilization for the rather large (comparatively with simple ions), charged molecules of SRB. And before we begin to compare the phenomena of PA/Ca^{2+}-dependent permeability transitions in artificial and mitochondrial membranes, let us set forth our considerations on the nature and the possible mechanism of PA/Ca^{2+}-induced SRB release from LUV.

4.1. The mechanism of PA/Ca^{2+}-induced SRB release from LUV in light of the theory of lipid pores

As it follows from the confocal fluorimetry experiments, LUV do not disintegrate upon influence of PA and Ca^{2+} (Table 4). In fact, this result is not surprising, if we take into consideration that the critical micelle formation for PA is equal to 2.8 mM [25]. Another noteworthy fact is that mentioned in the paper of Sultan and Sokolove [6]: the content of FFA in the lipid bilayer can achieve 60% (mol/mol), with the bilayer integrity being not violated, but on the contrary, being stabilized [26]. As for Ca^{2+} cations, their binding at the membrane surface may, of course, destabilize the bilayer, promoting the appearance of non-bilayer lipid phases and the membrane fusion [27]. However, these processes are relatively slow and require a higher content of lipid in the system comparatively to that used in the present work.

Thus, underlying SRB release from LUV should be a rupture of the liposomal membrane. But what does the term “membrane rupture” really mean? As a matter of fact, when applied to the lipid bilayer, this term implies the formation of lipid pores. Indeed, any process of membrane rupture begins with the appearance of hydrophilic pores in the bilayer; these pores grow in size and if their radius exceeds a certain critical value, the membrane will burst like in a soap bubble [28]. Adopting such interpretation of “rupture process”, we shall consider the permeabilization of liposomal membranes for SRB in light of the theory of lipid pores [28,29]. We have even more grounds to do so as Antonov et al. [28,30–32] have already observed formation of lipid pores under similar conditions: upon binding of bivalent cations to BLM formed from pure phosphatidic acid. The mechanism of pore formation proposed by Antonov et al. can well be realized in our system. According to this mechanism, lipid pores arise from the reduction of area of lipid monolayers, which is induced by the binding of Ca^{2+} to PA molecules. As PA/Ca^{2+} complexes are predominantly formed from the outer side of the liposomal membrane, their formation should lead to an imbalance of the surface tension at different membrane sides. Such an imbalance can result in the lipid bilayer losing its integrity, followed by the appearance of lipid pores. But once pores have arisen, the imbalance will be removed and pores will tighten if their radius has not exceeded the critical value by this moment. This is an important point, as the ability of lipid pores to tighten can explain why we observe SRB release only at the very first moment after addition of Ca^{2+}. The lifetime of lipid pores formed upon binding of bivalent cations to the membrane from phosphatidic acid was demonstrated to be about a few seconds [28], this well-corresponding to the kinetics of SRB release from LUV in our experiments.

Whether a lipid pore in a liposome would tighten or would result in the liposome to burst—this seems to be a probabilistic alternative as the same imbalance in a liposome can be resolved, for example, by the appearance of several small pores or by the formation of a large single pore whose growth will result in the bursting of the membrane. From this point of view, the quantity of SRB released would depend on the number, size and lifetime of pores, as well as on the number of burst LUV. We guess, that all these parameters would contribute in the overall release of SRB from LUV and any "extreme case" could hardly take place. Say, 30% release of SRB cannot be interpreted, in our opinion, as though 30% of LUV burst while the remaining 70% retain all SRB inside. Such an "all or nothing" interpretation will imply that our system is heterogeneous in a parameter that determines whether SRB will be released from a liposome or not. However, the suspension of LUV is rather homogeneous inherently and the only parameters that may be nonuniformly distributed in the LUV population would be, in our opinion, the number and the size of pores in a vesicle. So to adopt the "all or nothing" interpretation, we have to suppose that only large pores causing LUV to burst are formed. But this supposition seems to us improbable. The pore critical radius calculated for the liquid–crystalline phosphatidylcholine bilayer is about 9 nm [28], this being a rather large value. So it is unclear why the pores with a smaller radius should not be formed under our conditions if at the same time, they are proven to arise in the phosphatidic acid BLM upon binding of bivalent cations [28,30–32]. Moreover, the fact that this BLM does not burst indirectly indicates that in our case, hardly should bursting of LUV contribute significantly in the overall SRB release.

4.2. Comparison of the phenomena of PA/Ca^{2+}-induced LUV permeabilization and NCPT

Although we do not know how identical are the processes, underlying the permeabilization of LUV and the NCPT, a number of parallels between these two phenomena can well be drawn. Of course, we should take into consideration some differences that originate from the mitochondria being a more complex system than liposomes and the necessity of NCPT triggers to be transferred to the matrix side of the mitochondrial membrane.

1. Both permeabilization of LUV and NCPT are induced by the same concentrations of PA (Fig. 2). The roughly estimated PA/lipid ratio, however, is several times higher in the case of maximal development of LUV permeabilization comparing to that in the experiments of Sultan and Sokolove [6]. Nevertheless, there is no a large discrepancy here, in our mind, because first, LUV permeabilization is still developed at lower PA/lipid ratios; second, the PA content in the contact sites of the inner mitochondrial membrane may, in fact, be higher due to the activation of phospholipase A_2 [33–36]; and third, the differences in the lipid composition between mitochondria and LUV might also be of matter.
2. The concentrations of Ca^{2+} used to trigger NCPT are lower than those which caused the maximal effect on LUV. But in the case of NCPT, the acting Ca^{2+} concentration is the matrix one [6], which will be higher in Ca^{2+}-loaded mitochondria comparatively to the concentration in the external medium.
3. Both the NCPT and the effect observed on LUV are induced by the long-chain saturated FFA (Table 3, [6,7]), this correlates to their high affinity to Ca^{2+} [11]. The unsaturated FFA, having a low affinity to Ca^{2+} [11], are ineffective in the induction of LUV permeabilization (Table 3) and do not always trigger the NCPT [7].
4. Not only Ca^{2+} but some other bivalent cations are also able to trigger the permeabilization of LUV and NCPT (Table 2, [6]). In the case of NCPT, it depends on the ability of a cation to be accumulated in the mitochondrial matrix.
5. The pH optimum for the formation of FFA/Ca^{2+} complexes lies in the alkaline region [11], the same is proven for the effect of LUV permeabilization (Fig. 3) and probably for the NCPT (in the experiments of Sultan and Sokolove [6], pH of the external medium was 7.4).
6. Identifying the phenomenon of NCPT as the opening of a pore, Sultan and Sokolove [6] found that this pore was closed after the process of mitochondria swelling had been completed. Dealing with LUV, we see that permeabilization occurs at the very first moment of Ca^{2+} binding to PA, with no further release of SRB (Fig. 1A and B). As has been discussed in the previous section, such a picture is well explained in the light of theory of lipid pores.

Thus, there are several analogies between NCPT and PA/Ca^{2+}-induced LUV permeabilization, suggesting that the PA/Ca^{2+}-induced mitochondrial NCPT can well be a phenomenon of lipid nature. Further studies on both model and cell systems are necessary to give a definitive answer to this question, which is a key point in the understanding of the mechanism of PA-induced apoptosis.

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