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ARTICLE *in* JOURNAL OF MEMBRANE BIOLOGY · SEPTEMBER 2010

Impact Factor: 2.46 · DOI: 10.1007/s00232-010-9302-1 · Source: PubMed

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Palmitic Acid Induces the Opening of a Ca^{2+}-Dependent Pore in the Plasma Membrane of Red Blood Cells: The Possible Role of the Pore in Erythrocyte Lysis

Konstantin N. Belosludtsev · Alexander S. Trudovishnikov · Natalia V. Belosludtseva · Alexey V. Agafonov · Galina D. Mironova

Received: 26 April 2010 / Accepted: 17 August 2010 / Published online: 11 September 2010

Abstract Earlier we found that in the presence of Ca^{2+} palmitic acid (Pal) increases the nonspecific permeability of artificial (planar and liposomal) membranes and causes permeabilization of the inner mitochondrial membrane. An assumption was made that the mechanism of Pal/Ca^{2+}-induced membrane permeabilization relates to the Ca^{2+}-induced phase separation of Pal and can be considered as formation of fast-tightening lipid pores due to chemotropic phase transition in the lipid bilayer. In this article, we continue studying this pore. We have found that Pal *plus* Ca^{2+} permeabilize the plasma membrane of red blood cells in a dose-dependent manner. The same picture has been revealed for stearic acid (20 μM) but not for myristic and linoleic acids. The Pal-induced permeabilization of erythrocytic membranes can also occur in the presence of Ba^{2+} and Mn^{2+} (200 μM), but other bivalent cations (200 μM Mg^{2+}, Sr^{2+}, Ni^{2+}, Co^{2+}) are relatively ineffective. The formation of Pal/Ca^{2+}-induced pores in the erythrocytic membranes has been found to result in the destruction of cells.

Keywords Palmitic acid · Calcium · Lipid pore · Erythrocyte

K. N. Belosludtsev (✉) · A. S. Trudovishnikov · N. V. Belosludtseva · A. V. Agafonov · G. D. Mironova
Institute of Theoretical and Experimental Biophysics RAS, Institutskaya, 3, Pushchino, 142290 Moscow Region, Russia
e-mail: bekonik@gmail.com

N. V. Belosludtseva · A. V. Agafonov · G. D. Mironova
Pushchino State University, Prospekt Nauki, 3, Pushchino, 142290 Moscow Region, Russia

Abbreviations

Pal	Palmitic acid
FFA	Free fatty acids
SRB	Sulforhodamine B

Introduction

Free fatty acids (FFAs), which can be found in all biological membranes, carry out several important functions in the cell. Apart from their involvement in various metabolic processes, they also perform an important biophysical function: They modulate membrane permeability. It has been shown that palmitic acid (Pal), a known natural activator of apoptosis (Sparagna et al. 2000), can induce, at rather low concentrations, the opening of a nonspecific pore in the inner mitochondrial membrane, the effect being Ca^{2+}-dependent (Sultan and Sokolove 2001; Mironova et al. 2004; Belosludtsev et al. 2005). This pore differs from the well-known proteinaceous MPT pore by many features. As shown in our laboratory, the opening of the Pal/Ca^{2+}-induced pore can occur in both mitochondrial and artificial (planar and liposomal) lipid membranes, which suggests a lipid nature of this pore (Mironova et al. 2001; Agafonov et al. 2003, 2007; Belosludtsev et al. 2005). Our studies have demonstrated that underlying the mechanism of pore formation is the ability of Pal to bind Ca^{2+} with high affinity, which is one to two orders of magnitude higher than that of other FFAs and lipids. The experiments with liposomes have revealed that the pore is formed by the mechanism of chemotropic phase transition, when the binding of Ca^{2+} with Pal anions in the lipid bilayer leads to the segregation of Pal/Ca^{2+} complexes into separate membrane domains (Agafonov et al. 2007).

On the assumption of such a mechanism, one can suppose that the pore would open in any lipid membrane if the content of Pal in the lipid bilayer is increased and there are Ca^{2+} ions in the medium. The level of FFA (first of all, palmitic and stearic acids) in tissues and blood is known to rise in many pathologies, such as diabetes (Kenno and Severson 1985), myocardial infarction (Mironova et al. 2004), Refsum disease (Schönfeld and Struy 1999), HELLP syndrome and acute fatty liver of pregnancy (Lee and Brady 2009), cold stress (Brustovetsky et al. 1990), obesity (Shimabukuro et al. 1998), alcoholism (Laposata and Lange 1986), hyperoxia (Abe and Sevanian 1981) and others. The development of these pathologies can be accompanied by erythrocyte hemolysis (Abe and Sevanian 1981; Wrońska-Nofer et al. 1991; Lee and Brady 2009). In some cases, it might result from the formation of lipid pores in the membrane of erythrocytes, which could be induced by Pal and Ca^{2+}. In this connection, the objective of the present work was to examine the possibility of Pal/Ca^{2+} pore formation in the plasma membrane of red blood cells. As shown in the earlier works, high concentrations of Pal exert a lytic effect on the membrane of erythrocytes (Zavodnik et al. 1991). A hypothesis, which was made on the basis of mathematical models, assumed the formation of nonspecific pores in the membrane, the nature of which was not clearly defined (Kaler et al. 1986).

The present study shows that (1) Pal *plus* Ca^{2+} cause a nonspecific permeabilization of the erythrocytic plasma membrane to the molecules of sulforhodamine B (SRB), with the effect depending on the fatty acid and Ca^{2+} concentration as well as on temperature, and (2) the Pal/Ca^{2+}-induced permeabilization of the erythrocytic membrane leads to the lysis of cells.

Materials and Methods

Isolation of Erythrocytes

The whole blood of a mature male Wistar rat (220–250 g) was collected in a tube containing 3 ml of 3.5% sodium citrate with EDTA (1 mg/ml) and centrifuged for 10 min at 500×*g* and 4°C. The plasma and leukocytes were removed, and erythrocytes were resuspended and washed three times in a quintupled volume of phosphate-buffered saline (PBS)–glucose buffer (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 5 mM glucose, pH 7.4), the conditions of centrifugation being the same and the pellet being rinsed with the buffer every time to remove the layer of leukocytes. The resulting erythrocyte mass was diluted with the PBS–glucose buffer to a final concentration of ~5–9 × 10^9 cells/ml.

Loading of Erythrocytes with the Fluorescent Dye SRB

Erythrocytes were loaded with SRB according to the "Preswell dilutional hemolysis" protocol (Ihler and Tsang 1987). A volume of erythrocyte mass was resuspended in a fivefold volume of a buffer containing 76 mM NaCl, 1.5 mM KCl, 5.5 mM Na_2HPO_4 and 2.7 mM glucose (pH 7.4). After incubation for 5 min at 0°C, the suspension was centrifuged for 10 min at 500×*g* and 4°C. The swelling procedure was repeated once.

The resulting cell suspension was diluted 1.5- to 2.0-fold with a hypotonic buffer containing 10 mM KH_2PO_4, 10 mM glucose, 1.5 mM ATP, 3 mM GSH, 2 mM $MgCl_2$ and 30 mM SRB (pH 7.4). The cells were allowed to permeabilize for 10 min at 0°C. Then, erythrocytes were incubated with resealing buffer containing 3% NaCl, 10 mM NaH_2PO_4, 1.5 mM ATP, 10 mM glucose, 10 mM pyruvate and 1 mM $MgCl_2$ for 30 min in the dark at room temperature. Then, the suspension was centrifuged for 10 min at 500×*g* and 4°C. The pellet was washed five to six times with a quintupled volume of the PBS–glucose buffer (10 min, 500×*g*, 4°C), and the final suspension was diluted to a final concentration of ~5–9 × 10^9 cells/ml.

Measuring Permeabilization of Erythrocytic Membranes

The nonspecific permeabilization of erythrocytes was assessed by the release of SRB from the dye-loaded cells using a USB 2000 spectrofluorimeter (Ocean Optics, Dunedin, FL). Measurements were carried out at excitation and emission wavelengths of 565 and 586 nm, respectively, and the release of SRB was determined by the growth of fluorescence intensity due to dilution of SRB in the external medium (Schwarz and Arbuzova 1995).

In the experiments, erythrocytes were resuspended in PBS–glucose buffer (final cell concentration 2–5 × 10^6 cells/ml) and fluorescence of the suspension was measured before and after various additions. At the end of each experiment, 0.1% Triton X-100 (TX-100) was added to the sample to estimate the maximal fluorescence level after the complete release of SRB into the external medium. In different experiments, the amount of erythrocytes injected into the measuring cell was adjusted so that the maximal fluorescence was always at the same level.

The amount of SRB released from erythrocytes in response to an experimental action (addition of a fatty acid, Ca^{2+}, their combination, etc.) was calculated in relation to the total SRB entrapped in erythrocytes:

$$R = \frac{kF - F_{\text{base}}}{F_{\text{max}} - F_{\text{base}}} \cdot 100\%$$

where R is the amount of SRB released from erythrocytes, a percent of the total entrapped in the cells; F_{base} is the base fluorescence level observed after the addition of erythrocytes; F is the level of fluorescence after an experimental action; F_{max} is the maximal fluorescence level observed after the addition of TX-100; k is the correction coefficient for F, which was used when the fluorescence of SRB was affected by the experimental action itself (in most cases, $k = 1$).

Counting Erythrocytes

Erythrocytes were counted under a Carl Zeiss (Göttingen, Germany) light microscope using a Goryaev's cell-counting chamber. The number of erythrocytes per milliliter of blood was calculated according to the following formula:

$$X = \frac{200a}{80b} \cdot 10^3$$

where X is the number of erythrocytes per milliliter of blood, a is the total number of erythrocytes in the chamber, b is the volume of a mesh in the Goryaev chamber (2.5×10^{-4} µl), 200 is blood dilution and 80 is the number of meshes in the chamber.

Results

Pal and Ca^{2+} Induce Nonspecific Permeabilization of the Erythrocyte Plasma Membrane

In this work, we checked the possibility that Pal and Ca^{2+} would open pores in the plasma membrane of erythrocytes. As seen in Fig. 1, the separate addition of either Pal (50 µM) or Ca^{2+} (0.5 mM) to erythrocytes does not induce SRB release. However, the successive addition of, first, Pal and, then, Ca^{2+} results in a sharp jump of fluorescence. Fluorescence rises to a certain level, right after the addition of Ca^{2+}, and then stabilizes and remains steady (Fig. 1a). If we reverse the order of additions—first Ca^{2+} and then Pal—fluorescence will grow only a little (Fig. 1b). Probably, when Pal is added into the Ca^{2+}-containing medium, their complexes are formed already in the aqueous phase, and this will have no effect on the membrane. An identical picture was observed in our earlier experiments with liposomes (Agafonov et al. 2003), in which we studied the effect of Pal and Ca^{2+} on the permeability of azolectin membranes. Evidently, these phenomena have a similar nature.

The dependences of the Pal/Ca^{2+}-induced permeabilization of erythrocytes on the concentration of Pal and Ca^{2+} are given in Fig. 2. As the figure shows, the maximal amount of SRB released in the process of membrane permeabilization reaches ~80% of the total; this level is

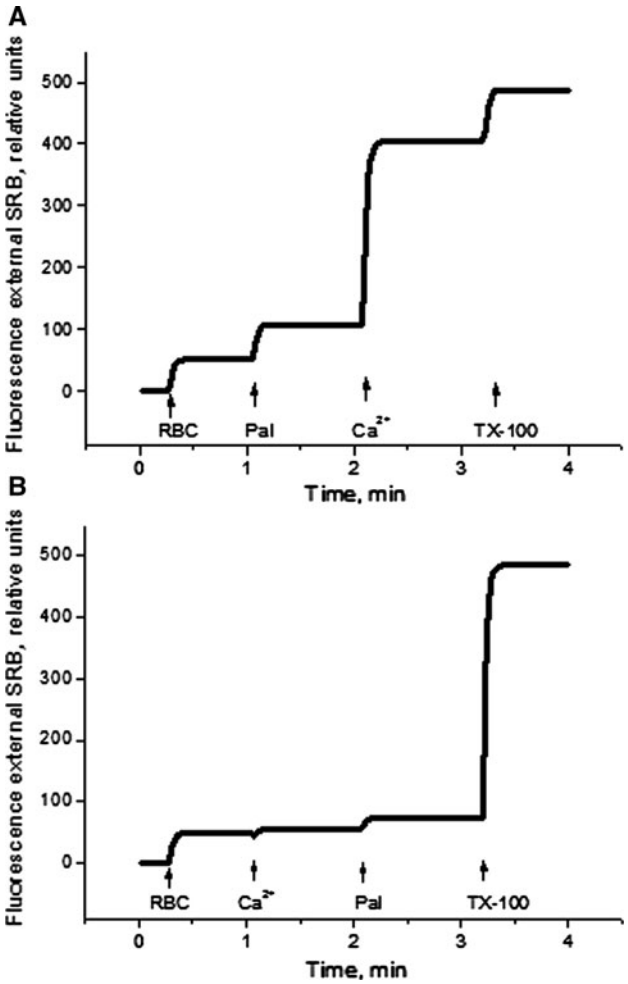

Fig. 1 Fluorescence changes in the suspension of SRB-loaded erythrocytes (*RBC*) upon the addition of Pal and Ca^{2+}. Medium composition: 138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 5 mM glucose, pH 7.4. Additions: **a** 50 µM Pal, 0.5 mM Ca^{2+}, 0.1% TX-100 in the direct order (first Pal, then Ca^{2+}); **b** 0.5 mM Ca^{2+}, 50 µM Pal, 0.1% TX-100 in the reverse order (first Ca^{2+}, then Pal)

observed at Pal concentrations of 50 µM and higher. The half-maximal effect can be seen at a Pal concentration of 25 µM (Fig. 2a).

Figure 2b shows the dependence of the Pal/Ca^{2+}-induced release of SRB from erythrocytes on the concentration of Ca^{2+}. The concentration of Pal in those experiments was 50 µM. The curve comes to the plateau of maximal effect at the concentration of Ca^{2+} ~40 µM; the half-maximal effect is observed at ~10 µM Ca^{2+}.

Pal/Ca^{2+}-Induced Permeabilization of Erythrocytes Depends on Temperature

All of the experiments described above were carried out at room temperature (22–23°C). Raising temperature to 37°C (Fig. 3) resulted in maximal Ca^{2+}-induced SRB release (~95%) being observed already at a Pal concentration of

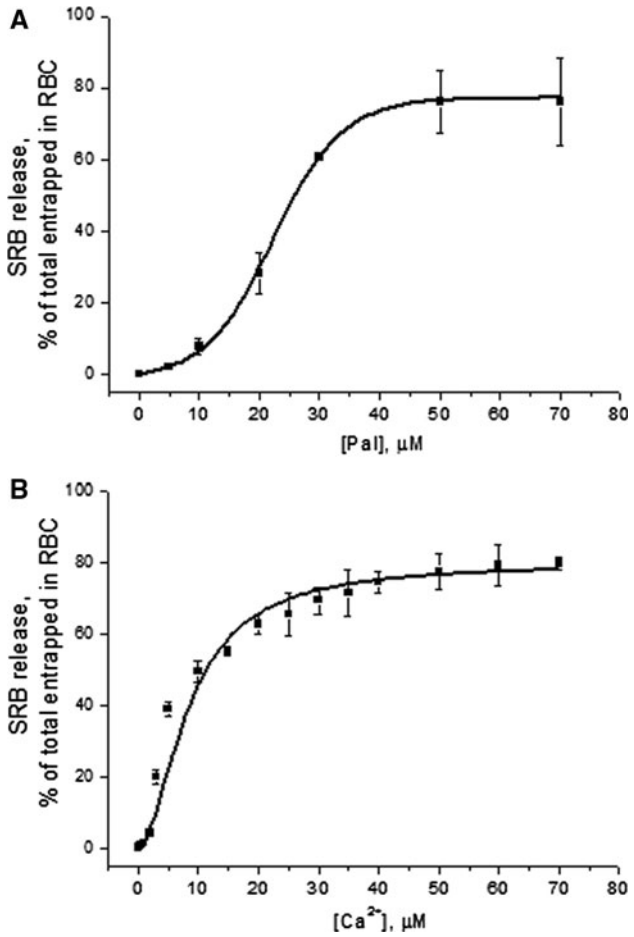

Fig. 2 Dependence of SRB release from erythrocytes (*RBC*) on the concentration of Pal (**a**) and Ca^{2+} (**b**). Medium composition was the same as in Fig. 1. SRB release was induced by 5–70 μM Pal and 0.5 mM Ca^{2+} (**a**) and 50 μM Pal and 2–70 μM Ca^{2+} (**b**). Mean values ± SD are presented ($n = 5$)

30 μM (the concentration of Ca^{2+} in that experiment was 0.1 mM).

Nonspecific Permeabilization of the Erythrocytic Membrane Can Also Be Induced by Other Fatty Acids and Divalent Cations

As in the experiments with mitochondria and liposomes, the Ca^{2+}-dependent permeabilization of erythrocytes can be observed not only with Pal but also with other FFAs (Table 1). The highest degree of membrane permeabilization was observed in the case of 20 μM palmitic and stearic acids; oleic acid was much less effective, and myristic and linoleic acids were not effective at all. At the same time, raising the concentration of oleic and linoleic acids to 50 μM resulted in marked release of SRB from erythrocytes even without Ca^{2+} (data not shown).

As can be seen from Table 2, other divalent cations, like Ca^{2+}, are able to induce the release of SRB from the

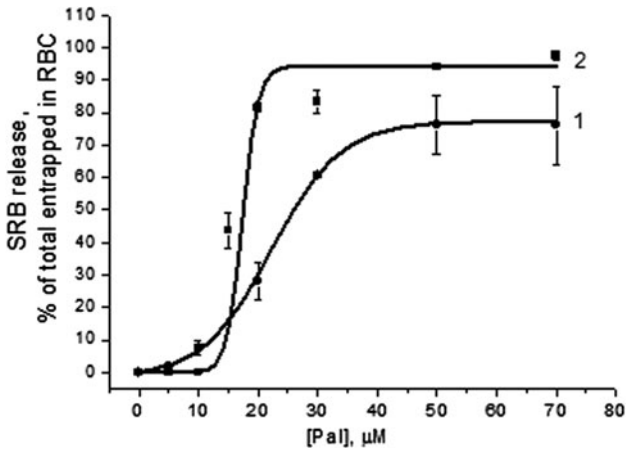

Fig. 3 Dependence of the Pal/Ca^{2+}-induced SRB release from erythrocytes measured under 25°C (*1*) and 37°C (*2*) on the concentration of Pal. SRB release was induced by 5–70 μM Pal and 0.5 mM Ca^{2+}. Medium composition was the same as in Fig. 1. Mean values ± SD are presented ($n = 3$)

Table 1 The ability of different fatty acids to induce Ca^{2+}-dependent release of SRB from erythrocytes

Fatty acid (20 μM)	SRB release (% of total entrapped in erythrocytes)
Palmitic	82.9 ± 6.64
Myristic	2.29 ± 0.1
Stearic	72.36 ± 5.36
Linoleic	3.37 ± 1.21
Oleic	31.83 ± 15.2

Additions: 20 μM FFAs and 100 μM Ca^{2+}. The medium composition was the same as in Fig. 1. Mean values ± SD are given ($n = 5$)

Table 2 The ability of bivalent cations to induce the release of SRB from erythrocytes in the presence of 20 μM Pal

Me^{2+}	SRB release (% of total entrapped in erythrocytes)
Ca^{2+}	67.63 ± 2.9
Sr^{2+}	18.83 ± 2.85
Ba^{2+}	62 ± 2.54
Mn^{2+}	44.29 ± 2.86
Mg^{2+}	14.68 ± 1.63
Ni^{2+}	19.38 ± 0.98
Co^{2+}	20.17 ± 0.87

Additions: 20 μM Pal and 200 μM Me^{2+}. The medium composition was the same as in Fig. 1. Mean values ± SD are given ($n = 5$)

Pal-containing erythrocytes. Among the cations examined, Ba^{2+} and Mn^{2+} (200 μM) demonstrated the highest ability to cause permeabilization of the erythrocytic membrane.

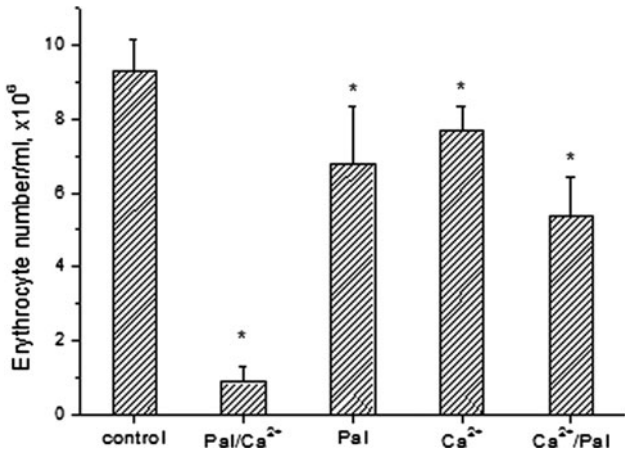

Fig. 4 Changes in the number of erythrocytes upon the addition of Pal and Ca^{2+}. Additions: 20 μM Pal and 100 μM Ca^{2+}. Medium composition was the same as in Fig. 1. Mean values ± SD are presented ($n = 5$)

Pal/Ca^{2+}-Induced Permeabilization of the Erythrocytic Membrane Leads to Cell Lysis

As Fig. 4 shows, addition of Pal and Ca^{2+} to the suspension of erythrocytes, at the pore-opening concentrations, resulted in cell lysis. At the same time, when added separately or in the reverse order, Pal and Ca^{2+} did not reduce the number of living erythrocytes.

Serum Proteins Suppress the Pal/Ca^{2+}-Induced Lysis of Erythrocytes

The experiments described above were carried out in a phosphate–saline buffer. When the buffer was replaced with serum, the number of cells subjected to Pal/Ca^{2+}-induced lysis decreased substantially (Table 3). A marked reduction in cell number was observed only at the Pal concentration of 100 μM.

The presence of FFA-binding proteins—in particular, albumin—in serum is an obvious and the most probable cause of the suppression of Pal/Ca^{2+}-induced lysis of erythrocytes. As shown in Table 3, the addition of BSA (10 mg/ml) to the PBS resulted in marked inhibition of pore opening in the presence of 20 μM Pal and 100 μM Ca^{2+}.

Table 3 Erythrocyte number changes in serum and in the presence of bovine serum albumin (BSA) upon the addition of Pal and Ca^{2+}

	Erythrocyte number (ml), $\times 10^6$		
	Serum	5 mg/ml BSA	10 mg/ml BSA
Control	5.0 ± 0.7	4.9 ± 0.6	5.3 ± 0.6
Pal/Ca^{2+}	3.8 ± 0.6	3.5 ± 0.7	5.0 ± 0.7
Pal	4.2 ± 0.8	4.6 ± 0.6	5.2 ± 0.6
Ca^{2+}	4.3 ± 0.5	4.7 ± 0.3	4.9 ± 0.2
Ca^{2+}/Pal	4.6 ± 0.6	4.5 ± 0.6	5.2 ± 0.3

Additions: 20 μM Pal and 100 μM Ca^{2+}. Medium composition was the same as in Fig. 1 (in experiments with BSA). Mean values ± SD are given ($n = 5$)

Discussion

The effect of Pal/Ca^{2+}-induced membrane permeabilization has been studied in our laboratory for many years (Mironova et al. 2001, 2004, 2007; Belosludtsev et al. 2005, 2006, 2009; Agafonov et al. 2003, 2007). As shown earlier, the mechanism of permeabilization is based not upon the well-known detergent-based properties of FFA (Agafonov et al. 2003) but rather on the separation of solid Pal/Ca^{2+} phase in the lipid bilayer (Agafonov et al. 2007). The separation is supposedly accompanied by fluctuations of lipid density in the membrane leaflets, and if a transient mismatch in the area of these coupled leaflets is too large, this may result in the loss of membrane integrity and formation of a hydrophilic lipid pore (for more details on the mechanism, see Agafonov et al. 2003, 2007).

The specificity of the Pal/Ca^{2+}-induced membrane permeabilization is primarily based on the fact that at alkaline pH long-chain saturated FFA has a very high affinity to Ca^{2+}, which is an order of magnitude higher than that of other FFAs and lipids (Mironova et al. 2001). The mechanism should, therefore, work under the conditions that promote binding of Ca^{2+} to Pal anions at one of the membrane sides. An example is the Pal/Ca^{2+}-induced permeability of inner mitochondrial membrane, which is driven by the accumulation of Pal/Ca^{2+} complexes on the matrix side of the inner mitochondrial membrane (Sultan and Sokolove 2001; Belosludtsev et al. 2005).

Although requiring some specific conditions, the Pal/Ca^{2+}-induced membrane permeabilization can, at the same time, take place in membranes of various phospholipid content (total brain phospholipids [Mironova et al. 2001], azolectin [Agafonov et al. 2003], mitochondrial lipids [Mironova et al. 2004]). The lipid content did not seem to be important for permeabilization to occur, which poses a question: Can the mechanism work in other biological membranes, apart from the mitochondrial one?

In this respect, the plasma membrane of erythrocytes is an interesting object. Among the factors that could affect the phase-transitional mechanism of membrane permeabilization in erythrocytes are the following:

- a high content of membrane cholesterol (Leidl et al. 2008), which is very different from the mitochondrial membrane

- a domain structure of the lipid bilayer:cholesterol and sphingomyelin form rafts with a roughly 1:1 molar ratio (Koumanov et al. 2005)
- an elastic, spectrin-based membrane skeleton network, which, apart from stabilizing the entire shape of the membrane system, contributes to the balance of lateral forces in the bilayer (Mohandas and Gallagher 2008)

What is interesting, though, is that none of these factors seems to influence the process of membrane permeabilization triggered by the addition of Ca^{2+}. In response to a sharp rise of external Ca^{2+} concentration, Pal-enriched erythrocytic membranes show the same phenomenon of permeabilization that was observed earlier for the artificial (Agafonov et al. 2003) and mitochondrial (Belosludtsev et al. 2005) membranes. It can be summarized as follows.

1. There exists a range of Pal and Ca^{2+} concentrations (Figs. 1, 2) at which none of the agents alone has a significant effect on membrane permeability, but the successive addition of, first, Pal and, then, Ca^{2+} leads to the loss of membrane integrity. The addition of the agents in the reverse order has no effect.
2. The Pal/Ca^{2+}-induced effect can be generalized for long-chain saturated fatty acids (Table 1) and a number of divalent cations (Table 2).

Thus, the mechanism of Pal/Ca^{2+}-induced membrane permeabilization can be triggered in such a cholesterol-rich membrane as erythrocytic membrane. This fact indicates, in our mind, that the separation of the Pal/Ca^{2+} phase, which the mechanism is supposedly based upon, outweighs any other factors, such as possible segregation of Pal/cholesterol in rafts. Moreover, our earlier experiments showed that the enrichment of mitochondrial membranes with cholesterol even facilitated the opening of the Pal/Ca^{2+}-induced pore in mitochondria (Belosludtseva et al. 2009). A possible explanation is that the supposed pre-separation of Pal with cholesterol would make the subsequent separation of the Pal/Ca^{2+} phase faster, by reducing the time of lateral diffusion of Pal molecules (a rate-limiting step). A faster separation of the Pal/Ca^{2+} phase would mean a greater disbalance of lateral pressure/tension forces in the membrane and a higher probability that pores would open.

The enhanced Pal/Ca^{2+}-induced permeabilization at 37°C (Fig. 3), when the erythrocytic membrane becomes much more fluid (Galla and Luisetti 1980; Gordon and Mobley 1984), can also be considered under the angle of "greater disbalance of lateral forces." The lateral diffusion of Pal molecules in a more fluid membrane should be faster, and the area change upon separation of solid Pal/Ca^{2+} domains from a more fluid phase should be larger. As a result, the formation of lipid pores would be facilitated.

Hemolysis of erythrocytes, following Pal/Ca^{2+}-induced membrane permeabilization (Fig. 4), is an obvious result assuming that the erythrocytic membrane becomes permeable, like the mitochondrial one, to any solutes of low molecular weight (<1,500 Da) (Sultan and Sokolove 2001). It can be supposed that the instant collapse of transmembrane ion gradients will lead to rapid osmotic swelling of erythrocytes, detachment of the plasma membrane from the cytoskeleton and, after exceeding a certain threshold of the cell volume/surface area ratio, the membrane rupture and release of cell contents into the outer medium.

Acknowledgements We are grateful to Dr. Natalia Venediktova (Institute of Theoretical and Experimental Biophysics RAS, Russia) for technical assistance in the preparation of SRB-loaded erythrocytes. This work was supported by a grant from the Russian Foundation for Basic Research, to K. N. B. (09-04-01024a), and the program Development of High School Scientific Potential from the Russian Ministry for Education, to G. D. M. (3840).

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