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Ca^{2+}-Induced Phase Separation in the Membrane of Palmitate-Containing Liposomes and Its Possible Relation to Membrane Permeabilization

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Abstract A Ca^{2+}-induced phase separation of palmitic acid (PA) in the membrane of azolectin unilamellar liposomes has been demonstrated with the fluorescent membrane probe nonyl acridine orange (NAO). It has been shown that NAO, whose fluorescence in liposomal membranes is quenched in a concentration-dependent way, can be used to monitor changes in the volume of lipid phase. The incorporation of PA into NAO-labeled liposomes increased fluorescence corresponding to the expansion of membrane. After subsequent addition of Ca^{2+}, fluorescence decreased, which indicated separation of PA/Ca^{2+} complexes into distinct membrane domains. The Ca^{2+}-induced phase separation of PA was further studied in relation to membrane permeabilization caused by Ca^{2+} in the PA-containing liposomes. A supposition was made that the mechanism of PA/Ca^{2+}-induced membrane permeabilization relates to the initial stage of Ca^{2+}-induced phase separation of PA and can be considered as formation of fast-tightening lipid pores due to chemotropic phase transition in the lipid bilayer.

Keywords Calcium · Palmitic acid · Nonyl acridine orange · Liposome · Lipid bilayer · Phase separation · Permeability transition · Lipid pore

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Introduction

Free fatty acids (FFAs), whose content in tissues may increase greatly at various pathologies (Gremlich, Roduit and Thorens 1997; Korge, Honda and Weiss 2003; Mironova et al. 2004; Zhou et al. 2000), were shown to induce cell death (Bernardi, Penzo and Wojtczak 2002; de Pablo et al. 1999; Kong and Rabkin 2000; Scorrano et al. 2001; Sparagna et al. 2000), with the mechanism of induction based on the mitochondrial permeability transition (PT). Two types of FFA/Ca^{2+}-induced PT were described: classical PT, which is inhibited by cyclosporin A (CsA) (Bernardi et al. 2002; Penzo et al. 2002), and CsA-insensitive PT (Belosludtsev, Belosludtsev and Mironova 2005; Mironova et al. 2004; Sultan and Sokolove 2001a, b). The latter is mainly induced by long-chain saturated FFA, such as palmitic acid (PA) or stearic acid.

While the classical CsA-sensitive PT in mitochondria is generally accepted to be due to opening of a protein pore

(Bernardi 1999; Crompton 2004; Crompton et al. 2002; Fontaine and Bernardi 1999; Forte and Bernardi 2005; Halestrap and Brennerb 2003; Halestrap, McStay and Clarke 2002; Pfeiffer et al. 2001; Zoratti and Szabo 1995; Zoratti, Szabo and De Marchi 2005), the nature of the CsA-insensitive FFA/Ca^{2+}-induced PT is not clear. This PT appears to be insensitive not only to CsA but also to other protein-targeting agents known to be modulators of classical PT (Sultan and Sokolove 2001b). At the same time, evidence has been obtained that the CsA-insensitive FFA/Ca^{2+}-induced PT results from complexation of Ca^{2+} with PA anions on the matrix side of the inner mitochondrial membrane (Sultan and Sokolove 2001b). This conclusion, which attributes CsA-insensitive FFA/Ca^{2+}-induced PT in mitochondria to the formation of PA/Ca^{2+} complexes in the membrane, is in good agreement with the earlier results of Mironova et al. (1997, 2001). They isolated a hydrophobic Ca^{2+}-binding component from the lipid fraction of the mitochondrial membranes, and this component turned out to be a mixture of PA and stearic acid (Mironova et al. 1997). It was later shown that these two acids had the highest affinity to Ca^{2+} among a large number of other fatty acids and phospholipids and formed channels in black-lipid membranes in the presence of Ca^{2+} (Mironova et al. 2001).

Earlier we performed a more detailed investigation of the mechanism of PA/Ca^{2+}-induced membrane permeabilization using the model of unilamellar liposomes loaded with the fluorescent dye sulforhodamine B (SRB) (Agafonov et al. 2003). Our experiments showed that binding of Ca^{2+} to PA-containing SRB-loaded liposomes led to the instantaneous release of a part of SRB, which was followed by restoration of membrane integrity. We seemed to deal with a transition-type process in the lipid bilayer, leading to a short break of membrane. Additional tests confirmed that the phenomenon was, indeed, not a disruption of a part of liposomes but a short loss of membrane integrity (Agafonov et al. 2003).

Since membrane permeabilization observed upon addition of Ca^{2+} to the PA-containing liposomes had a transitional character, we supposed it was due to transition in the phase state of the lipid bilayer. In the present study, this supposition was further tested with a technique based on self-quenching of the fluorescent membrane probe nonyl acridine orange (NAO).

Materials and Methods

Chemicals

NAO was purchased from Molecular Probes (Eugene, OR); all other chemicals, including azolectin and SRB, were products of Sigma-Aldrich (St. Louis, MO).

Preparation of Large Unilamellar Liposomes

Large unilamellar liposomes were obtained by a conventional extrusion technique. Dry azolectin (5 mg) was hydrated in 0.5 ml of buffer for several hours with periodic stirring in a vortex mixer. The buffer contained 10 mM Tris-HCl (pH 8.5), 40 mM KCl and 50 μM ethylenediaminetetraacetic acid (EDTA). After five cycles of freezing/thawing at −10/+30°C, the suspension of multilamellar liposomes was pressed 11 times through a 0.1-μm polycarbon membrane using an Avanti microextruder (Avanti Polar Lipids, Birmingham, AL). All operations (excluding the freezing/thawing procedure) were carried out at room temperature. Liposomes were stored at +4°C and used within 2–3 days.

Preparation of SRB-Loaded Liposomes

Large unilamellar SRB-loaded liposomes were prepared from azolectin 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 EDTA and 40 mM KCl.

Fluorescence Measurements

NAO fluorescence was measured with a Kontron spectrofluorimeter (Kontron Instruments, Milan, Italy) (excitation wavelength 495 nm, emission wavelength 519 nm, bandwidth 10 nm). SRB release from PA-containing liposomes was measured as described earlier (Agafonov et al. 2003).

In most experiments, the medium contained 10 mM Tris-HCl buffer (pH 8.5), 50 μM EDTA and 40 mM KCl. The formal concentration of liposomal lipids was 50 μM.[1] FFA and NAO were dissolved in ethanol and added to the suspension of liposomes just before measurements. The final concentrations of ethanol in the medium did not exceed 1% (v/v). After addition of an amphiphilic compound (NAO or FFA), the sample was left for several minutes to let amphiphilic molecules equilibrate between water and lipid phase. FFAs are known to incorporate into lipid

[1] In Agafonov et al. (2003), the roughly estimated molar concentration of lipid in liposome samples was reported to be 10 μM. We revised this value after determination of the average azolectin molar mass (923 ± 14 Da as assayed by the technique of Bartlett [1959]). Recalculation of the lipid molar concentration in our samples gave a value of ~50 μM. It should be stressed that in the experiments on Ca^{2+}-induced phase separation of PA we used the same azolectin preparation and weighed the same amount of lipid as in our previous experiments on PA/Ca^{2+}-induced SRB release from liposomes in order to be sure that the concentration curves of the two effects could be compared.

membranes very rapidly (Bell, Brown and Baker 1992; Bell et al. 1995, 1996; Bent and Bell 1995; Jain, Yu and Kozubek 1989), and NAO equilibrates between lipid and water phase within 2–5 min (*data not shown*). All substances added to the suspension of liposomes were checked for their own effects on the parameters measured, and only Triton X-100 used in the experiments with SRB release was found to influence dye fluorescence. Correspondingly, a corrective coefficient was introduced in the formula for calculation of SRB release (Agafonov et al. 2003).

Acoustic Measurements

The parameters of the main phase transition in the membrane of dipalmitoylphosphatidylcholine/PA (DPPC/PA) liposomes were measured with a temperature-scanning differential ultrasonic interferometer (Kharakoz and Shlyapnikova 2000). The measured quantity was the relative change in sound velocity ($\Delta u/u$) caused by the dispersed lipid of the concentration, c: $[u] = \Delta u/uc$. The reproducibility of sound velocity was 4×10^{-4}% at the temperature scan rate -0.5 K/min (cooling mode). Liposomes were obtained as described above except that (1) DPPC was used instead of azolectin, (2) the concentration of DPPC in samples was higher (4.07 mM) and (3) all operations were carried out at 45°C. PA was added to the suspension of DPPC liposomes as an ethanolic solution. The medium contained 10 mM Tris-HCl buffer (pH 8.5), 50 μM EDTA and 40 mM KCl.

Results

Concentration-Dependent Quenching of NAO Fluorescence in the Membrane of Azolectin Liposomes

NAO, an aliphatic derivative of acridine, is an amphiphilic probe which, being added to a suspension of membranes, incorporates into the lipid bilayer (Garcia Fernandez, Ceccarelli and Muscatello 2004). In cell biology applications, NAO is mostly known as a marker for cardiolipin, owing to its ability to form a specific complex with this mitochondrial lipid (Garcia Fernandez et al. 2004; Gohil et al. 2005; Mileykovskaya and Dowhan 2000; Mileykovskaya et al. 2001; Petit et al. 1992, 1994). The complex fluoresces in the far red region, at 640 nm. To avoid misunderstanding, we should emphasize that we did not use this specific feature of NAO in the present work. Here, we dealt with NAO monomers;[2] the parameters of their fluorescence are much closer to those of acridine. In Figure 1, the emission spectra of NAO in the suspension of azolectin liposomes are given; at a low NAO concentration, the emission maximum is about 520 nm, which is close to the maximum of NAO emission in methanol (Mileykovskaya et al. 2001; Wiosetek-Reske, Wysocki and Bak 2005).

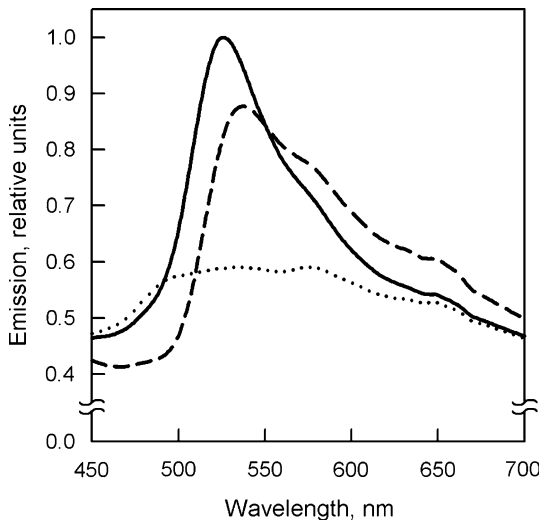

Fig. 1 Emission spectra of NAO in the membrane of azolectin unilamellar liposomes. *Dotted line*, 50 μM azolectin liposomes; *solid line*, 50 μM liposomes + 2 μM NAO; *dashed line*, 50 μM liposomes + 10 μM NAO. The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl and 50 μM EDTA

Like other acridine probes, NAO reveals a self-quenching of fluorescence (Figs. 1 and 2), which results from the association of probe molecules (Mileykovskaya et al. 2001; Murakami et al. 1986). The degree of association will depend on the probe concentration and environment. In water phase, amphiphilic molecules of NAO tend to aggregate and NAO fluorescence in the pure buffer is therefore low (Fig. 2). In the suspension of liposomes, NAO enters the liposomal membranes, leading to dissociation of probe aggregates and a corresponding increase in fluorescence intensity. However, as the concentration of NAO in the lipid phase increases, quenching of fluorescence develops (Figs. 1 and 2), which results from equilibrium shifting in the direction of NAO association.

The concentration-dependent quenching of NAO fluorescence in lipid bilayer enables one to judge changes of NAO concentration in the membrane by following the corresponding changes in fluorescence intensity. In general, NAO concentration in lipid phase may vary either because of changing the volume of this phase or due to the redistribution of NAO between water and lipid phase. The latter would occur if the NAO lipid/water partition coefficient is not high enough. However, in two-phase systems, NAO seems to be almost completely concentrated in the apolar phase (Garcia Fernandez et al. 2004). To check this, we titrated NAO with

[2] In cardiolipin-related works, NAO is usually used at quite high concentrations when almost no free NAO monomers are available in the system.

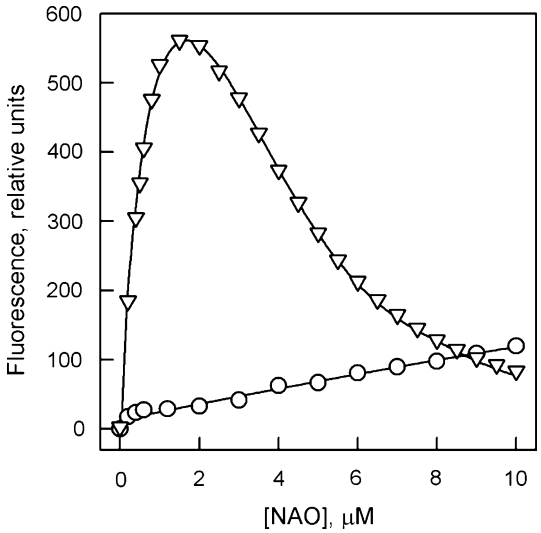

Fig. 2 Concentration dependence of NAO fluorescence in the buffer (*circles*) and in the suspension of 50 μM azolectin liposomes (*triangles*). The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl and 50 μM EDTA. Curves were obtained by titration of the corresponding samples with NAO

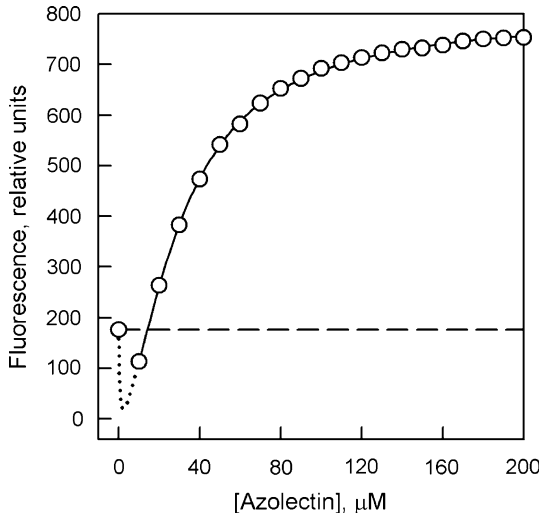

Fig. 3 Titration of 1 μM NAO with azolectin liposomes. The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl and 50 μM EDTA. *Dashed line*, level of NAO fluorescence in pure buffer; *dotted line*, approximation of the titration curve to the point of zero lipid concentration

lipid, i.e., with liposomes (Fig. 3). The shape of the titration curve (Fig. 3) indicates that even at high NAO/lipid ratios practically all NAO molecules remain in the lipid phase. Indeed, there are two extreme situations at the near-zero point of lipid concentration: (1) all NAO is in water and some NAO monomers are available (this corresponds to the [0, A] point, where A is a certain positive value) and (2) all NAO is in a very small (theoretically, near-zero) volume of lipid phase, when there are no monomers at all (the [0, 0] point). If not all NAO goes into the lipid phase, a part of it would remain in water. In this case, the level of NAO monomers must be lower compared to the first situation. This would correspond to the (0, B) point, where $0 < B < A$. The curve, however, is approximated not to such a (0, B) point but to the (0, 0) point. Thus, the right assumption is that even at low lipid concentrations practically all NAO goes into the lipid phase. In other words, NAO redistribution between water and lipid phase, even if occurs, would be too negligible to affect the overall fluorescence intensity.

Calculation of the Coefficient of NAO Fluorescence Quenching

The quenching coefficient (or quenching degree), q, a measure of NAO fluorescence quenching in the membrane, can be defined as follows:

$$q = \left(1 - \frac{F}{F_{\max}}\right).100\% \quad (1)$$

where F is the observed fluorescence at a given point of NAO and lipid concentrations and $F_{\max}$ is the estimated level of maximal (unquenched) NAO fluorescence at the same point (as it would be provided that all NAO is in the monomeric form). In this case, $F_{\max}$ would be proportional to the molar concentration of NAO in the membrane (C):[3]

$$F_{\max} = fC = f\frac{Q}{V} \quad (2)$$

where f is the molar coefficient of NAO fluorescence in the membrane, Q is the molar quantity of NAO in the system and V is the membrane volume. Instead of V, we can use a parameter of N, the number of aliphatic chains in the lipid bilayer (V is proportional to N):

$$F_{\max} = f'\frac{Q}{N} \quad (3)$$

where f' can be defined as an apparent molar coefficient of NAO fluorescence in the membrane.

Combining (1) and (3), we get the final equation:

$$q = \left(1 - \frac{F}{f'}.\frac{N}{Q}\right).100\% \quad (4)$$

This equation allows us to calculate q. The value of f' can be estimated from the initial, linear part of the curve in Figure 2. Under our conditions, f' was equal to 93,300.

[3] We assume that practically all NAO molecules are incorporated in the liposomal membranes and no redistribution of NAO between lipid and water phase occurs; see arguments above.

Changes in NAO Fluorescence upon Addition of PA to Azolectin Liposomes

As mentioned above, addition of ethanolic solutions of PA to the suspension of liposomes will result in rapid incorporation of PA molecules in the liposomal membranes (Bell et al. 1992, 1995, 1996; Bent and Bell 1995; Jain et al. 1989). Presumably, that should cause fluorescence to rise since the concentration of NAO in lipid (and correspondingly the degree of fluorescence quenching) decreases.

The dependence of NAO fluorescence on the concentration of PA added to the suspension of liposomes is given in Figure 4. Looking at the figure, we can state the following: (1) up to a certain point, the addition of PA to liposomes results in an increase of fluorescence; (2) after this point (at PA/lipid molar ratio about 1:2), the curve deviates from the expected pattern. Instead of increasing and gradually reaching a plateau, fluorescence decreases a little and then levels off.

The maximum at the PA/lipid ratio of 1:2 probably indicates the beginning of a transition process in the lipid bilayer. Apparently, this is phase separation of a part of PA. The fluorescence changes that follow this turning point can be understood if we assume that NAO remains in the lipid domains after phase separation. Indeed, the emerging PA phase must have a rather tight, highly ordered packing. This implies that any impurities (including NAO) would be excluded from these ordered domains. So NAO should be forced out of the PA phase into the lipid domains, accompanied by a corresponding quenching of fluorescence. Similar techniques for detection of phase separation, which are based on self-quenching of membrane probes, were used earlier (Hoekstra 1982a, b; MacDonald 1990).

To be sure that NAO fluorescence changes reflect only alterations of NAO concentration in the lipid phase, we compared *q vs. N/Q* plots for pure azolectin and azolectin/PA liposomes (*q* was calculated using Eq. 4). The parameter *N/Q* is an inverse measure of NAO concentration in the lipid phase, and *q vs. N/Q* plots will therefore show how much fluorescence quenching is determined by NAO concentration. As can be seen in Figure 5, points calculated from the data in Figure 4 (i.e., points obtained for azolectin/PA liposomes) are superimposed on the curve constructed from the data of Figure 2 (i.e., points obtained for pure azolectin liposomes). Hence, the level of NAO fluorescence seems to depend only on the concentration of NAO in the membrane, without regard to its lipid composition and related factors.

Using NAO to Detect the Ca^{2+}-Induced Phase Separation in PA-Containing Azolectin Liposomes

Our next step was to check the possibility of phase separation in azolectin/PA liposomes being induced by Ca^{2+}. It seemed reasonable that binding of Ca^{2+} to PA anions would lead to segregation of PA/Ca^{2+} complexes. On the one hand, Ca^{2+} has long been known to cause phase separation in mixtures with negatively charged lipids (Jacobson and Papahadjopoulos 1975; Tamura-Lis et al. 1986);

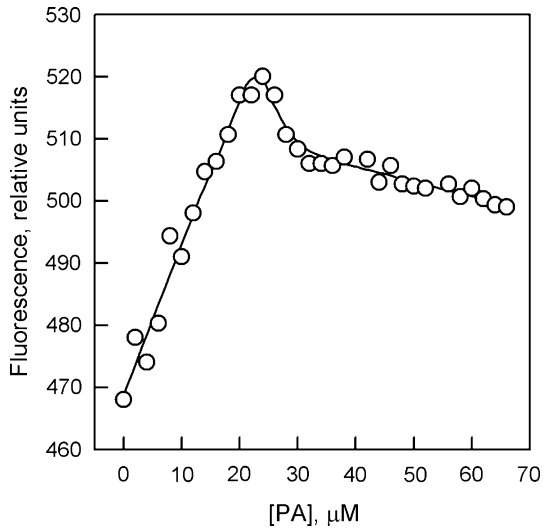

Fig. 4 NAO fluorescence changes upon addition of PA to azolectin liposomes. The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl and 50 μM EDTA. Samples, containing 50 μM liposomes, were supplemented with 2 μM NAO and various concentrations of PA, followed by measurement of fluorescence intensity

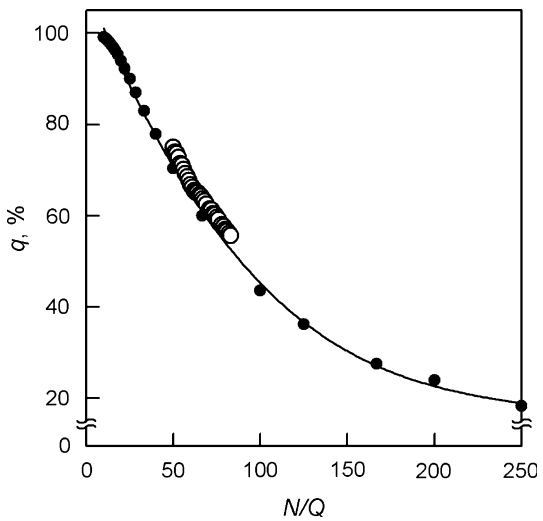

Fig. 5 Dependence of *q* (coefficient of NAO fluorescence quenching) on the ratio of *N* (number of hydrocarbon chains in the lipid matrix, mol) to *Q* (quantity of NAO in the system, mol). *Filled circles*, curve constructed from the data of Figure 2 (from the curve of NAO fluorescence in liposomes); *open circles*, points calculated from the data of Figure 4

on the other hand, the ability of Ca^{2+} to bind tightly to PA was demonstrated earlier in our laboratory (Mironova et al. 2001).

Figure 6 shows traces of NAO fluorescence changes in the suspension of azolectin or azolectin/PA liposomes after addition of Ca^{2+}. In the pure azolectin liposomes, Ca^{2+} did not alter fluorescence intensity, but fluorescence dropped when Ca^{2+} was added to PA-containing liposomes. Presumably, this drop reflects separation of PA/Ca^{2+} complexes in distinct membrane domains, the mechanism of NAO fluorescence quenching being the same as described above. However, the data of Figure 6 raise a question: Why does fluorescence fall below the initial (prior to the addition of Ca^{2+}) level? An answer may be that not only PA but also a part of the lipid is segregated. PA might form a compound with some lipids of the azolectin preparation, as it does with DPPC (Cevc et al. 1988; Inoue et al. 2001; Koynova et al. 1988; Schullery et al. 1981). The separating PA/Ca^{2+} phase could contain this compound.

Detection of Ca^{2+}-Induced Phase Separation in DPPC/PA Liposomes With Ultrasonic Interferometry

The ability of Ca^{2+} to induce phase separation of PA in the lipid bilayer was also tested with the ultrasonic interferometry approach. This approach allows one to measure parameters of the thermotropic phase transition in a lipid system. Azolectin could not be used in these studies because, being a complex mixture of natural lipids, it has no discernible thermotropic phase transition. We used DPPC instead, which is a well-studied model phospholipid widely used in phase transition experiments.

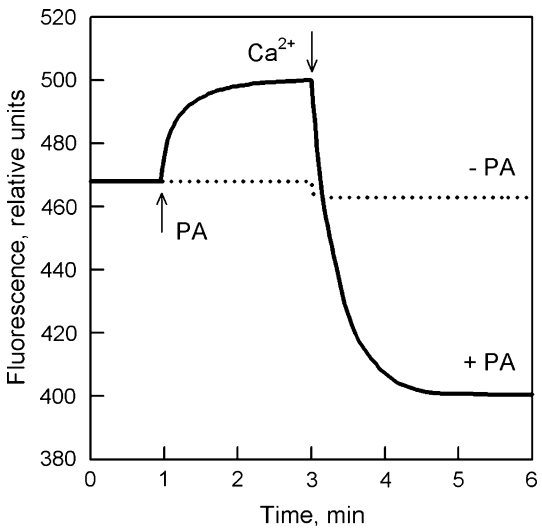

Fig. 6 Quenching of NAO fluorescence in the membrane of PA-containing azolectin liposomes after addition of Ca^{2+}. The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl and 50 μM EDTA. *Solid line*, a sample of 50 μM liposomes was supplemented with 2 μM NAO and 50 μM PA, followed by addition of 0.5 mM Ca^{2+}; *dotted line*, the same but without PA

Figure 7A shows the temperature dependence of ultrasonic velocity in the suspension of DPPC/PA liposomes at different PA contents and Ca^{2+} concentrations. The S-shaped region of the temperature curves represents the main (thermotropic) phase transition. Added to DPPC liposomes, PA increased the temperature of transition and expanded its range, corresponding to the literature data (Cevc et al. 1988; Inoue et al. 2001; Koynova et al. 1988; Ortiz and Gomez-Fernandez 1987; Schullery et al. 1981). These effects are more visible on the phase diagram (Fig. 7B), where the liquidus and solidus points at different PA contents are shown.

Addition of Ca^{2+} to PA-containing DPPC liposomes abolished changes induced by PA. The shape of the curves is restored to that observed for pure DPPC liposomes (Fig. 8A): liquidus and solidus points shifted back, close to the position where they were before the addition of PA (Fig. 7B). In other words, Ca^{2+} causes emergence of a pure DPPC phase, which is evidently a result of phase separation of the initial DPPC/PA mixture. We can only observe liquid/gel transitions in this DPPC phase; the PA/Ca^{2+} phase cannot be seen because it seems to melt at a temperature, which is higher than the upper working limit of our device.

Comparing the Effects of Ca^{2+}-Induced Phase Separation of PA and PA/Ca^{2+}-Induced Membrane Permeabilization

Earlier we found that Ca^{2+} induced permeabilization of PA-containing liposomal membranes (Agafonov et al. 2003). Here, we compared these two effects, phase separation and membrane permeabilization, examined under similar conditions. As a measure of phase separation, we calculated Δq, the difference between the levels of NAO fluorescence quenching before and after addition of Ca^{2+}. The measure of membrane permeabilization was the amount of SRB released after addition of Ca^{2+} (in relation to the total SRB entrapped in liposomes).

Figure 8 shows the dependence of the two effects on the concentration of PA and Ca^{2+}. It can be seen that NAO fluorescence changes develop in the same PA/Ca^{2+} concentration range as those of SRB fluorescence. The concentration curves of the effects (expressed as percent of maximum) are close to each other.

Our previous experiments showed that PA/Ca^{2+}-induced SRB release occurred at pH > 7.0 (Agafonov et al. 2003). The same is true for the Ca^{2+}-induced effect in NAO-labeled, PA-containing liposomes (Fig. 9). It, however, should be noted that the pH optimum of phase separation is shifted to higher pH values compared to the optimum of

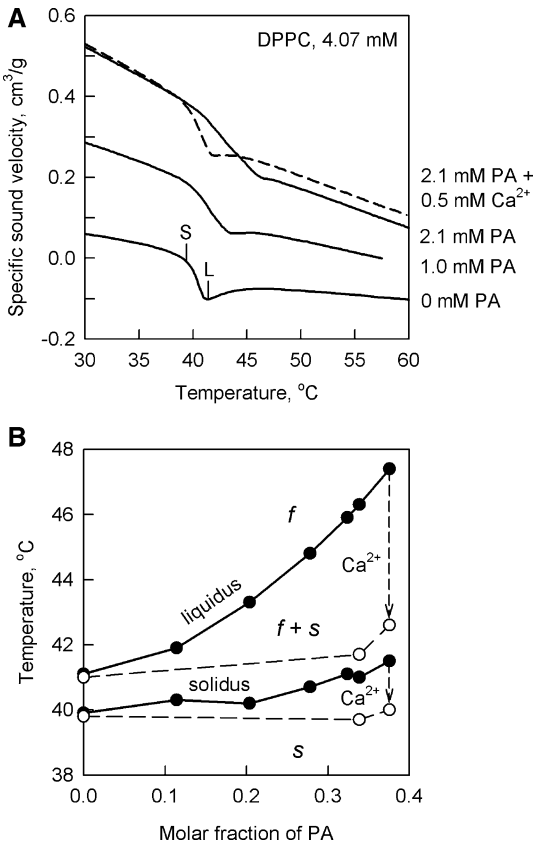

Fig. 7 Effect of Ca^{2+} on phase behavior of DPPC/PA mixture. The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl and 50 μM EDTA. (*A*) Temperature curves of specific sound velocity at different contents of PA in the system (specific sound velocity is defined as $[u - u_o]/u_o c$, where c is the specific lipid concentration and u and u_o are the sound velocities in the suspension of liposomes and buffer, respectively). The points of solidus (*S*) and liquidus (*L*) are defined as the points of maximal curvature. The difference between curves along the ordinate is due to the different concentrations of ethanol, which was introduced into the suspension with PA (at the concentrations used, ethanol enhances the sound velocity but does not affect the character of phase transition). (*B*) A fragment of the phase diagram constructed from the results of acoustic measurements in the suspension of DPPC/PA liposomes in the absence (*filled circles*) or presence (*open circles*) of Ca^{2+}. *Dashed arrows* indicate changes caused by addition of 0.5 mM Ca^{2+}. Letters *f* and *s* denote the fluid and solid states of lipid in the Ca^{2+}-free medium. The molar part of PA is defined as [PA]/([PA] + [DPPC])

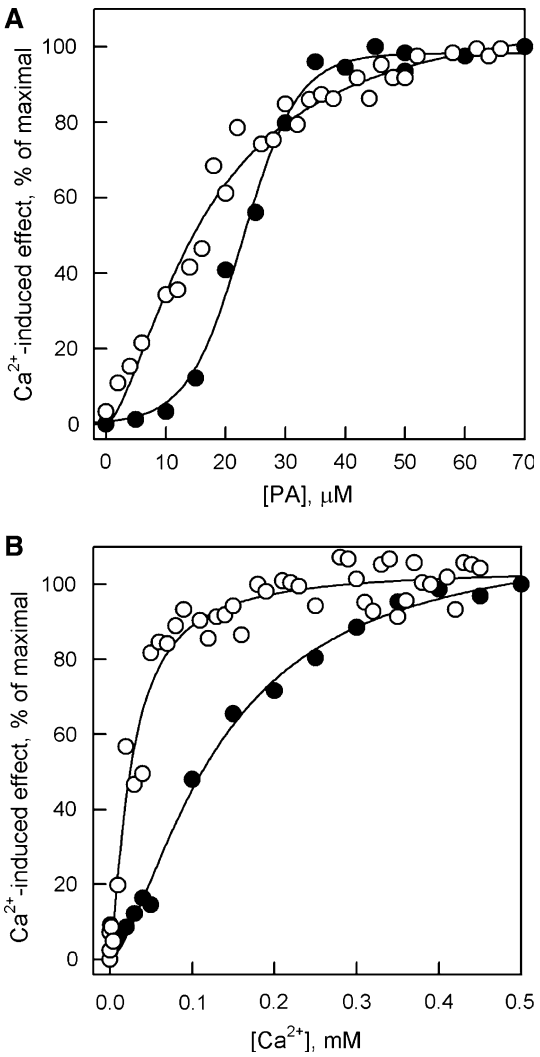

Fig. 8 Concentration curves of two Ca^{2+}-induced effects: phase separation of PA in the lipid bilayer (*open circles*) and permeabilization of PA-containing liposomal membranes (*filled circles*). The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl, 50 μM EDTA (in case of phase separation) or EGTA (in case of membrane permeabilization) and 50 μM azolectin liposomes (either containing 2 μM NAO or loaded with 50 mM SRB). (*A*) Dependence of the effects on PA concentration (concentration of Ca^{2+} was 0.5 mM in case of phase separation and 1 mM in case of membrane permeabilization). (*B*) Dependence of the effects on the concentration of Ca^{2+} (PA concentration was 50 μM)

membrane permeabilization. Anyway, the pH profiles of both these effects evidently depend on the pH profile of PA/Ca^{2+} complexation: the latter was also shown to occur in the alkaline pH range (Mironova et al. 2001).

We demonstrated earlier that the release of SRB from PA-containing liposomes can be induced not only by Ca^{2+} but also by Ba^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+} and Sr^{2+}, with the effect slightly diminishing in this cation series but being large enough (Mironova et al. 2001). Mg^{2+} was also able to cause membrane permeabilization; however, its effect (at the saturating concentration of 1 mM) was less than one-third that of Ca^{2+}. Here, we present analogous data on the ability of divalent cations to induce phase separation of PA in liposomes (Table 1). The table does not contain data on the effect of Ni^{2+} and Co^{2+}, which were found to affect fluorescence of NAO itself and excluded from further consideration. As for Mn^{2+}, Ba^{2+} and Sr^{2+}, they turned out to be quite effective inducers of phase separation. Again, the effect of Mg^{2+} was sufficiently lower than that of other divalent cations.

Long-chain saturated FFAs (PA, stearic acid) were found to have much higher affinity to Ca^{2+} than other FFAs (unsaturated and saturated with shorter or longer chain

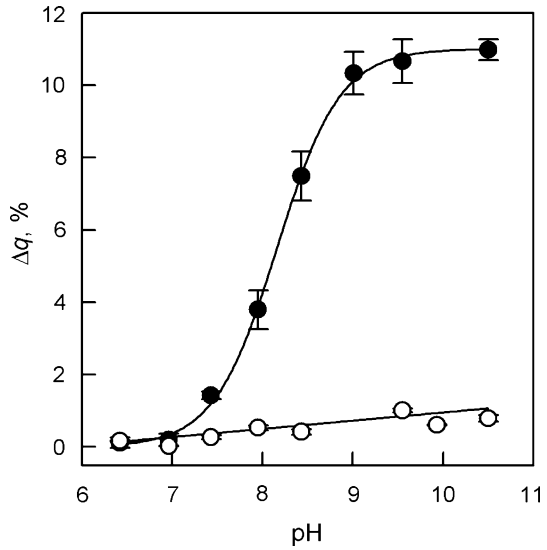

Fig. 9 The pH profile of Ca^{2+}-induced phase separation of PA in the membrane of azolectin liposomes. The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl and 50 μM EDTA. *Open circles*, 50 μM liposomes without PA; *filled circles*, 50 μM liposomes + 50 μM PA

Table 1 Ability of some divalent cations to induce phase separation of PA in the membrane of azolectin liposomes

Cation	Δq (%)
Ca^{2+}	1.54 ± 0.18
Mn^{2+}	1.46 ± 0.18
Ba^{2+}	1.11 ± 0.16
Sr^{2+}	0.76 ± 0.18
Mg^{2+}	0.52 ± 0.09

The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl and 50 μM EDTA. Phase separation was triggered by addition of a cation (0.1 mM) to the 50 μM azolectin liposomes labeled with 2 μM NAO and containing 50 μM PA

lengths) (Mironova et al. 2001). Something similar was observed in the case of cyclosporin-insensitive FFA/Ca^{2+}-induced PT in mitochondria (Sultan and Sokolove 2001a). The PT occurs only in the presence of saturated FFA, and its dependence on the length of FFA tails has a distinct bell-shaped pattern, with a peak at 14 carbon atoms. Studying PA/Ca^{2+}-induced SRB release from liposomes, we tried other FFAs, stearic and palmitoleic, as substitutes for PA (Agafonov et al. 2003). As expected, the effect of stearic acid was similar to that of PA, whereas palmitoleic acid was ineffective.

In the experiments on Ca^{2+}-induced phase separation, several FFAs were tested (Table 2). These experiments revealed the same regularity: the effect depended on FFA length. What was different is that unsaturated FFAs segregated after addition of Ca^{2+} too, although the effect was

Table 2 Ca^{2+}-induced phase separation of some fatty acids in the membrane of azolectin liposomes

FFA (50 μM)	Δq (%)
Lauric (12:0)	1.90 ± 0.22
Myristic (14:0)	3.41 ± 0.27
Palmitic (16:0)	6.40 ± 0.58
Stearic (18:0)	4.33 ± 0.74
Eicosanoic (20:0)	4.74 ± 0.38
Linoleic (18:2)	3.71 ± 0.13
Palmitoleic (16:1)	2.57 ± 0.27

The medium contained 10 mM Tris-HCl buffer (pH 8.5), 40 mM KCl and 50 μM EDTA. Phase separation was triggered by addition of Ca^{2+} (0.5 mM) to the 50 μM azolectin liposomes labeled with 2 μM NAO and containing 50 μM fatty acid

rather moderate. It is possible, however, that the Ca^{2+}-induced separation of unsaturated FFAs is related to their peroxidation.

Discussion

Using NAO to Detect Phase Separation of PA in the Membrane of Azolectin Liposomes

The results of our study show that in lipid membranes NAO fluorescence is quenched in a concentration-dependent manner. Hence, one can use NAO to register changes in membrane volume or, if the membrane has a domain structure, in the volume of the probe-containing domains. This means that in particular the probe can be used to detect phase separation in multicomponent lipid membranes. Indeed, if NAO is initially distributed all over the multicomponent membrane and after phase separation it is concentrated in one of the new phases, fluorescence quenching would increase. Following this principle, we measured phase separation in azolectin/PA liposomal membranes. The experiments revealed that, first, phase separation occurred upon the concentration of PA in lipid reaching a certain threshold and, second, it could be induced by Ca^{2+}.

Although NAO is mostly known as a marker for cardiolipin (Garcia Fernandez et al. 2004; Gohil et al. 2005; Mileykovskaya and Dowhan 2000; Mileykovskaya et al. 2001; Petit et al. 1992, 1994), the field of its use need not be restricted to this specific application. NAO seems to be quite suitable as a self-quenched probe when the matter concerns model membranes. In artificial lipid bilayers, the interpretation of NAO fluorescence changes is not as complicated as it is in biological membranes, where NAO can specifically interact with certain lipids or proteins. In

addition, some features of NAO facilitate its use as a self-quenched membrane probe. First, acridine fluorophore has rather high molar absorption and emission coefficients. Second, the maxima of NAO absorption and emission spectra (495 and 519 nm, respectively) are close to each other, increasing the efficiency of fluorescence self-quenching. Third, NAO's nonyl tail guarantees the probe to have a high lipid/water partition coefficient. At the same time, NAO is not so hydrophobic as to create problems with its addition into aqueous media. It is soluble in ethanol and, being added to the suspension of liposomes, will be distributed between water and lipid within a few minutes.

A Possible Relation between Phase Separation and Membrane Permeabilization Caused by Ca^{2+} in PA-Containing Liposomes

The results of comparing the two Ca^{2+}-induced effects, phase separation and membrane permeabilization, indicate that they are certainly related to each other; but this does not seem to be a simple cause-effect relation. There are overall correlations between the two effects, yet also there are differences. It is obvious that both phase separation and membrane permeabilization are associated with the formation of PA/Ca^{2+} complexes; this is the foundation for correlations between these phenomena. The question, however, is whether phase separation can play any role in the induction of membrane permeabilization.

First of all, it should be noted that only the earliest stage of phase separation could have a causal relation to the loss of membrane integrity. This follows from comparing the dynamics of the two processes. Membrane permeabilization occurs within fractions of a second[4] (Agafonov et al. 2003), whereas phase separation develops within minutes (Fig. 6).

Phase separation in a two-component lipid system will begin with the formation of nuclei of a new phase, and only then do these nuclei grow in size and fuse, giving rise to larger domains. The formation of small nuclei at the first stage of phase separation was, e.g., visualized by atomic force microscopy upon the activation of phospholipase A_2 on the supported phospholipid bilayers (Nielsen et al. 1999, 2002). In our case, the addition of quite concentrated Ca^{2+} to PA-containing liposomes resulted in the instantaneous formation of a large number of PA/Ca^{2+} complexes all over the liposomal surface. These complexes would segregate and form nuclei of the PA/Ca^{2+} phase.

The thesis that membrane permeabilization relates to the first (nucleation) stage of phase separation allows us to explain differences observed between the two processes in our experiments. Indeed, the method we used to detect phase separation ''senses'' mainly the late stages of the process. The extent of NAO fluorescence quenching would depend on the average distance between probe molecules floating in the lipid phase. Thus, NAO would begin to concentrate only after the domain of the emerging NAO-depleted phase has grown larger than this average distance. The parameter Δq is therefore a measure of the final result of phase separation, the state when separation is completed. However, this final result may not be coupled to characteristics of the nucleation stage. For example, the kinetics of the separation process in this first stage can vary without any effect on the equilibrium parameters of the final state. Besides, there might be a situation when equilibrium will be reached early, well before the domains of the new phase have grown large.

What happens at the initial stage of phase separation that could cause the membrane to break? We suppose it gets permeabilized because of an abrupt change in the packing order of hydrocarbon tails in the outer membrane leaflet, which would occur at the initial moment of phase separation. The packing order must change considerably since Ca^{2+} would induce not only phase separation of PA but also the liquid-to-gel phase transition in the PA domains. The melting temperature of pure PA is about 63°C, and Ca^{2+} would only shift it to a higher value (Cevc et al. 1988; Inoue et al. 2001; Koynova et al. 1988; Ortiz and Gomez-Fernandez 1987; Schullery et al. 1981). This also explains differences between the effects of saturated and unsaturated FFAs. Unsaturated FFAs seem to segregate upon addition of Ca^{2+} (Table 2), but this is not accompanied by membrane permeabilization (Agafonov et al. 2003). Since unsaturated FFAs usually melt at temperatures below 0°C, their domains should not solidify under our conditions. This indicates that liquid-to-gel transition is an important step in the mechanism of FFA/Ca^{2+}-induced membrane permeabilization. The whole mechanism can therefore be described in terms of a more general theory, which considers formation of lipid pores upon phase transition in the lipid bilayer (Antonov and Shevchenko 1995). As applied to our case, the mechanism will look as follows (Fig. 10).

1. The separation of PA/Ca^{2+} domains in the outer monolayer of the liposomal membranes is accompanied by their transition from the liquid-crystalline to gel phase (a so-called chemotropic phase transition, CPT).
2. The solidification of PA/Ca^{2+} domains results in the reduction of their area due to a tighter packing of fatty acid tails. As PA/Ca^{2+} domains are mainly formed in the outer membrane leaflet, the outer monolayer

[4] We cannot even say what is the characteristic time of membrane permeabilization because its kinetics is beyond the time resolution of the spectrofluorimeter used.

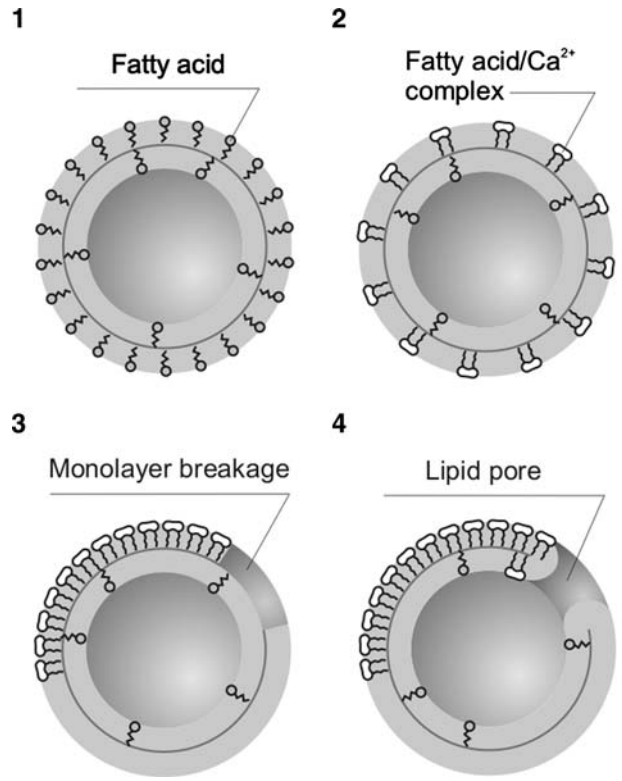

Fig. 10 Scheme for the mechanism of PA/Ca^{2+}-induced membrane permeabilization. It is suggested that binding of Ca^{2+} to PA anions in the outer monolayer of liposomal membranes results in the separation PA/Ca^{2+} complexes into domains of the solid PA/Ca^{2+} phase. This process leads to unbalance of lateral pressure/tension forces on different membrane sides, which is resolved by the breakage of the outer monolayer. After that, the inner monolayer also breaks, and a hydrophilic lipid pore appears, which then rapidly tightens

stretches out, whereas the inner one becomes condensed. The membrane turns into a stressed state.

3. This stressed state, resulting from unbalance of lateral pressure/tension forces in the inner and outer monolayers, is resolved by the emergence of gaps in the outer membrane leaflet. Following this, the inner leaflet also breaks in the region of gaps. Monolayers fuse, enclosing hydrophobic gap edges, and a hydrophilic lipid pore appears. The pore then tightens and closes, which was shown to be energetically favorable (Antonov and Shevchenko 1995).

Thus, the key factor in the mechanism of the CPT-based membrane permeabilization is the unbalance of lateral pressure/tension forces on different sides of the membrane. This unbalance would be maximal at the initial moment of phase separation, a moment of massive association of PA/Ca^{2+} complexes into nuclei of the new PA/Ca^{2+} solid phase. At that moment, the unbalance will grow too fast to be resolved smoothly and, upon reaching a certain critical point, will trigger membrane permeabilization.

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