ELSEVIER

Biochimica et Biophysica Acta 1509 (2000) 373–384

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.elsevier.com/locate/bba

Stabilization of O-pyromellitylgramicidin channels in bilayer lipid membranes through electrostatic interaction with polylysines of different chain lengths

Andrey V. Krylov [a,b], Elena A. Kotova [a], Alexander A. Yaroslavov [b], Yuri N. Antonenko [a,*]

[a] *A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russia*
[b] *Department of Polymer Sciences, School of Chemistry, Moscow State University, Moscow 119899, Russia*

Received 5 June 2000; received in revised form 17 August 2000; accepted 22 August 2000

Abstract

Functioning of membrane proteins, in particular ionic channels, can be modulated by alteration of their arrangement in membranes. We addressed this issue by studying the effect of different chain length polylysines on the kinetics of ionic channels formed in a bilayer lipid membrane (BLM) by O-pyromellitylgramicidin carrying three negative charges at the C-terminus. The method of sensitized photoinactivation was applied to the analysis of the channel association–dissociation kinetics (characterized by the exponential factor of the curve describing the time course of the flash-induced decrease in the transmembrane current, τ). Addition of polylysine to the bathing solutions of BLM led to the deceleration of the photoinactivation kinetics, i.e. to the increase in τ. It was shown here that for a series of polylysines differing in their chain lengths, the value of τ grew as their concentration increased above a threshold level until at a certain concentration of each polylysine τ reached maximum. At higher polylysine concentrations τ began to decrease and finally became close to the control level observed in the absence of polylysine. With lengthening of the polylysine chain the maximum value of τ increased, the concentration dependence became steeper, and the threshold concentration decreased. The increase in the ionic strength of the medium shifted the concentration dependence of τ to higher polylysine concentrations and decreased the maximum value of τ. It was concluded that the increase in τ was caused by the formation of domains of O-pyromellitylgramicidin molecules induced by binding of polylysines. This can be related to functional aspects of polycation-induced sequestering of negatively charged transmembrane peptides in neutral membranes.

Keywords: Planar bilayer lipid membrane; Domain formation; Polyelectrolyte; Electrostatic interaction; Gramicidin channel; Reactive oxygen species; Photodynamic action

1. Introduction

Recently the concept of recruitment of lipid-anchored proteins into lipid microdomains, called lipid rafts, has been considered as the basis for sorting in polarized cells and signal transduction [1–6]. Although the existence of phase-separated regions

Abbreviations: DPhPC, diphytanoylphosphatidylcholine; DPhPG, diphytanoylphosphatidylglycerol; gA, gramicidin A; OPg, O-pyromellitylgramicidin; OSg, O-succinylgramicidin; $AlPcS_3$, aluminium trisulfophthalocyanine; BLM, bilayer lipid membrane; PLL, poly-L-lysine

* Corresponding author. Fax: +70-95-939-3181; E-mail: antonen@genebee.msu.su

0005-2736/00/$ – see front matter
PII: S0005-2736(00)00320-5

(lipid microdomains) has been demonstrated in artificial membranes by using diverse biophysical techniques [7–10], the process of segregation of integral membrane proteins, in particular, of ionic channels, has not been studied in detail in model membranes.

According to the literature [11–14], clustering of ionic channels in biological membranes is of great importance for their functioning. A study of model channels incorporated into BLM can be useful for elucidation of the mechanisms of channel clustering. The pentadecapeptide gramicidin A (gA) is one of the best studied channel formers [15–18] with a simple structure and many derivatives that are used for different research purposes [19]. In particular, O-pyromellitylgramicidin (OPg), a gramicidin analogue bearing three negative charges at its C-terminus [20,21], has been used in our previous paper [22] to study the effect of a basic polypeptide, polylysine, on the association–dissociation channel kinetics using the sensitized photoinactivation method. This method developed in our recent papers [23,24] is based on the fact that a flash-induced transient of the gramicidin-mediated current through BLM observed in the presence of a photosensitizer reflects the process of equilibration of channel assembly and dissociation reactions after a sudden distortion of the equilibrium as a result of damage to gramicidin molecules by reactive oxygen species generated in the presence of the photosensitizer. As shown previously [22], polylysine exerts a dramatic deceleration of the photoinactivation kinetics of OPg channels. It has been supposed that the effect of polylysine is associated with clustering of OPg. Here, a comparison of the effects of polylysines differing in chain lengths is shown as evidence in favor of polylysine-induced segregation of lateral domains enriched in negatively charged OPg molecules. This process was shown to determine the kinetic behavior of gramicidin channels in the system under study.

The present work is relevant to studies of the influence of charged peptide motifs on the structure of lipid membranes, resulting, in particular, in segregation of charged lipids into domains [25–31]. As shown in the literature, domain formation in membranes can be induced by binding of a series of physiologically active peptides and proteins, e.g. prothrombin and blood coagulation factors [32,33], cytochrome *c* [34–37], myelin basic protein [38–40], annexins [41–44], protein kinase C [45–47], the basic peptide corresponding to the effector region of the myristoylated alanine-rich C kinase substrate (MARCKS) [30,48–50], histones [50–52], human C-reactive protein (C1q) [53], the C2 domain of synaptotagmin [54], cardiotoxin [55,56], the peptide fragment of the envelope glycoprotein of human immunodeficiency virus type I [57], the envelope-associated proteins of vesicular stomatitis virus [58,59] the v-Src-oncoprotein [30], polymyxin B [60–64], the cationic lipopeptide analogue of bacterial lipoproteins [65], and the signal sequence of the bacterial protein LamB [66]. A large body of evidence has shown that interaction of synthetic charged polymers, in particular synthetic polyelectrolytes, with model membranes containing oppositely charged lipids causes their lateral phase separation [48,53,67–81].

On the other hand, it is evident that in biological membranes local concentration and/or aggregation of integral membrane proteins exposing their charged residues on the membrane surface can be involved in the processes of domain formation which is triggered by interaction with water-soluble charged peptides. In fact, such polycations as polylysines were shown to induce membrane protein clustering in biological membranes [82–85]. The present study of polylysine-induced formation of gramicidin channel clusters in bilayer lipid membranes is aimed at elucidation of the mechanism of clustering of integral membrane proteins, in particular, ion channels, in cellular membranes occurring upon binding of charged species.

2. Materials and methods

BLMs were formed from a solution of 2% diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, Alabaster, AL, USA) or a mixture with diphytanoylphosphatidylglycerol (DPhPG, Avanti Polar Lipids, Alabaster, AL, USA) in *n*-decane (Merck, Darmstadt, Germany) by the brush technique [86] on a 0.55-mm diameter hole in a Teflon partition separating two compartments of a cell containing aqueous solutions of 100 mM (unless otherwise stated) KCl (Fluka, Buchs, Switzerland), 10 mM MES (Sigma, St. Louis, MO, USA), 10 mM Tris (Sigma, St. Louis, MO, USA) and 0.05 mM EDTA at pH 7.0.

OPg chemically synthesized as in [21] and O-succinyl-gramicidin (OSg) (both were generous gifts of Prof. R.E. Koeppe II, University of Arkansas) were added from stock solutions in ethanol to the bathing solutions at both sides of the BLM and routinely incubated for 15 min with constant stirring. Poly-L-lysine (PLL) hydrobromides with molecular weights equal to 3800 (degree of polymerization (DP) = 18, PLL_{18}); 12 000 (DP = 60, PLL_{60}); 21 500 (DP = 100, PLL_{100}); 50 000 (DP = 240, PLL_{240}) or 100 000 (DP = 480, PLL_{480}) (Sigma, St. Louis, MO, USA) were added to both compartments of the cell. Experiments were carried out at room temperature (20–22°C). Aluminium trisulfophthalocyanine ($AlPcS_3$) was from Porphyrin Products, Logan, UT, USA. $AlPcS_3$ was added to the bathing solution at the trans-side (the cis-side is the front side with respect to the flash lamp). The electric current (I) was recorded under voltage-clamp conditions. The currents were measured by means of a U5-11 amplifier (Moscow, Russia), digitized by using a DT2814 (Data Translation, Marlboro, MA, USA) and analyzed using a personal computer. Ag–AgCl electrodes were placed directly into the cell and a voltage of 30 mV was applied to the BLM. BLMs were illuminated by single flashes produced by a xenon lamp with flash energy of about 400 mJ/cm^2 and flash duration <2 ms. A glass filter cutting off light with wavelengths <500 nm was placed in front of the flash lamp. To avoid electrical artifacts the electrodes are covered by black plastic tubes.

Typical current traces after a flash of light have two components with characteristic times of <10 ms and about 1 s [23,87]. The fast phase which normally comprises less than 10% of the photoresponse apparently reflects inactivation of conducting dimers, while the main slow phase corresponds to the monomer–dimer equilibration after damage to gramicidin monomers (see scheme in Fig. 1). We believe that the process of the gramicidin damage includes generation of reactive oxygen species (ROS) due to interaction of excited photosensitizer molecules with oxygen and the attack by ROS of tryptophan residues in gramicidin molecules [88]. The lifetime of ROS is less than 10 μs [89–91], and the time for gramicidin damage [92] is substantially less than the characteristic time of the major photoinactivation component which is below the characteristic time of photoinactivation τ (about 1 s). Therefore, the current decay represents the relaxation after a concentration jump.

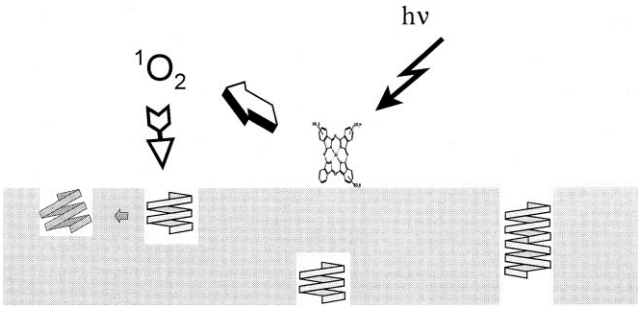

Fig. 1. A scheme for gramicidin photoinactivation in a lipid membrane in the presence of a photosensitizer. The process of gramicidin photodamage includes generation of reactive oxygen species ($^1O_2$) after excitation of the photosensitizer with a flash of visible light (hν) followed by their attack on tryptophan residues in gramicidin.

According to our experimental data τ appears to be close to the gramicidin channel lifetime under a variety of conditions and exhibits similar changes. For example, both the characteristic time of photoinactivation and the single channel lifetime decrease upon increasing the temperature or substituting squalane for decane in the membrane-forming solution [23]. These parameters also display similar changes in response to variations of the membrane dipole potential by phloretin or ketocholestanol [24,93].

3. Results

It is generally accepted that the transmembrane dimer formed by head-to-head association of monomers represents the channel state of gramicidin [94]. Recently a new method [23] based on the phenomenon of sensitized photoinactivation [88,95,96] has been developed which allows one to examine the kinetics of formation and disappearance of gramicidin channels, i.e. the kinetics of gramicidin dimerization and dissociation, respectively [23]. This method comprises the analysis of the time course of the decrease in the gramicidin-mediated current through BLM induced by a light flash in the presence of a photosensitizer. The decrease in current has been shown to follow a monoexponential decay with an exponential factor (τ, the characteristic time of photoinacti-

vation) being in good correlation with the single channel lifetime of gramicidin under different conditions [23].

As found by Krylov and coworkers [22], the addition of polylysine causes an increase in τ, i.e. the deceleration of the OPg channel kinetics, whereas the kinetics of uncharged gA remains unaltered by polylysine. In the present work continuing the study of the polylysine effect on the photoinactivation kinetics, all the experiments were performed with OPg. Fig. 2 illustrates the typical time courses of the decrease in the OPg-mediated transmembrane current after a flash (at zero time) in the presence of phthalocyanine for two PLLs with molecular weights of 12 000 (PLL_{60}) and 50 000 (PLL_{240}) at 18 nM and 4 nM, respectively, that is at nearly equal concentrations of lysine residues (1 μM). In the rest of the paper PLL concentrations are given as the concentration of lysine residues which is equal to the concentration of the polymer chains multiplied by the degree of polymerization (DP), and PLL index in subscript corresponds to the DP of the PLL. It is seen that PLL_{240} brings about a considerable deceleration of the photoinactivation kinetics in contrast to PLL_{60}, which is practically inactive at this concentration. It should be noted that in Fig. 2A the top time scale corresponds to the kinetics of PLL_{240} (curve 2), whereas the bottom time scale corresponds to the kinetics of the control (curve 1) and PLL_{60} (curve 3). In Fig. 2B the kinetic curves are presented in the semilogarithmic plot, which improves their comparison, because the slope of the logarithm of a monoexponential decay curve is proportional to the reciprocal characteristic time.

It is seen from Fig. 2A that PLL_{240} not only slows down the kinetics of photoinactivation, but also decreases its relative amplitude. This effect is produced also by PLL_{60} if added at higher concentrations (data not shown). As it was discussed in our previous paper [22], the reduction of the photoinactivation amplitude can hardly be accounted for by quenching of reactive oxygen species by polylysines, but rather is associated with interaction between polylysines and negatively charged photosensitizer molecules. As it has been shown by Rokitskaya et al. [23], the characteristic time and the amplitude of photoinactivation represent two independent parameters; for instance, variation of the photosensitizer concentration

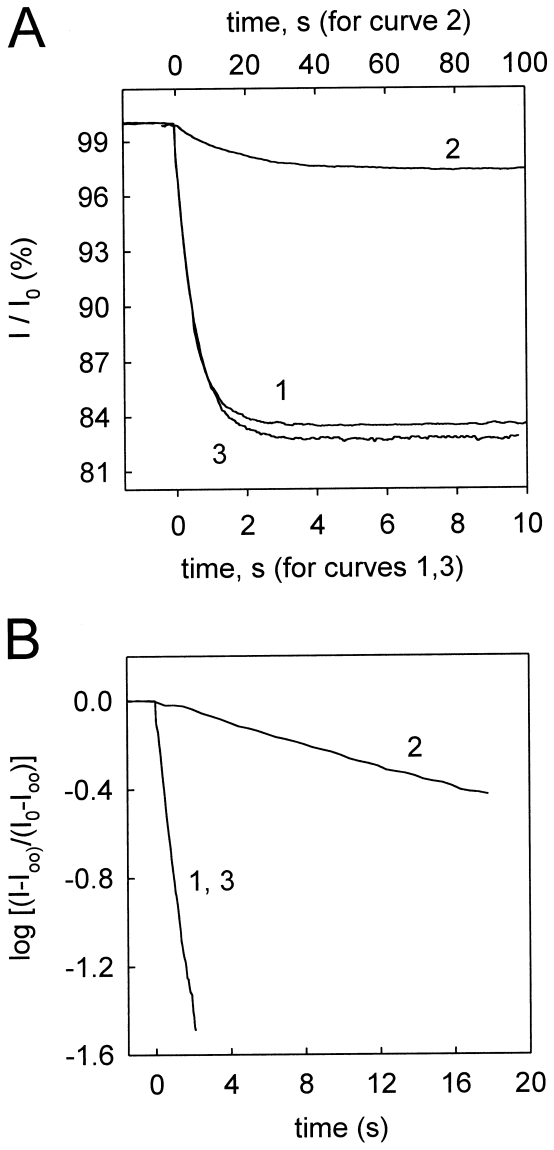

Fig. 2. (A) The effect of polylysines (PLL) of different degree of polymerization on the kinetics of the decrease in the OPg-mediated current (I) through BLM after a flash of visible light (at zero time) in the presence of 1 μM $AlPcS_3$. Polylysines were added to both sides of the BLM at concentrations corresponding to 1 μM of lysine monomer units (PLL_{240}, curve 2, and PLL_{60}, curve 3). Curve 1 shows the control without polylysine. The normalized values of the current (I/I_0) are plotted versus the time. The bottom time axis corresponds to the data of curves 1 and 3 while the top time axis, to curve 2. The initial value of the current (I_0) was approximately 3 μA. (B) The same data presented as $\log(I-I_\infty)/(I_0-I_\infty)$ versus the time where I_∞ is the stationary value of the current.

alters markedly the amplitude of photoinactivation, but does not change its characteristic time. The present study deals with the effect of polylysines on the characteristic time of photoinactivation.

In Fig. 3 the characteristic time of OPg photoinactivation is plotted versus the concentration of polylysines with different degrees of polymerization. It is seen that for all polylysines studied the dependencies of τ on concentration contain three regions. At low concentrations polylysines did not produce any effect on τ. Upon exceeding a certain (threshold) PLL concentration τ began to grow progressively and finally reached a maximum value. Further raising of the PLL concentrations led to a gradual decrease in τ. The higher DP of a polylysine, the lower threshold PLL concentration and the steeper concentration dependence of τ. The position of maximum was shifted towards higher concentrations for shorter polylysines (Fig. 3).

It should be noted that the absence of the effect on τ at low PLL concentrations was not a result of the predominant adsorption of PLL on the walls of the experimental cell. The perfusion of the cell with the solution of PLL_{60} at the pre-threshold concentration (0.5 μM) did not display noticeable changes in τ compared to the addition of 0.5 μM PLL_{60} to the bathing solution. Therefore, the adsorption of polylysines on the walls of the experimental cell did not perturb the bulk concentration of the added polylysine and the absence of the effect on τ was due to the poor binding of PLL with the OPg-containing BLM under these experimental conditions.

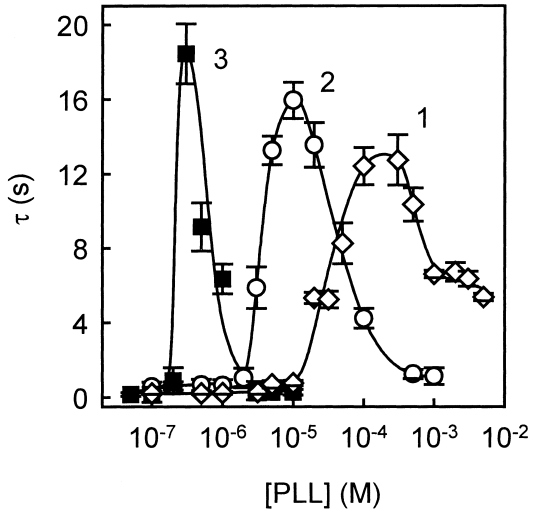

Fig. 3. The dependence of the characteristic time of OPg photoinactivation (τ) measured in the presence of polylysines with different degree of polymerization on their concentration in monomer units. Polylysines (PLL_{18}, curve 1; PLL_{60}, curve 2; and PLL_{480}, curve 3) are added at both sides of the BLM. The initial value of the current for each measurement of τ was approximately 3 μA.

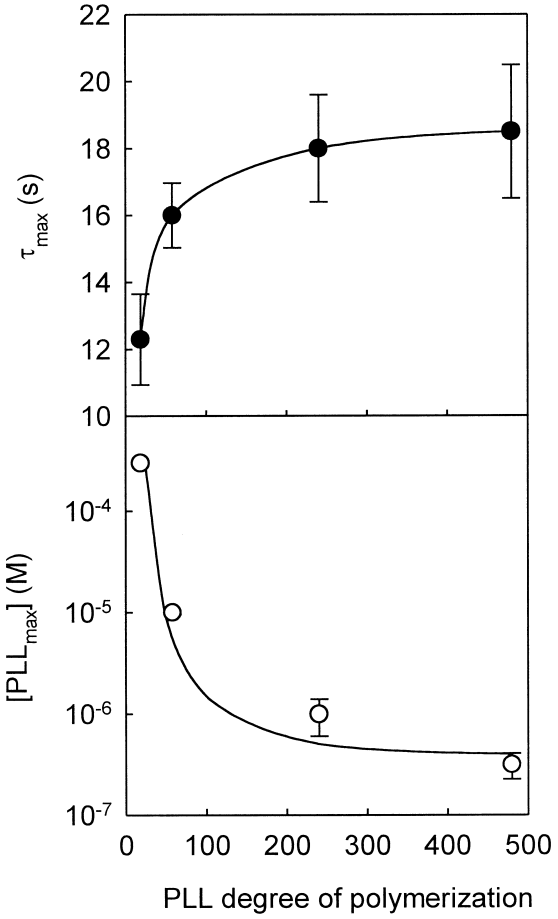

Fig. 4. The dependence of the maximum characteristic time of OPg photoinactivation (τ_{max}, closed circles, top) and of the polylysine concentration corresponding to the maximum value of τ (PLL_{max}, open circles, bottom) on the polylysine degree of polymerization. The initial value of the current for each measurement of τ was approximately 3 μA.

The bottom graph of Fig. 4 illustrates the dependence of the PLL concentration corresponding to the maximum τ ($[PLL_{max}]$) on the PLL degree of polymerization. It is seen that $[PLL_{max}]$ decreases with the increase in DP. The top graph shows the dependence of τ_{max} on the DP. The increase in τ_{max} amounts to 60% in the range of polylysine DP studied.

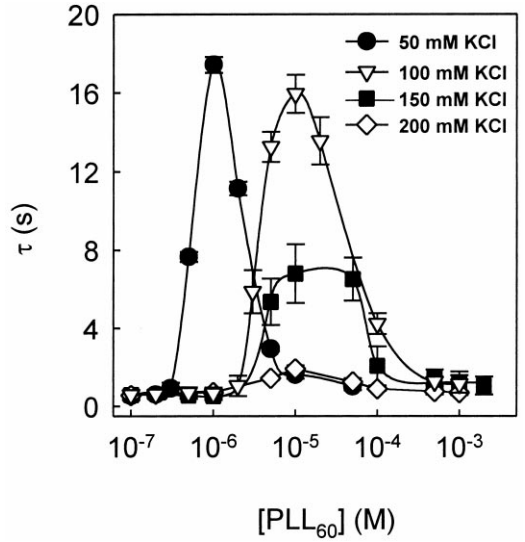

Fig. 5. The dependence of the characteristic time of OPg photoinactivation (τ) on the PLL_{60} concentration (in monomer units) measured at different concentrations of KCl (50 mM, 100 mM, 150 mM and 200 mM) in the bathing solution. The initial value of the current for each measurement of τ was approximately 2 μA at 50 mM KCl, 3 μA at 100 mM KCl, 4.5 μA at 150 mM KCl and 5 μA at 200 mM KCl.

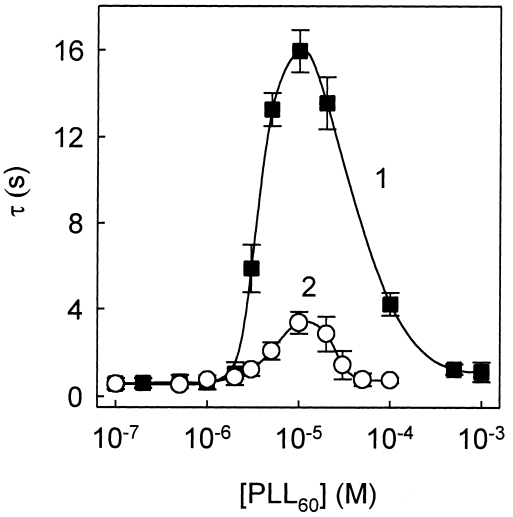

Fig. 6. The dependence of the characteristic time of OPg photoinactivation (τ) on the PLL_{60} concentration (in monomer units) in the absence (curve 1) and in the presence of 5 mM $CaCl_2$ (curve 2). The initial value of the current for each measurement of τ was approximately 3 μA. Other conditions were as in Fig. 2.

It is reasonable to assume that the effect of polylysines on τ is dependent on the ionic strength of the bathing solution. We tested this assumption in the experiments with PLL_{60}. Fig. 5 shows the dependence of τ on the concentration of PLL_{60} at four different concentrations of potassium chloride: 50 mM, 100 mM, 150 mM, 200 mM (note that the experimental results presented in all the other figures are obtained at 100 mM KCl). It was seen that the increase in ionic strength led to the reduction of the polylysine effect on τ, namely to a decrease in τ_{max} and to a shift of $[PLL_{max}]$ towards higher concentrations. This shift is especially pronounced if we compare the concentration dependence measured at 50 mM and 100 mM KCl (Fig. 5). The addition of 5 mM calcium chloride to the 100 mM KCl solution (Fig. 6) simulates the effect of an increase in the concentration of potassium chloride.

The addition of 0.5 μM low molecular weight polylysine, PLL_{18}, which did not change the photoinactivation kinetics if added alone, suppressed the increase in τ caused by the high molecular weight polylysine, PLL_{480}, at the same concentration of lysine residues (Fig. 7). If PLL_{18} was added to the bathing solutions before PLL_{480}, the deceleration of the kinetics produced by the latter was prevented. In contrast to the effect of the increase in KCl concentration, this phenomenon can hardly be attributed to the variation of the ionic strength of the solution

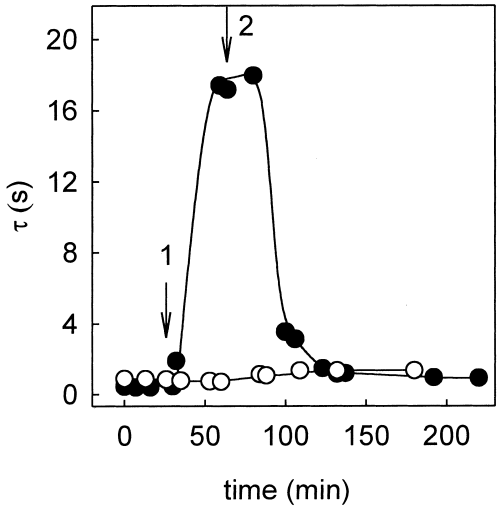

Fig. 7. The dependence of the characteristic time of OPg photoinactivation (τ) on the time after the addition of PLL_{480} (0.5 μM, arrow 1) and the subsequent addition of PLL_{18} (0.5 μM, both concentrations in monomer units, arrow 2). Open circles show the experiment with the reversed order of addition of polylysines (initially PLL_{18}, arrow 1; then PLL_{480}, arrow 2).

because of the low PLL concentration (even multiplied by the square of the number of its positive charges) as compared to that of KCl.

As shown in Fig. 8, the incorporation of the negatively charged lipid DPhPG into the neutral BLM formed of DPhPC resulted in a reduction of the polylysine effect on the OPg photoinactivation kinetics: the increase in τ was observed at higher concentrations of polylysine and the maximum magnitude of the effect was considerably lower. For the negatively charged BLM, τ rose gradually from 1.1 s to 6 s, as the concentration of PLL_{60} was increased from 5 μM to 100 μM (Fig. 8, curve 2) in contrast to the steep increase in τ from 0.6 s to 16 s in a rather narrow range of PLL_{60} concentrations observed in the absence of DPhPG (Fig. 8, curve 1). It should be noted that the addition of negatively charged lipids led to an increase in the characteristic time of OPg photoinactivation in the absence of polylysine, which is in agreement with the data on the effect of charged lipids on the single channel lifetime of gramicidin channels [97,98].

It has been shown in our previous work [22] that polylysines do not affect the kinetics of electrically neutral gA. To ascertain whether all three negative charges are required for the effect of polylysine, we performed experiments with OSg carrying only one negative charge at its C-terminus (at pH 7.0). Fig. 9 shows the kinetics of OSg photoinactivation in the absence (curve 1) and in the presence (curve 2) of PLL_{60}. It appears that there is practically no difference between the values of the characteristic time of OSg photoinactivation in these two cases (1.3 s and 1.4 s for curves 1 and 2, respectively). The effect of PLL_{60} was absent in a wide range of concentrations of PLL_{60} and OSg. Thus, it is evident that attachment of one negatively charged group to a gramicidin molecule is not sufficient for polylysine to produce its effect on the gramicidin channel kinetics.

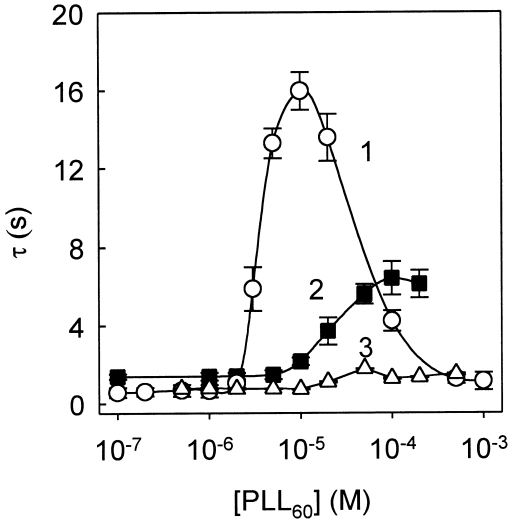

Fig. 8. The concentration dependence of the effect of PLL_{60} on the characteristic time of OPg photoinactivation for BLM formed from: pure DPhPC (open circles, curve 1), a mixture (90:10% by weight) of DPhPC and DPhPG (closed squares, curve 2), and a mixture (50:50% by weight) of DPhPC and DPhPG (open triangles, curve 3).

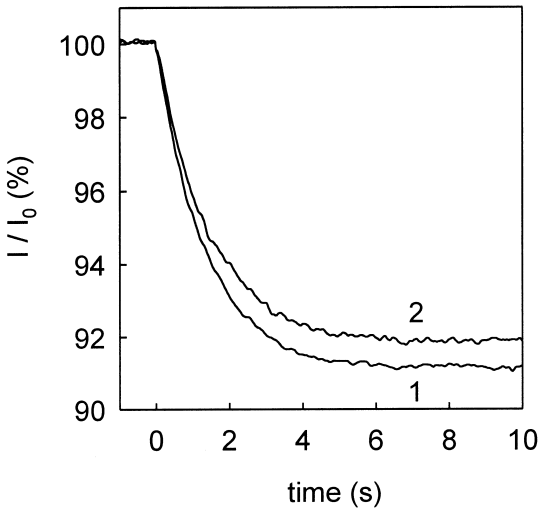

Fig. 9. The kinetics of the decrease in the OSg-mediated current (I) through BLM in the presence of 1 μM $AlPcS_3$ after a flash of visible light (at zero time) in the absence (curve 1) and in the presence of 10 μM PLL_{60} (curve 2). The normalized values of the current (I/I_0) are plotted versus the time, the initial value of the current (I_0) was approximately 3 μA.

4. Discussion

There is a large body of evidence in the literature indicating that ionic channels are frequently arranged as clusters in biological membranes [11–14]. It is relevant that under certain conditions gA channels tend to form aggregates in lipid bilayer membranes ([17] and references therein, [99–102]). Here we studied the process of formation of OPg clusters in BLM induced by the addition of polylysines of different DP.

As shown earlier [23], the characteristic time of the photosensitized decrease in the gramicidin-mediated

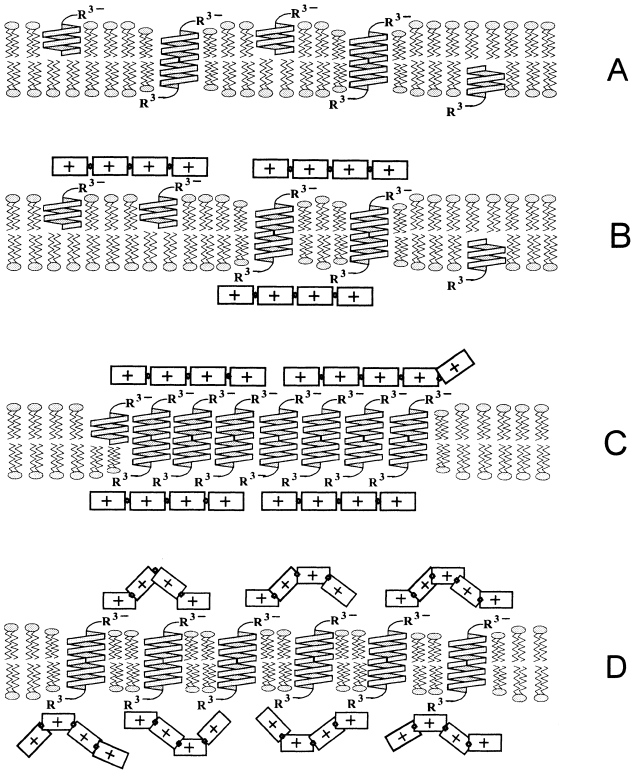

Fig. 10. A scheme for the channel formation by OPg in the absence (A) and in the presence of low (B), medium (C), and high (D) concentrations of polylysine. The increase in PLL concentration leads to formation of clusters of OPg channels (B) and OPg domains (aggregates of clusters, C). Further increase in the PLL concentration results in the electrostatic repulsion of PLL loops under conditions of PLL competition for OPg charges which leads to loosening of compact charged domains by including additional molecules of neutral lipid.

current after a light flash in the absence of polylysine is determined by the time for the establishment of an equilibrium between transmembrane dimers and monomers of gramicidin after inactivation of some of them. In a previous paper [22], polylysine-induced clustering of OPg was proposed to account for the effect of polylysine on the characteristic time of photoinactivation (Fig. 10B). The experiments performed in the present work required development of this hypothesis. In particular, it can be supposed that the characteristic time of photoinactivation (τ) was related to the number of OPg molecules bound to the same polylysine chain, because an increase in the latter apparently lowers the dissociation rate constant of the transmembrane OPg dimers. This number can be considered as related to the size of a cluster. In view of this assumption, the bell-shaped concentration dependence of τ (Fig. 3) can be interpreted as modulation of the size of the OPg clusters which takes place as more and more PLL chains are adsorbed on the membrane containing OPg. The maximum τ corresponds to the involvement of a maximum number of the OPg molecules in the formation of ionic bonds with PLL chains. The decrease in τ reflects a reduction in the size of a cluster as a result of the competition of the increased number of PLL chains interacting with the constant number of OPg molecules incorporated in the membrane. Other properties of the PLL effect on OPg channel kinetics, namely the sensitivity to ionic strength, the presence of calcium ions and negatively charged lipids, can be also explained in terms of this model, because all these properties are associated with weakening of ionic interaction between PLL and OPg.

However, it should be noted that the dependence of τ on the PLL concentration was essentially nonlinear in the region of low PLL concentrations (about the threshold concentration). This nonlinearity was most pronounced with polylysines of high DP (Fig. 3). The nonlinear character of the concentration dependence argues against a simple mechanism of the PLL effect. Actually, it is known that binding of different polylysines to negatively charged lipids is characterized by a linear dependence on the concentration of the added peptides and exhibits saturation at high peptide concentrations [103–105]. It is reasonable to assume that the size of a cluster depends linearly on the amount of polycations adsorbed on the membrane at low PLL concentrations, i.e. when OPg is present in excess.

Another striking point which contradicts the cluster hypothesis was provided by the data on the competition between the long chain and the short chain polylysines (Fig. 7). This experiment clearly shows that the lack of effect of the low molecular weight polymer at low concentrations was not a result of the absence of its binding to the membrane. Otherwise, it could not prevent the effect of the long chain polylysine when added first (Fig. 7, open circles curve). The binding of PLL_{18} should lead to the formation of OPg clusters and an increase in the photoinactivation time even at low concentration (0.5 μM per polymer unit). Therefore, further modification of the above model is required to explain the pattern of

concentration dependence observed in the experiments.

This modification can be based on a proposal that the increase in the characteristic time of photoinactivation is related to formation of aggregates of OPg–polylysine clusters, i.e. τ is a function of sequestering of OPg molecules into domains floating in the matrix of neutral phosphatidylcholine molecules. In fact, it is known that binding of basic peptides to mixed lipid bilayers induces the formation of two-dimensional domains enriched in negatively charged lipids [30,48–50,53–57,60–76,79–81]. This process, often called the phase separation of lipids, is suggested to result from electrostatic interaction between cationic peptides and negatively charged lipids [67,106,107]. In particular, it has been shown that the phase separation is suppressed by increasing the ionic strength of the solution [48]. The ability of polycations (polylysines) to provoke lipid phase separation, however, is enhanced by increasing the degree of polymerization [72,73,75]. Remarkably, it has also been shown that domains formed at intermediate concentrations of polyelectrolytes disappear as their concentration is increased further [42,54,80,108]. The mechanism of this phenomenon has not been studied in detail.

Recently, the validity of the phenomenon of lipid domain formation induced by short polylysines was questioned on an experimental basis [109]. However, this does not mean that the concept of domain formation should be completely reevaluated. Rather, these results indicate that the application of fluorescence microscopy to the study of domain formation in membranes should be more carefully implemented. In fact, Macdonald et al. [80] report that they do not see domain formation by pentalysine using NMR, while with polylysine of higher molecular weight they do. The reason for fluorescence microscopy discrepancy may be the size of the actual domains which is normally beyond the resolution achieved by an optical microscope. This is demonstrated in [2] by the application of fluorescence depolarization techniques for the case of GPI-anchored protein domains.

We use the description of lipid domain formation given in the literature to explain some kinetic properties of OPg channels in the presence of polylysine. In this case OPg molecules play a similar role to charged lipids, with DPhPC as the neutral lipid (Fig. 10). The scheme for the process of domain formation (Fig. 10C) includes the intermediary state of cluster formation (Fig. 10B) which, however, does not affect the channel kinetics of the OPg. The validity of this model is based on common properties of polylysine-induced changes in OPg channel characteristics on the one hand, and formation of charged lipid domains, on the other hand. These properties comprise the similar chain length dependence of the polylysine effect on OPg channel kinetics and the efficacy of polylysines in provoking phase separation of lipids, the inhibitory action of the increase in ionic strength and the bell-shaped dependence on the polyelectrolyte concentration for both cases. Stabilization of OPg channels by polylysines upon domain formation is associated presumably with restriction of OPg mobility in BLM, similar to motional restriction of negatively charged lipids observed upon polylysine binding [105].

According to our experimental results, the increase in ionic strength led to a reduction of the PLL effect on τ. A similar action on the PLL effect was produced by calcium ions. In our opinion, the action of calcium ions also can be considered as a consequence of an increase in ionic strength. In fact, it is known that calcium and other divalent cations added at millimolar concentrations produce the same effect on a number of processes in biological membranes as monovalent cations at a concentration of about 100 mM [110]. The competitive action of calcium ions on lipid domain formation induced by polymyxin B was observed in [61]. The reduction of the PLL effect on the OPg photoinactivation kinetics provoked by an increase in ionic strength apparently results from weakening of the electrostatic interaction between PLL and OPg. These data, as well as the reversal of the PLL effect by polyacrylate observed previously [22] strongly support the electrostatic nature of the phenomenon under study.

The most vivid proof of the validity of the 'domain' hypothesis was provided by the data on the competition between the long chain and the short chain polylysines (Fig. 7). In fact, the suppression of the long chain PLL effect caused by the addition of the short chain PLL cannot be explained in terms of the alternative 'cluster' hypothesis, because both of these polylysines should produce the formation of gramicidin clusters. On the contrary, according to

the 'domain' hypothesis, binding of the long chain PLL produces segregation of tightly packed domains of OPg channels (with a high value of τ). The subsequent binding of the short chain PLL which competitively occupies some part of ion contacts with OPg would lead to loosening of the compact domains and destabilization of gramicidin dimers. In the case of reversed order of additions, the initial addition of 0.5 μM short chain polylysine induces cluster formation which is not accompanied by an increase in τ. Moreover, the subsequent addition of the long chain polylysine does not lead to formation of tightly packed domains. Thus, obviously the suppression of the long chain PLL-induced domain formation in the presence of short chain PLL can be accounted for by the molar excess of short chains compared to long chains at equal concentrations of monomer units (lysine residues) of these two polylysines.

The experiments with membranes containing negatively charged lipid, DPhPG (Fig. 8), was another example of competition with polylysine in influence on OPg channel operation. The reduction of the maximal τ value in the presence of DPhPG in BLM as compared to the control BLM formed of only neutral lipids (DPhPC) can be attributed to the formation of combined OPg–DPhPG domains with lower surface density of OPg molecules which would not lead to considerable restriction of OPg mobility in BLM.

One of the most interesting results of the present work was the reversal of the PLL-induced OPg deceleration at high PLL concentrations. This reversal can be accounted for by the recovery of lateral homogeneity in a mixture of two components (neutral and charged, see [80]). However, in our opinion this reversal most probably results from the electrostatic repulsion of PLL loops under conditions of PLL competition for OPg charges which leads to loosening of charged domains by including additional molecules of neutral lipid into them (Fig. 10D).

It can be concluded that the present work has demonstrated that sequestering of OPg molecules by adsorbed PLL can lead to essential functional consequences which are manifested in the marked deceleration of OPg channel operation. We believe that the simple model system, OPg channel clustering induced by PLL binding to BLM, will be useful for elucidation of more complex mechanisms (e.g. attachment to cytoskeletal elements [5,111,112]) governing the clustering of channels in cell membranes.

Acknowledgements

We are indebted to Prof. R.E. Koeppe, II for the gift of O-pyromellitylgramicidin and O-succinylgramicidin. We are grateful to Prof. O.S. Andersen, Prof. R.E. Koeppe, II and Dr. T.I. Rokitskaya for valuable discussions. This work has been partially supported by the grants 00-04-48299 and 99-03-33460 of the Russian Foundation for Basic Research.

References

[1] K. Simons, E. Ikonen, Nature 387 (1997) 569–572.
[2] R. Varma, S. Mayor, Nature 394 (1998) 798–801.
[3] T. Friedrichson, T.V. Kurzchalia, Nature 394 (1998) 802–805.
[4] D.A. Brown, E. London, Annu. Rev. Cell Dev. Biol. 14 (1998) 111–136.
[5] S. Oliferenko, K. Paiha, T. Harder, V. Gerke, C. Schwarzler, H. Schwarz, H. Beug, U. Gunthert, L.A. Huber, J. Cell Biol. 146 (1999) 843–854.
[6] S. Manes, E. Mira, C. Gomez-Mouton, R.A. Lacalle, P. Keller, J.P. Labrador, A. Martinez, EMBO J. 18 (1999) 6211–6220.
[7] G. Cevc, Chem. Phys. Lipids 57 (1991) 293–307.
[8] S.W. Hui, Mol. Membr. Biol. 12 (1995) 45–50.
[9] R. Mendelsohn, D.J. Moore, Chem. Phys. Lipids 96 (1998) 141–157.
[10] O.G. Mouritsen, K. Jorgensen, Chem. Phys. Lipids 73 (1994) 3–25.
[11] V.I. Geletyuk, V.N. Kazachenko, Biochim. Biophys. Acta 981 (1989) 343–350.
[12] E.H. Joe, K. Angelides, Nature 356 (1992) 333–335.
[13] E. Kim, M. Niethammer, A. Rothschild, Y.N. Jan, M. Sheng, Nature 378 (1995) 85–88.
[14] M.R. Kaplan, A. Meyer-Franke, S. Lambert, V. Bennett, I.D. Duncan, S.R. Levinson, B.A. Barres, Nature 386 (1997) 724–728.
[15] S.B. Hladky, D.A. Haydon, Curr. Top. Membr. Transp. 21 (1984) 327–372.
[16] O.S. Andersen, R.E. Koeppe, Physiol. Rev. 72 (1992) S89–S158.
[17] J.A. Killian, Biochim. Biophys. Acta 1113 (1992) 391–425.
[18] D.D. Busath, Annu. Rev. Physiol. 55 (1993) 473–501.
[19] R.E. Koeppe, O.S. Andersen, Annu. Rev. Biophys. Biomol. Struct. 25 (1996) 231–258.

[20] H.J. Apell, E. Bamberg, H. Alpes, P. Läuger, J. Membr. Biol. 31 (1977) 171–188.
[21] A.S. Cifu, R.E. Koeppe, O.S. Andersen, Biophys. J. 61 (1992) 189–203.
[22] A.V. Krylov, Y.N. Antonenko, E.A. Kotova, T.I. Rokitskaya, A.A. Yaroslavov, FEBS Lett. 440 (1998) 235–238.
[23] T.I. Rokitskaya, Y.N. Antonenko, E.A. Kotova, Biochim. Biophys. Acta 1275 (1996) 221–226.
[24] T.I. Rokitskaya, Y.N. Antonenko, E.A. Kotova, Biophys. J. 73 (1997) 850–854.
[25] W. Knoll, G. Schmidt, H. Rotzer, T. Henkel, W. Pfeiffer, E. Sackmann, S. Mittler-Neher, J. Spinke, Chem. Phys. Lipids 57 (1991) 363–374.
[26] J.F. Tocanne, L. Cezanne, A. Lopez, B. Piknova, V. Schram, J.F. Tournier, M. Welby, Chem. Phys. Lipids 73 (1994) 139–158.
[27] R. Welti, M. Glaser, Chem. Phys. Lipids 73 (1994) 121–137.
[28] P.K. Kinnunen, A. Koiv, J.Y. Lehtonen, M. Rytomaa, P. Mustonen, Chem. Phys. Lipids 73 (1994) 181–207.
[29] D. Marsh, Mol. Membr. Biol. 12 (1995) 59–64.
[30] C.A. Buser, J. Kim, S. McLaughlin, R.M. Peitzsch, Mol. Membr. Biol. 12 (1995) 69–75.
[31] A. Raudino, Adv. Colloid Interface Sci. 57 (1995) 229–285.
[32] L.D. Mayer, G.L. Nelsestuen, Biochemistry 20 (1981) 2457–2463.
[33] L.D. Mayer, G.L. Nelsestuen, H.L. Brockman, Biochemistry 22 (1983) 316–321.
[34] G.B. Birrell, O.H. Griffith, Biochemistry 15 (1976) 2925–2929.
[35] L.R. Brown, K. Wuthrich, Biochim. Biophys. Acta 468 (1977) 389–410.
[36] D.M. Haverstick, M. Glaser, Biophys. J. 55 (1989) 677–682.
[37] T. Heimburg, B. Angerstein, D. Marsh, Biophys. J. 76 (1999) 2575–2586.
[38] J.M. Boggs, D.D. Wood, M.A. Moscarello, D. Papahadjopoulos, Biochemistry 16 (1977) 2325–2329.
[39] B. Maggio, J.M. Sturtevant, R.K. Yu, J. Biol. Chem. 262 (1987) 2652–2659.
[40] M.B. Sankaram, P.J. Brophy, D. Marsh, Biochemistry 28 (1989) 9699–9707.
[41] M.D. Bazzi, G.L. Nelsestuen, Biochemistry 31 (1992) 10406–10413.
[42] M. Junker, C.E. Creutz, Biochemistry 32 (1993) 9968–9974.
[43] O. Saurel, L. Cezanne, A. Milon, J.F. Tocanne, P. Demange, Biochemistry 37 (1998) 1403–1410.
[44] L. Cezanne, A. Lopez, F. Loste, G. Parnaud, O. Saurel, P. Demange, J.F. Tocanne, Biochemistry 38 (1999) 2779–2786.
[45] M.D. Bazzi, G.L. Nelsestuen, Biochemistry 30 (1991) 7961–7969.
[46] J.W. Orr, A.C. Newton, Biochemistry 31 (1992) 4661–4667.
[47] L. Yang, M. Glaser, Biochemistry 35 (1996) 13966–13974.
[48] L. Yang, M. Glaser, Biochemistry 34 (1995) 1500–1506.
[49] M. Glaser, S. Wanaski, C.A. Buser, V. Boguslavsky, W. Rashidzada, A. Morris, M. Rebecchi, S.F. Scarlata, L.W. Runnels, G.D. Prestwich, J. Chen, A. Aderem, J. Ahn, S. McLaughlin, J. Biol. Chem. 271 (1996) 26187–26193.
[50] R.M. Epand, C. Stevenson, R. Bruins, V. Schram, M. Glaser, Biochemistry 37 (1998) 12068–12073.
[51] M. Rytomaa, P.K. Kinnunen, Biochemistry 35 (1996) 4529–4539.
[52] E.M. Goldberg, D.B. Borchardt, R. Zidovetzki, Eur. J. Biochem. 258 (1998) 722–728.
[53] A.J. Bradley, E. Maurer-Spurej, D.E. Brooks, D.V. Devine, Biochemistry 38 (1999) 8112–8123.
[54] A.K. Hinderliter, P.F. Almeida, R.L. Biltonen, C.E. Creutz, Biochim. Biophys. Acta 1448 (1998) 227–235.
[55] A. Desormeaux, G. Laroche, P.E. Bougis, M. Pezolet, Biochemistry 31 (1992) 12173–12182.
[56] M.A. Carbone, P.M. Macdonald, Biochemistry 35 (1996) 3368–3378.
[57] K. Gawrisch, K.H. Han, J.S. Yang, L.D. Bergelson, J.A. Ferretti, Biochemistry 32 (1993) 3112–3118.
[58] P. Luan, M. Glaser, Biochemistry 33 (1994) 4483–4489.
[59] P. Luan, L. Yang, M. Glaser, Biochemistry 34 (1995) 9874–9883.
[60] W. Hartmann, H.J. Galla, E. Sackmann, Biochim. Biophys. Acta 510 (1978) 124–139.
[61] F. Sixl, H.J. Galla, Biochim. Biophys. Acta 643 (1981) 626–635.
[62] A. Theretz, J.L. Ranck, J.F. Tocanne, Biochim. Biophys. Acta 732 (1983) 499–508.
[63] F. Sixl, A. Watts, Biochemistry 24 (1985) 7906–7910.
[64] P. Kubesch, J. Boggs, L. Luciano, G. Maass, B. Tummler, Biochemistry 26 (1987) 2139–2149.
[65] J. Gonzalez-Christen, I. Vergne, R. Sussmuth, S. Sidobre, M. Prats, J.F. Tocanne, G. Laneelle, Biochim. Biophys. Acta 1368 (1998) 97–107.
[66] L. Voglino, S.A. Simon, T.J. Mcintosh, Biochemistry 38 (1999) 7509–7516.
[67] H.J. Galla, E. Sackmann, Biochim. Biophys. Acta 401 (1975) 509–529.
[68] H.J. Galla, E. Sackmann, J. Am. Chem. Soc. 97 (1975) 4114–4120.
[69] W. Hartmann, H.J. Galla, E. Sackmann, FEBS Lett. 78 (1977) 169–172.
[70] W. Hartmann, H.J. Galla, Biochim. Biophys. Acta 509 (1978) 474–490.
[71] R. Krbecek, C. Gebhardt, H. Gruler, E. Sackmann, Biochim. Biophys. Acta 554 (1979) 1–22.
[72] D. Carrier, J. Dufourcq, J.F. Faucon, M. Pezolet, Biochim. Biophys. Acta 820 (1985) 131–139.
[73] D. Carrier, M. Pezolet, Biochemistry 25 (1986) 4167–4174.
[74] R.N. McElhaney, Biochim. Biophys. Acta 864 (1986) 361–421.
[75] G. Laroche, D. Carrier, M. Pezolet, Biochemistry 27 (1988) 6220–6228.
[76] S. Mittler-Neher, W. Knoll, Biochem. Biophys. Res. Commun. 162 (1989) 124–129.
[77] P. Mitrakos, P.M. Macdonald, Biochemistry 35 (1996) 16714–16722.
[78] P. Mitrakos, P.M. Macdonald, Biochemistry 36 (1997) 13646–13656.

[79] A. Raudino, F. Castelli, Macromolecules 30 (1997) 2495–2502.
[80] P.M. Macdonald, K.J. Crowell, C.M. Franzin, P. Mitrakos, D.J. Semchyschyn, Biochem. Cell Biol. 76 (1998) 452–464.
[81] P. Lasch, C.P. Schultz, D. Naumann, Biophys. J. 75 (1998) 840–852.
[82] A. Elgsaeter, D.M. Shotton, D. Branton, Biochim. Biophys. Acta 426 (1976) 101–122.
[83] M.J. Dufton, R.J. Cherry, J.W. Coleman, D.R. Stanworth, Biochem. J. 223 (1984) 67–71.
[84] M.J. Clague, R.J. Cherry, Biochim. Biophys. Acta 980 (1989) 93–99.
[85] O.M. Zakharova, A.A. Rosenkranz, A.S. Sobolev, Biochim. Biophys. Acta 1236 (1995) 177–184.
[86] P. Mueller, D.O. Rudin, H. Ti Tien, W.C. Wescott, J. Phys. Chem. 67 (1963) 534–535.
[87] E.A. Kotova, T.I. Rokitskaya, Y.N. Antonenko, Membr. Cell Biol. 13 (2000) 411–420.
[88] L. Kunz, U. Zeidler, K. Haegele, M. Przybylski, G. Stark, Biochemistry 34 (1995) 11895–11903.
[89] D.P. Valenzeno, M. Tarr, in: J.F. Rabek (Ed.), Membrane Photomodification and Its Use to Study Reactive Oxygen Effects, Vol. III, CRC Press, Boca Raton, 1991, pp. 137–191.
[90] A.A. Krasnovsky Jr., Membr. Cell Biol. 12 (1998) 665–690.
[91] B. Ehrenberg, J.L. Anderson, C.S. Foote, Photochem. Photobiol. 68 (1998) 135–140.
[92] D.D. Busath, E. Hayon, Biochim. Biophys. Acta 944 (1988) 73–78.
[93] Y.N. Antonenko, T.I. Rokitskaya, E.A. Kotova, Membr. Cell Biol. 13 (1999) 111–120.
[94] O.S. Andersen, H.J. Apell, E. Bamberg, D.D. Busath, R.E. Koeppe, F.J. Sigworth, G. Szabo, D.W. Urry, A. Woolley, Nat. Struct. Biol. 6 (1999) 609–612.
[95] M. Strässle, G. Stark, Photochem. Photobiol. 55 (1992) 461–463.
[96] T.I. Rokitskaya, Y.N. Antonenko, E.A. Kotova, FEBS Lett. 329 (1993) 332–335.
[97] J.A. Lundbæk, P. Birn, J. Girshman, A.J. Hansen, O.S. Andersen, Biochemistry 35 (1997) 3825–3830.
[98] S. Mittler-Neher, W. Knoll, Biochim. Biophys. Acta 1152 (1993) 259–269.
[99] H.A. Kolb, E. Bamberg, Biochim. Biophys. Acta 464 (1977) 127–141.
[100] G. Stark, M. Strässle, Z. Takacz, J. Membr. Biol. 89 (1986) 23–37.
[101] O.V. Kolomytkin, A.O. Golubok, D.N. Davydov, V.A. Timofeev, S.A. Vinogradova, S.Y. Tipisev, Biophys. J. 59 (1991) 889–893.
[102] J.X. Mou, D.M. Czajkowsky, Z.F. Shao, Biochemistry 35 (1996) 3222–3226.
[103] J. Kim, M. Mosior, L.A. Chung, H. Wu, S. McLaughlin, Biophys. J. 60 (1991) 135–148.
[104] B. de Kruijff, A. Rietveld, N. Telders, B. Vaandrager, Biochim. Biophys. Acta 820 (1985) 295–304.
[105] J.H. Kleinschmidt, D. Marsh, Biophys. J. 73 (1997) 2546–2555.
[106] G. Denisov, S. Wanaski, P. Luan, M. Glaser, S. McLaughlin, Biophys. J. 74 (1998) 731–744.
[107] P.M. Macdonald, K.J. Crowell, C.M. Franzin, P. Mitrakos, D.J. Semchyschyn, Solid State Nucl. Magn. Reson. 16 (2000) 21–36.
[108] M. Subramanian, J.M. Holopainen, T. Paukku, O. Eriksson, I. Huhtaniemi, P.K.J. Kinnunen, Biochim. Biophys. Acta 1466 (2000) 289–305.
[109] D. Murray, A. Arbuzova, G. Hangyas-Mihalyne, A. Gambhir, N. Ben Tal, B. Honig, S. McLaughlin, Biophys. J. 77 (1999) 3176–3188.
[110] J. Barber, J. Mills, A. Love, FEBS Lett. 74 (1977) 174–181.
[111] F. Bradke, C.G. Dotti, Biochim. Biophys. Acta 1404 (1998) 245–258.
[112] T. Harder, K. Simons, Eur. J. Immunol. 29 (1999) 556–562.