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pH-control of the miscibility properties of a binary lipid alloy and its influence on the ion transport by gramicidin

Silvia Mittler-Neher and Wolfgang Knoll

Max-Planck-Institut für Polymerforschung, Mainz (F.R.G.)

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We studied the coupling of a membrane function (the transport of ions by the pore forming polypeptide gramicidin) to chemically driven phase changes in black membranes of binary lipid mixtures. In particular, we investigated the influence of the aqueous pH value on the fluid-fluid demixing effect of Ca^{2+} to phosphatidylcholine/phosphatidylglycerol bilayers. It is found that one can switch, under certain conditions, between a homogenously mixed and a phase separated membrane by changing the pH. We interpret this as being caused by the change in the degree of dissociation of one of the lipid components.

Introduction

Phase separation processes in mixed lipid membranes [1–3] together with selective lipid–protein interaction [4] have been proposed as a general concept for the control of the distribution and performance of functional (super-)structures in biomembranes [5,6]. On the model-membrane level, there are already numerous examples for phase separation phenomena in binary mixed lipid alloys among which the chemically induced domain formations (through, e.g., Ca^{2+} [1–3,7–10] or polylysine [11,12] are the most interesting ones because they allow for an isothermal control of lateral structure formation. Moreover, phase changes could be triggered by these mechanisms in selected areas of a biomembrane by only local and transient variations of the concentration of the demixing agents.

In this note we report the experimental evidence for another phase separating mechanism, namely the change of the pH in the electrolyte solution. We demonstrate this by analysing the single-channel conductance fluctuations of gramicidin, a well-characterized pore-forming polypeptide [13,14] which is incorporated into bimolecular lipid membranes (BLM) made out of a mixture of phosphatidylcholine and phosphatidylglycerol. These systems have been shown recently to be well-suited for investigations of the coupling of a membrane function, e.g., the translocation of ions across the membrane, to the lateral organization of the lipid matrix [9]: homogeneous distributions of the lipid components manifest themselves in a single, narrow conductance histogram whereas a demixed membrane with two coexisting phases can be identified by a bimodal channel distribution. Phase separation and its influence on an integral model-protein can be studied, therefore, very directly.

Materials and Methods

Black membranes were prepared according to the method described by Mueller et al. [15] from 1% (w/v) lipid solutions in *n*-decane (Fluka, purum). 1,2-Dioleoyl-*sn*-glycero-3-phosphatidylcholine (PC) and 1,2-dioleoyl-*sn*-glycero-3-phosphatidylglycerol (PG) were obtained from Avanti (Birmingham, AL) and used without further treatment. The electrolyte solutions contained 0.5 M CsCl and 10^{-4} M $CaCl_2$ (both p.A. quality, Merck, Darmstadt). Different pH values were adjusted by adding NaOH and HCl and were checked before and after completion of each experiment. The temperature was 22°C. Gramicidin (a commercial mixture of A, B, and C) was added from methanolic stock solutions as needed.

Single-channel current fluctuations were recorded as described before and analysed with respect to conductance, Λ, and lifetime, τ, in a way that allowed also for the analysis of sub-populations thereby looking for correlations between Λ and τ [9]. It was thus possible to derive in a demixed membrane with two different channels not only their mean conductance, $\overline{\Lambda}_i$, but also their mean lifetime, $\bar{\tau}_i$.

Correspondence: W. Knoll, Max-Planck-Institut für Polymerforschung, Ackermannweg 10, D-6500 Mainz, F.R.G.

Results

All experiments in this study were performed with equimolar mixtures of PC and PG. This was chosen because in an extended study of Ca^{2+}-induced phase separation (unpublished results) we found that in the presence of 10^{-4} M Ca^{2+} at pH 6 the 1:1 mixture is right in the middle of a Ca^{2+}-induced miscibility gap and therefore two channel populations can be well distinguished in the conductance histogram. The mean conductance values are $\Lambda_1 = 80$ p and $\Lambda_2 = 25$ pS, the channel life times are $\bar{\tau}_1 = 4$ s and $\bar{\tau}_2 = 0.75$ s, respectively [16].

Upon decreasing the pH in the electrolyte solution virtually nothing changes for pH 5 and pH 4. The latter case is depicted in Fig. 1. This example of a current fluctuation is chosen to show the simultaneous appearance of two different, but coexisting types of channels which are well-separated by their different conductance increments. Their statistical analysis is given in Fig. 2 (upper frame) and shows a clear bimodal conductance histogram indicative of a phase separated system with two coexisting domains, both doped with gramicidin though, obviously, with different partition coefficients.

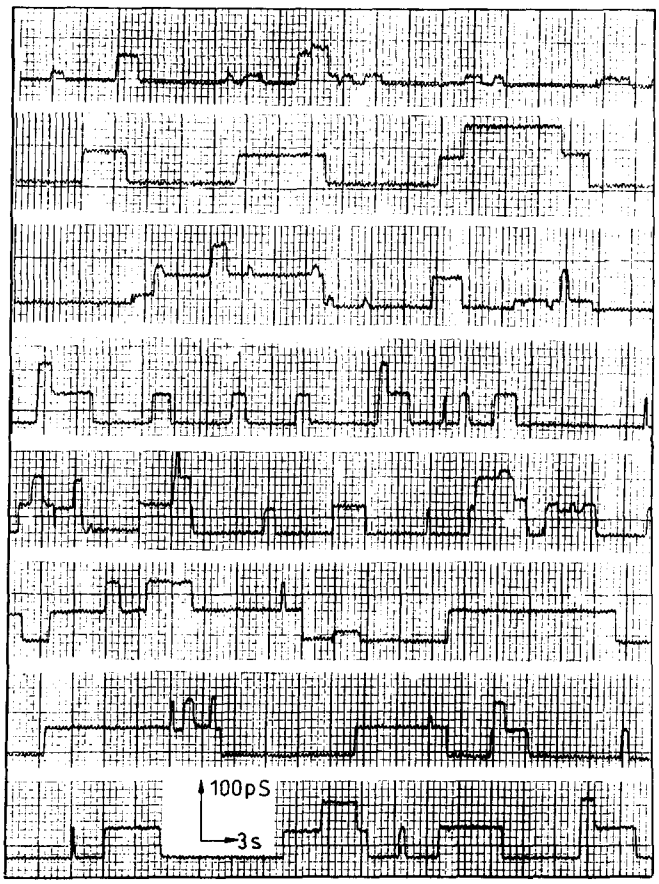

Fig. 1. Record of conductance fluctuations of gramicidin in a 1:1 mixed membrane of PC and PG. The electrolyte contained 0.5 M CsCl and 10^{-4} M $CaCl_2$. $T = 22°$ C, pH was adjusted to pH 4. The full bars indicate the conductance scale of the ordinate and the time scale of the abscissa, respectively. The applied membrane voltage was $U = 100$ mV.

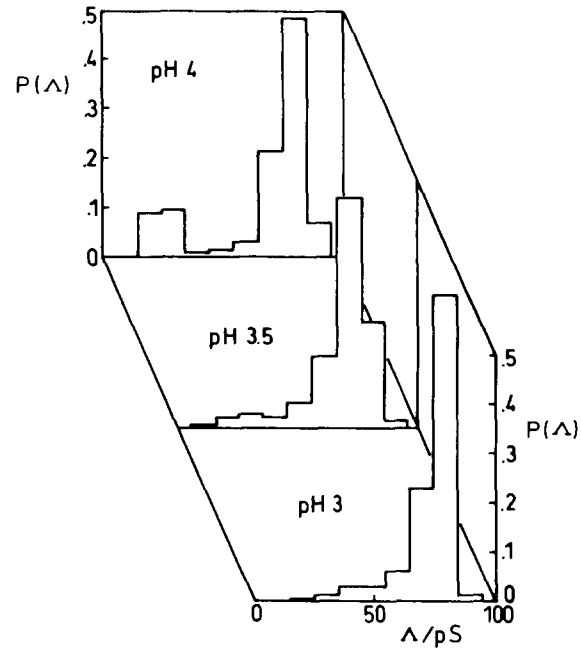

Fig. 2. Normalized conductance histograms P(Λ) of 1:1 mixed PC/PG-membranes at various pH-values as indicated in the different frames. 0.5 M CsCl, 10^{-4} M $CaCl_2$, $T = 22°$ C, $U = 100$ mV.

If, however, the pH is further lowered to 3.5 a significant difference in the channel characteristic is evident (see Fig. 2, middle frame): a much broader conductance histogram is found covering the whole

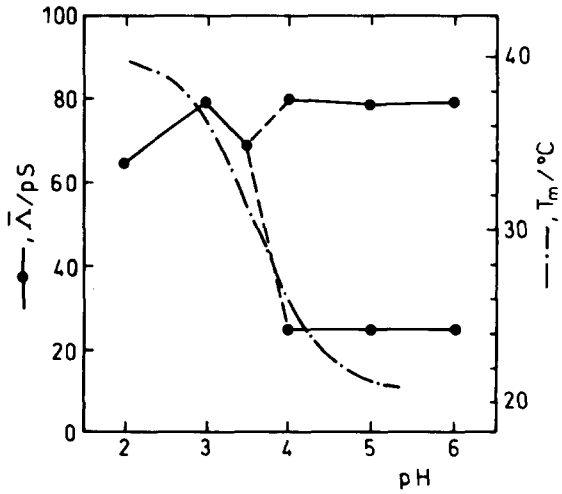

Fig. 3. Full circles: mean conductance increments, $\bar{\Lambda}$, by a single gramicidin pore in mixed or demixed 1:1 PC/PG membranes derived from histograms like the ones shown in Fig. 2 at different pH values of the electrolyte (0.5 M CsCl, $U = 100$ mV, $T = 22°$ C). Full lines are only guides to the eye. Dash-dotted curve is reproduced from Ref. 17 and shows the decrease of the main phase transition temperature, T_m, of dimyristoylphosphatidylglycerol with increasing pH.

range from the small pores (Λ_2) to the large ones (Λ_1). In this respect, the histogram is very similar to those found for phosphatidylcholine/phosphatidic acid-mixed membranes at low Ca^{2+}-content (10^{-6}–10^{-5} M) close to a critical demixing point [9].

Upon further increasing the proton concentration, i.e., lowering the pH to pH 3 only one single rather narrow histogram is found (Fig. 2, lower frame) indicating a monophasic behavior of this lipid mixture at that pH. A similar histogram is found at pH 2 though with a slightly reduced mean conductance, $\bar{\Lambda}$. All mean conductance data of the different channel populations at various pH values are summarized in Fig. 3.

Discussions

One of the striking consequences of the strong electrostatic interactions between the polar (zwitterionic or charged) lipid headgroups and the ions in the surrounding aqueous medium are the phase changes in the bilayer that can be induced by changing the electrolyte condition. Examples for this are shifts in the phase transition temperatures of liposomal membranes by varying the pH [17] or the ionic strength [18] or by binding of divalent ions like Ca^{2+} or charged polypeptides, e.g., polylysine [19]. Similar effects have been reported for monolayers at the water/air interface where the transition pressure behaves in a corresponding way [20]. Particularly sensitive to ionic changes are lipid systems close to the pK value(s) of their dissociable headgroup(s) [18,20]: already a minor variation of an external parameter can induce major structural changes in the membranes. For phosphatidylglycerol, the lipid that we used in our study, it was shown that the main phase transition temperature, T_m, decreases by about 20 degrees between pH 2.5 and pH 4.5. Within this pH range the proton of the phosphate function dissociates so that the lipid headgroup changes from neutral to negatively charged. This is schematically added to Fig. 3 (dash-dotted line, after Ref. 17 for dimyristoyl-PG).

The coincidence between this apparent pK of PG and the onset of phase separation in PC/PG-mixed BLMs then suggests the following mechanism: at pH 2 most of the PG is neutral and mixes homogeneously with PC: we find only one narrow channel population whose mean conductance might be somewhat increased relative to a fully neutral membrane due to an interfacial potential-enhanced Cs^+ concentration according to a Gouy-Chapman model [21]. (For comparison, the mean conductance of gramicidin in a PC membrane at pH 6 is $\bar{\Lambda} = 40$ pS [15]). Increasing the pH to pH 3 leads to a further dissociation of PG, but no change in the miscibility behavior is found yet. Only the even more enhanced interfacial Cs^+-concentration results in an increased mean channel conductance of $\bar{\Lambda} = 80$ pS. The situation is similar to a PC-membrane at pH 6 where the addition of little PG^- ($x_{PG} < 0.20$) increases the mean conductance $\bar{\Lambda}$ without enhancing the Ca^{2+} concentration at the interface up to a level where blocking occurs [22].

Now, at pH 3.5 a degree of dissociation, $\alpha \cong 0.5$, is reached where about half of the PG-molecules bear one negative charge. Here, a highly dynamical equilibrium is reached with large fluctuations at different levels, as expressed by the following equations:

$$PG \rightleftharpoons PG^- + H^+ \tag{1}$$

$$2\,PG^- + Ca^{2+} \rightleftharpoons PG_2^{2-}\,Ca^{2+} \tag{2}$$

$$n(PG_2^{2-}\,Ca^{2+}\cdot PC) \rightleftharpoons nPG_2^{2-}\,Ca^{2+} + nPC \tag{3}$$

Eqn. 1 describes the association/dissociation reaction of the phosphate proton. Eqn. 2 takes into account some Ca^{2+} 'binding' which does occur because in the absence of any divalent impurities (either by adding EDTA or using ultrapure CsCl for the electrolyte) no changes occur. Eqn. 3, finally, symbolizes the demixing of the PG/PC membrane induced by Ca^{2+} into a PC-rich and a PG-rich phase but is meant to describe this process only very schematically.

Reactions 1 and 2 are presumably very fast so that these equilibria are always established. This explains why, for a given open pore, we don't observe any further fluctuations in the channel current during the on-state despite the broad distribution, $P(\Lambda)$ of pores with different mean conductance (see Fig. 2). The second process is much slower, lateral diffusion limited. A relevant time window is given by the mean lifetime of the pores $\bar{\tau} \approx 0.25$ s. With $D = 10^{-7}$ cm^2/s for the diffusion coefficient of lipid molecules in fluid membranes [4] this would mean that according to

$$\Delta x \approx \sqrt{4D \cdot \bar{\tau}} \tag{4}$$

relatively large domains with correlation lengths of about $\Delta x = 1$ μm must be present in the membranes. This seems somewhat high, however, it is well-conceivable, that the gramicidin itself stabilizes the fluctuations and/or that additional critical slowing-down effect are operational.

Finally, at pH 4 two stable coexisting phases are built both doped with gramicidin, although there is a remarkable difference for the ionophores in the two phases: in all cases, the number of dimerization events is much smaller in the low-conductance state. This state is formed in the PC-rich areas which we know unequivocally from the determination of the whole phase diagram [15]. We attribute this probability difference to a different partition coefficient of gramicidin although other reasons might be also envisaged.

In conclusion, we have shown that the Ca^{2+}-induced demixing of binary lipid alloys is dependent on the

ionization state of the headgroups. Since their degree of dissociation depends on the pH of the aqueous medium (but also, of course, on the concentration and the nature of divalent cations present in the electrolyte) phase separation processes can be controlled by the variation of the (local) proton concentration. The framework of the Poisson-Boltzmann description of the coupling of the pH, the membrane charge density and the (bulk and interfacial) ion concentrations provides, therefore, a whole variety of control mechanisms of membrane lateral organization and its influence on the membrane functions like the transport of ion across the hydrophobic barrier.

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