Biochimica et Biophysica Acta, 1152 (1993) 259–269

BBAMEM 76114

Ca^{2+}-induced lateral phase separation in black lipid membranes and its coupling to the ion translocation by gramicidin

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(Received 29 March 1993)
(Revised manuscript received 13 July 1993)

Key words: Phase separation; Calcium ion concentration; Gramicidin; Bimolecular lipid membrane; Single-channel current; Ion translocation

We analyze the single-channel current fluctuations of gramicidin incorporated into bimolecular lipid membranes (BLM) of binary mixtures of phosphatidylcholine (PC) and phosphatidylglycerol (PG) as a function of the Ca^{2+} concentration in the electrolyte (0.5 M CsCl, pH 6) solution. At low Ca^{2+} levels ($c_{Ca^{2+}} < 10^{-6}$ M) a monomodal conductance histogram and a single average lifetime suggests a homogeneous mixture over the full range of composition ($PG_{(1-x)}PC_x$, $0 \le x \le 1$). At higher Ca^{2+} concentrations phase separation processes are inferred from the appearance of bimodal conductance histrograms. The two channel populations (in the two coexisting phases) can also be distinguished through their different average lifetimes. By a systematic variation of the mole fractions of the two lipid components we derive the respective phase boundaries and thus the full Ca^{2+} concentration–composition phase diagram.

Introduction

The lateral organization of the different biomembrane constituents and its physiological control is far from being well understood. The investigation of this order–function relationship is therefore still a major challange for membrane-biophysical research and, like in the case of the structure–function relation, a key-point for the understanding of the physical basis of membrane processes [1]. In this context, model membranes play an important role through their less complex interactions with certain components, and this is a necessary condition for systematic studies aimed at elucidating the physico-chemical principles underlying more complex membrane functions.

Along these lines, the phase behavior of lipid mixtures in general and phase separation phenomena in particular, have been studied extensively in the past and have been proposed as a possible mechanism that determines the lateral order even in multi-component biomembranes and thereby controls, e.g. the (transient) formation of functional superstructures [2,3]. A huge body of information, therefore, is available about the thermotropic and lyotropic polymorphism of binary (and to some extend ternary) lipid mixtures [4], and how this phase behavior is modified by external parameters like the pH or the concentration of divalent ions or peptides and proteins (charged and uncharged) in the aqueous medium.

Most of this knowledge was derived from studies with vesicular or liposomal model membranes and it was not until recently that it was shown that similar investigations are possible also with bimolecular lipid lamellas, so-called black lipid membranes (BLM) [5–7]. These model membranes, which have been successfully used in recent years to clarify the molecular mechanisms of the action of different ionophores that promote the transfer of ions across the hydrophobic membrane barrier [8], now allow also to study the coupling of such a membrane-function to the lateral organization of the lipid components in a binary mixed matrix. So far, examples had been given for the influence of Ca^{2+}-induced phase separation in phosphatidylcholine/phosphatidic acid mixed membranes on the translocation of hydrophobic ions [6], carriers [5] and pores [7].

This paper reports yet another lipid mixture, phosphatidylcholine/phosphatidylglycerol, that shows demixing into two coexisting phases under the influence of strikingly low Ca^{2+} concentrations thereby supporting the general validity of the concept of externally controlled lateral order as a concept to influence membrane function. We evaluate the full concentration–

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composition phase diagram by analysing the single-channel current fluctuations of gramicidin, a well-characterized pore-forming polypeptide [9,10].

Experimental

Black lipid membranes were either formed in the way described by Mueller et al. [11] or by the alternative first reported by Montal and Mueller [13] on a hole in the septum of a thermostated teflon cell. The bilayer area was about $5 \cdot 10^{-4}$ cm^2. The membrane forming solution contained 1,2-dioleoylphosphalidylcholine (PC) and 1,2-dioleoylphosphatidylglycerol (PG) in different molar ratios, x, covering the whole range of mixtures from pure PG ($x = 0$) to pure PC ($x = 1$). Both lipids were obtained from Avanti, Birmingham, AL, and were used without further treatment. Typical concentrations were 1–2% (w/v) in n-decane (Fluka).

The electrolyte solutions contained 0.5 M CsCl and various concentrations of $CaCl_2$ in ultrapure water (Millipore quality). The salts were analytical grade (Merck, Darmstadt). For some test measurements, 10^{-4} M ethylenediaminetetraacetate (EDTA) was added to remove traces of divalent ions. In another experiment ultrapure CsCl was used for the electrolyte preparation. The specified Ca^{2+} level was there in the ppb region. In all cases the pH was adjusted to pH 6, and the temperature was 22°C. Gramicidin (a commercial mixture of A, B, and C) was added from methanolic stock solution as needed.

Single-channel current fluctuations at typically 100 mV applied voltage were recorded and stored on magnetic tape about 5–10 min after the membrane has become black [12]. Statistical analysis was performed by digitizing the height (i.e., the conductance increment, Λ) and the width (i.e., the life time, τ) of individual events only. Thus, it was possible to either obtain from conductance histograms, $P(\Lambda)$, the mean channel conductance, $\bar{\Lambda}$, or from the life time probability distribution, $P(\tau)$, the mean lifetime, $\bar{\tau}$, or to even look for correlations between them, e.g., for channel-subpopulations. This was particularly important for demixed membranes where bimodal histrograms could be found from two types of channel with different conductivities. These two populations could then be analyzed separately also with respect to their channel lifetimes.

Results

In order to investigate the single-channel characteristics of gramicidin in mixed $PG_{(1-x)}PC_x$ membranes in the absence of even trace impurities of divalent ions first a series of current fluctuations with 10^{-4} M EDTA in addition to the 0.5 M CsCl in the electrolyte solution were recorded for bilayers of various mole fractions x. A typical example for a 1 : 1 ($x = 0.5$) mixed membrane is given in Fig. 1. The statistical analysis, $P(\Lambda)$ of the individual conductance increments, Λ, is shown in Fig. 2a together with the corresponding histrograms obtained for the pure lipid components, $PC_{(x=1)}$ and $PG_{(x=0)}$, respectively. All three examples show, like any other mixture examined, single-peaked conductance distributions typical for homogeneous (mixed) membranes. We therefore can characterize the conductance state of gramicidin in various PG/PC mixtures in the absence of Ca^{2+} by a respective single parameter, the mean conductance $\bar{\Lambda}$.

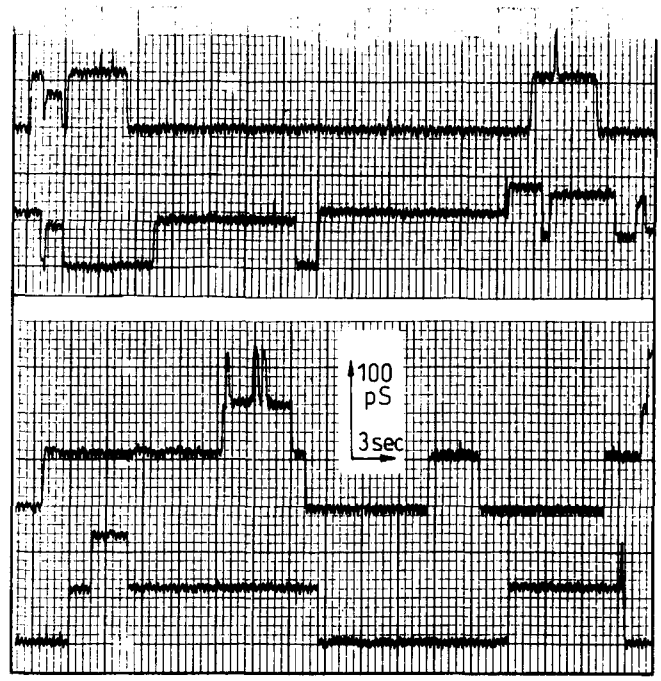

Fig. 1. Record of conductance fluctuations of gramicidin (mixture of A, B, and C) in a 1:1 mixed membrane of PC and PG. The electrolyte contained 0.5 M CsCl and 10^{-4} M EDTA. $T = 22$°C; pH 6; applied voltage 100 mV. The full arrows give the scale of the conductance and time coordinates, respectively.

The dependence of $\bar{\Lambda}$ as a function of the mole fraction, x, is summarized in Fig. 2b. Gramicidin in pure PC membranes ($x = 1$) shows a mean conductance increment of $\bar{\Lambda} = 40$ pS. By adding increasing amounts of PG this value steadily rises until for pure PG ($x = 0$) $\bar{\Lambda} = 80$ pS is obtained. Note that this increase is only a factor of 2 despite the fact that PC membranes are neutral whereas the charged PG membranes should give rise to a strong enhancement of the interfacial Cs^+ concentration. We will come back to this point later.

The second parameter that characterizes a single-channel is its lifetime, τ. As an example for the statistical analysis of a pore ensemble, the probability distribution $P(\tau)$ to find a channel with lifetime τ is given

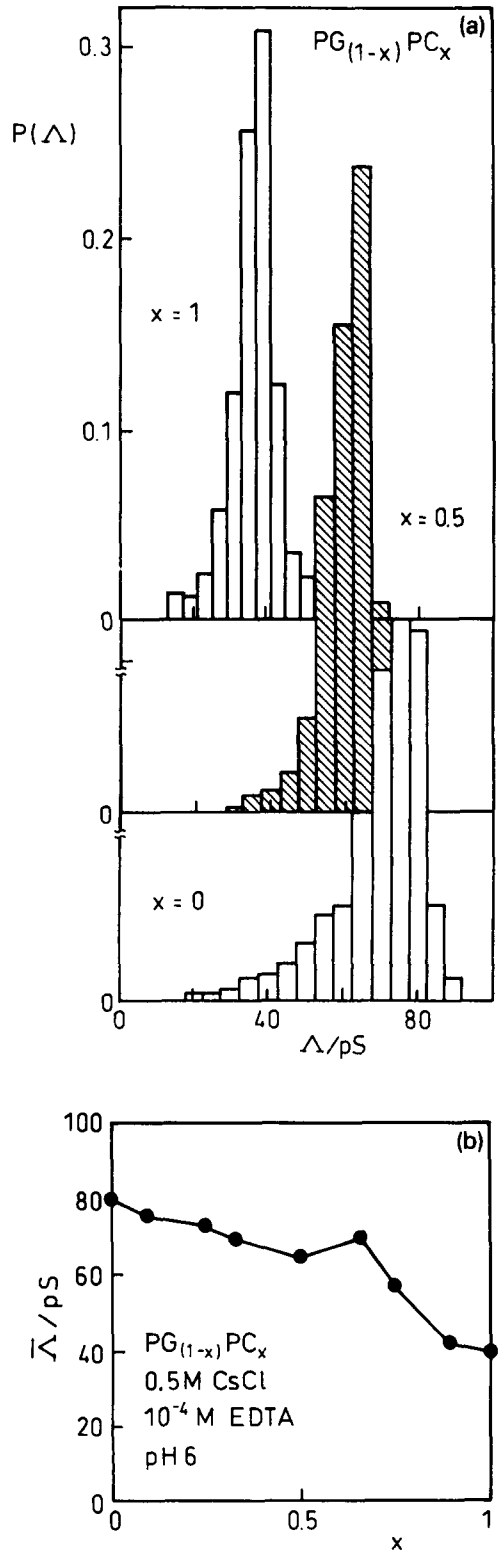

Fig. 2. (a) Normalized conductance histograms of mixed $PG_{(1-x)}PC_x$ membranes. Upper frame, $x = 1$; middle frame, $x = 0.5$ (shaded for better clarity); lower frame, $x = 0$. 0.5 M CsCl + 10^{-4} M EDTA, pH 6, $T = 22°C$, $U = 100$ mV. (b) Mean conductance increments, $\bar{\Lambda}$, by a single gramicidin pore in different mixed $PG_{(1-x)}PC_x$ membranes. $\bar{\Lambda}$ values were obtained from histograms like the ones given in (a).

in Fig. 3a for pure PC ($x = 1$) and for a 1:1 mixed PG/PC membrane. From the straight line fits of the ln $P(\tau)$ versus τ plots the mean channel lifetimes, $\bar{\tau}$, can be obtained. For all mixtures investigated no deviation from this exponential dependence was found. We therefore conclude again that the gramicidin channels in these lipid mixtures are all embedded in a homogeneous matrix indicating a monophasic behavior. This finding therefore supports the conclusion deduced from the conductance data.

The dependence of $\bar{\tau}$ on the membrane composition, x, is depicted in Fig. 3b. Note the substantial difference between $\bar{\tau} \approx 4$ s for pure PG and $\bar{\tau} = 0.5$ s for pure PC. Also very remarkable is the enhanced pore-stability for intermediate mixtures with a maximum at $x \approx 0.3$. As we will see it is this strong dependence of $\bar{\tau}$ on x which helps considerably to identify coexisting phases in demixed systems.

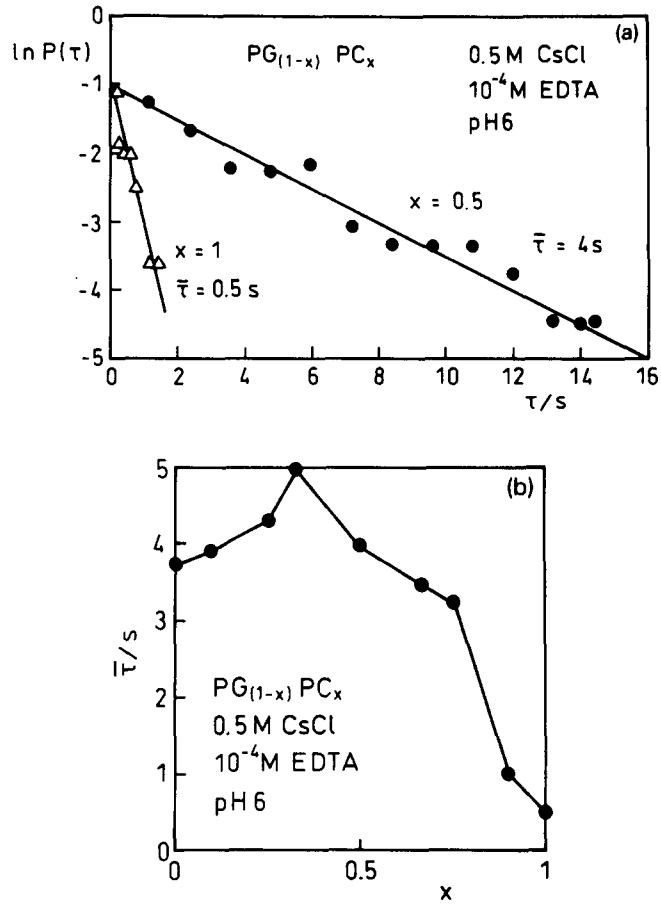

Fig. 3. (a) Semilogarithmic plot of the probability distribution, $P(\tau)$, of finding a channel of lifetime, τ, in mixed $PG_{(1-x)}PC_x$ membranes. Symbols represent experimental data, from the straight lines the mean channel lifetime, $\bar{\tau}$, is derived: △, $x = 1$ (pure PC), $\bar{\tau} = 0.5$ s; ●, $x = 0.5$, $\bar{\tau} = 4$ s. $U = 100$ mV, other parameters as indicated. (b) Mean channel lifetimes, $\bar{\tau}$, for gramicidin in $PG_{(1-x)}PC_x$ mixed membranes of different composition. Data are obtained from plots like the one presented in (a). The full line is a guide to the eye.

As a first example for a system that shows phase separation we investigated PG/PC mixed membranes over the full range of composition ($0 < x < 1$) in the presence of 10^{-3} M $CaCl_2$ in the electrolyte. A typical trace of current fluctuations is shown in Fig. 4 and demonstrates the simultaneous appearance of two types of pore with obviously different conductivities and, as we will see, also different lifetimes. An experimental difficulty for identifying phase separations arises from the fact that in all cases where we find two different channels the one with the lower conductance state has a considerably reduced formation probability which results in a bimodal, but highly asymmetric histogram. To demonstrate the somewhat gradual transition from a homogeneous to a heterogeneous system which also results from this probability difference, we show in Fig. 5 a whole series of histograms taken for various mixed membranes of different mole fraction, x. One can clearly see that mixtures rich in either of the two components, i.e., $x \gtrsim 0.80$ or $x \lesssim 0.20$, show a single peak in the histogram whereas intermediate mixtures show two peaks, though with different relative heights.

The mean conductance increments, $\bar{A}$, for the different lipid mixtures are all plotted in Fig. 6 as a function of the mole fraction, x, of PC. The full circles represent the experimental points, straight lines are only guides to the eye. Particularly interesting is the behavior found for PC-rich membranes. Adding only little PG to a PC membrane ($x \approx 0.90$) results in an increase of the mean conductance relative to pure PC, however, upon further increasing the PG content, the mean conductance increment decreases again until it merges into the value for the low-conductance state in the demixed regime. This then remains constant over a broad range of composition ($0.25 < x < 0.75$). The mean conductance of gramicidin in pure PG membranes is virtually unchanged by the presence of 10^{-3} M Ca^{2+} (c.f. Fig. 2) and only slightly decreases upon the addition of small amounts of PC. Again, this conducting state remains then constant over the same wide range of composition as the low-conductance state.

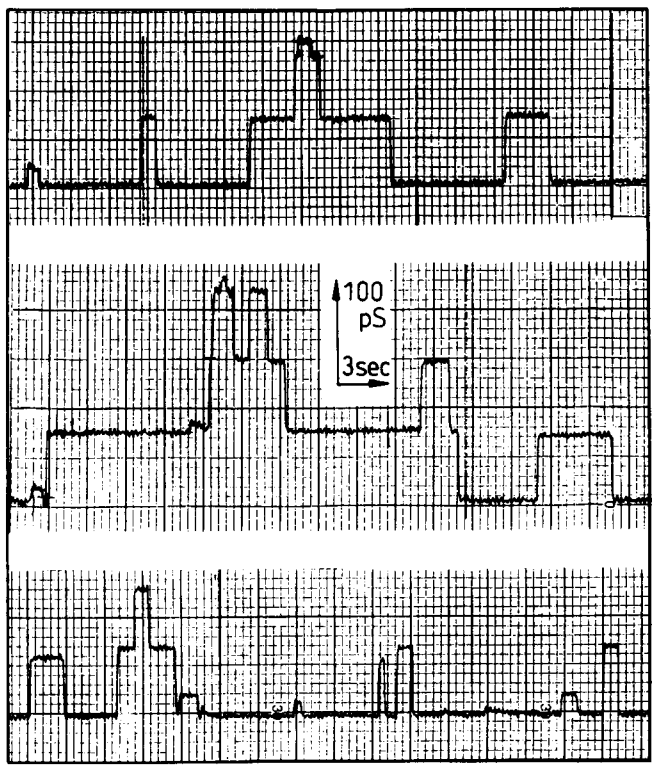

Fig. 4. Record of current fluctuations of gramicidin in a 1:1 (de)mixed PG/PC membrane after 10^{-3} M $CaCl_2$ were added to the electrolyte solution. Note the appearance of two types of channel with different conductance increments. Other parameters as in Fig. 1.

Another important piece of information can be derived from the statistical analysis of the channel lifetimes. Three examples of the probability distribution $P(\tau)$ are given in Fig. 7 for pure PC (Fig. 7a), pure PG (Fig. 7b) and a 1:1 mixture of both lipids (Fig. 7c). Some details are noteworthy: (1) The pure membranes show a probability distribution $P(\tau)$ indicating a homogeneous channel population (straight lines in Figs. 7a and 7b, respectively). (2) The mean lifetimes $\bar{\tau}$ thus derived are the same as found without Ca^{2+} (c.f. Fig. 3b). (3) The lifetime probability distribution of the gramicidin pores found in the $x = 0.5$ mixture was analysed separately for the low- and for the high-conductance state. Both populations give a straight line when $\ln P(\tau)$ is plotted versus τ although, of course, the low-conductance data have a considerable scatter due to the relatively low pore-formation probability.

The mean lifetimes, $\bar{\tau}$, thus derived are all plotted in Fig. 7d as a function of the PC mole fraction, x. It is interesting to note that for both pure lipids, when small fractions of the second component are added, the mean lifetimes first increase as in the case without Ca^{2+} (see Fig. 3b). Upon approaching the phase boundaries to the two-phase region a substantial destabilization of the gramicidin pores takes place. Within the coexistance range the two mean channel lifetimes remain constant within experimental error.

In order to test the influence of the solvent present in Mueller-Rudin type BLM [11] we performed a few test experiments with the virtually solvent free membranes prepared according to the Montal-Mueller technique [13]. The bilayers of the pure lipids showed only the well-known differences relative to the solvent-containing BLM. More important, and therefore presented in Fig. 8 for the case of a 1:1 mixed membrane under the influence of 10^{-3} M Ca^{2+}, is the finding that the phase separation phenomena are not depending on the presence of any solvent: We find the same bimodal conductance histogram though certainly with a higher fluctuation background (see Fig. 8 compared to Fig. 5) and possibly somewhat modified mean conductance values, $\bar{A}$. Also the mean channel life-

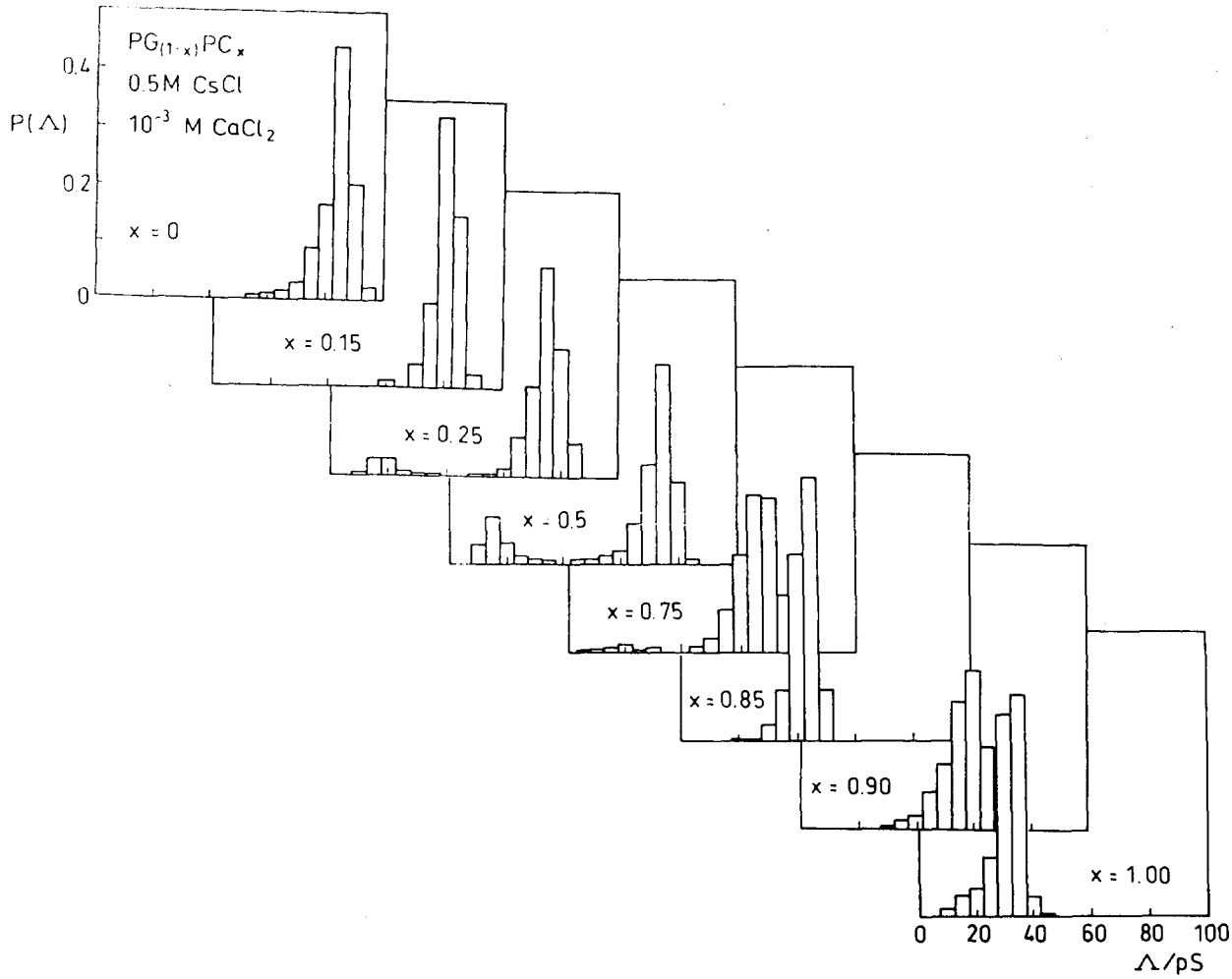

Fig. 5. Series of normalized conductance histograms of gramicidin in various $PG_{(1-x)}PC_x$ membranes taken in the presence of 10^{-3} M $CaCl_2$. Given is a selection of curves showing the monomodal channel distribution for membranes rich in either PC or PG. For intermediate mixtures, however, a (gradual) appearance of a bimodal distribution is evident.

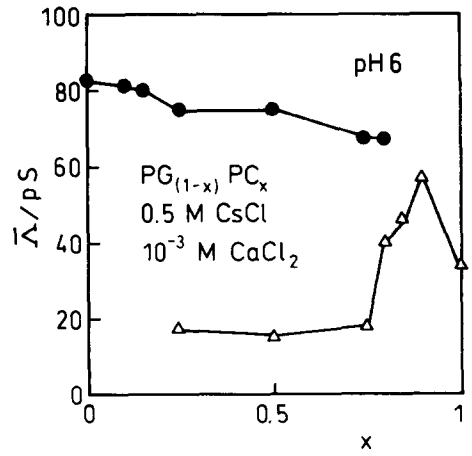

Fig. 6. Mean conductance increments, $\bar{\Lambda}$, by a single gramicidin pore in different PG/PC membranes in the presence of 10^{-3} M $CaCl_2$. Data are obtained from conductance histograms like the ones shown in Fig. 5 which give either a monomodal or a bimodal distribution. △, low-conductance state (pure PC, and PC-rich phases); •, high-conductance state (pure PG, and PG-rich phase). Lines are guides to the eye.

times were reduced: we found $\bar{\tau}_1 = 0.37$ s for the low-conductance state and $\bar{\tau}_2 = 0.7$ s for the high-conductance state, respectively. These minor differences found for the two membrane preparation techniques justify to concentrate on the Mueller-Rudin type membranes which are considerably easier to work with.

In order to elucidate the full Ca^{2+} concentration–composition phase diagram and in particular to find the onset concentration for phase separation we performed single-channel measurements as a function of the membrane composition for various Ca^{2+} concentrations. The mean conductance data derived from the statistical analysis of all these conductance increment measurements are depicted in Fig. 9: Fig. 9a shows the situation found for 10^{-2} M Ca^{2+} in the aqueous phase. All conductance values are clearly reduced relative to 10^{-3} M Ca^{2+} due to the onset of a blocking effect of Ca^{2+} as we will discuss in more detail below. Current fluctuations at 10^{-1} M Ca^{2+} could not be resolved anymore with our set-up. Another obvious difference

to the situation at 10^{-3} M Ca^{2+} is the increased range of composition for finding two channel populations: Phase separation is triggered already when only 10% of the second component are added to the pure membranes. When reducing the Ca^{2+} concentration to 10^{-4} M in the electrolyte (Fig. 9b) a phase separation behavior very similar to that at 10^{-3} M Ca^{2+} is found. The only obvious difference is a slight increase of $\bar{\Lambda}$ for the low conductance state.

The residual Ca^{2+}-impurity level of 0.5 M CsCl solutions (p.A. quality) is in the range of 10^{-6}–10^{-5} M. To reach this level we therefore only had to perform current fluctuation measurements without any extra Ca^{2+} but also without adding EDTA. The result which is obtained under these conditions is shown in Fig. 9c, and demonstrates a considerably reduced composition range where phase separation occurs: only between $x = 0.25$ and $x \approx 0.6$ two channel populations are found. $\bar{\Lambda}$ for the low-conductance state has further increased somewhat.

In order to bridge the gap between the results which show phase separation even at the lowest (p.A. impurity) Ca^{2+} concentrations and the data obtained in the presence of EDTA we performed a few measurements with suprapure CsCl salt. Its impurity level for Ca^{2+} is approx. 10^{-8} M. We depict in Fig. 10 only one example of a conductance histogram, obtained with an equimolar PC/PG membrane. A very broad conductivity distribution is found but no clear indication of a phase separated system. As in the case of earlier data with

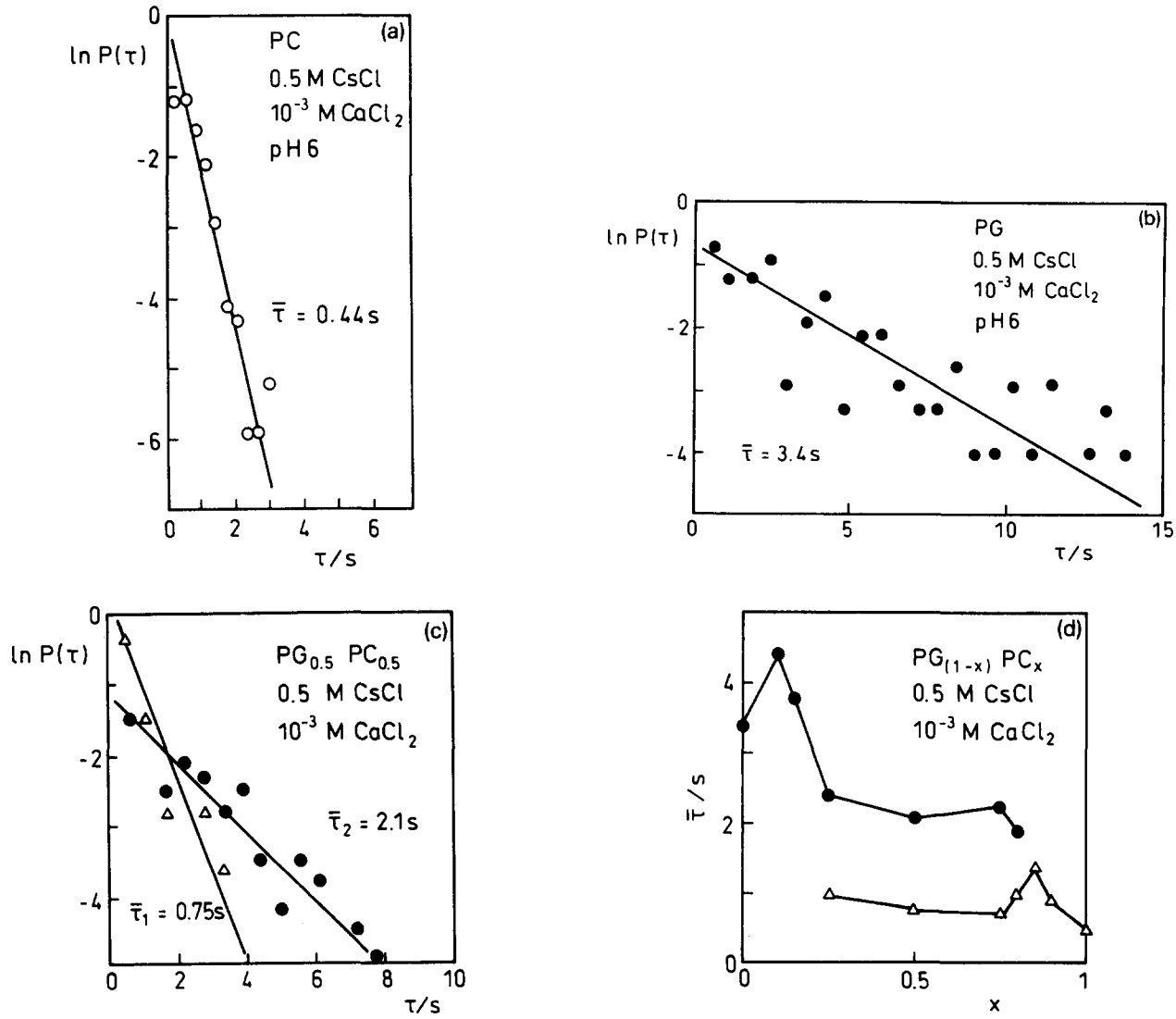

Fig. 7. (a)–(c) Semilogarithmic plots of the probability distribution, $P(\tau)$, to find a channel of lifetime, τ, in $PG_{(1-x)}PC_x$ membranes. From the straight line fit to the experimental data one obtains the mean lifetime, $\bar{\tau}$: (a) $x = 1$ (pure PC), $\bar{\tau} = 0.44$ s; (b) $x = 0$ (pure PG), $\bar{\tau} = 3.4$ s; (c) $x = 0.5$, $\bar{\tau}_2 = 2.1$ s and $\bar{\tau}_1 = 0.75$ s. (d) Mean channel lifetimes, $\bar{\tau}$, for gramicidin in partially demixed $PG_{(1-x)}PC_x$ membranes as obtained from fits like the ones presented in (a)–(c). Open triangles are for pure PC and PC-rich phases full circles for pure PG and PG-rich phases; full lines are guides to the eye.

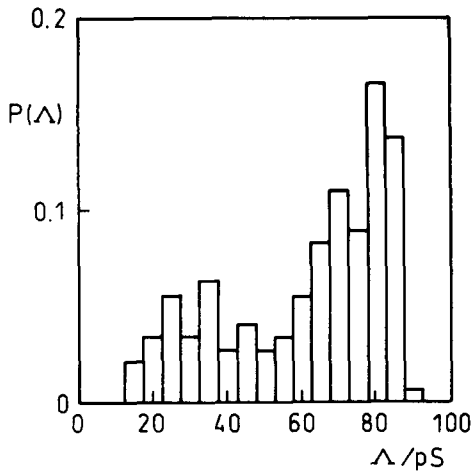

Fig. 8. Normalized conductance histogram of gramicidin current fluctuations in a 1:1 PG/PC membrane with 10^{-3} M $CaCl_2$ prepared by the Montal-Mueller technique [13]. Note the bimodal distribution which is, though, broader than found for Mueller-Rudin-type membranes [11] (c.f. Fig. 5).

mixed membranes of lecithin and charged phosphatidic acid we interpret this broadening as being caused by the induction of concentration fluctuations near the critical demixing point which marks the onset of a phase separated system [7]. A similar (second order) phase transition behavior has recently been found also in pH-dependent single-channel current fluctuation measurements [14].

Because of all these consistent results we are sure and also have proven that the gramicidin mixture (A, B and C) is able to act as a reliable and definite sensor for testing the lateral conformation of a membrane.

Discussion

(A) Homogeneous mixtures

In order to characterize the single-channel ion transport through a truely mixed binary PC/PG membrane we first carried out current fluctuation experiments in the absence of any divalent impurities by adding 10^{-4} M EDTA to the 0.5 M CsCl solution. As already deduced from small-angle neutron scattering experiments with fluid liposomal multilayers prepared from the same lipid mixture [3], in BLM, too, we find a complete miscibility over the full range of composition. This conclusion was inferred from the 'normal' conductance histograms found for all investigated mole fractions of the two mixed components. And also the single-exponential decrease of each of the channel lifetime probabilities pointed to this interpretation.

In view of the following conclusions concerning the Ca^{2+}-induced phase separation phenomena we need to discuss briefly the somewhat surprising result that the mean gramicidin conductance $\bar{\Lambda}$ in a pure PC membrane is approx. $\bar{\Lambda} = 40$ pS whereas in the highly negatively charged membrane of a PG-BLM only $\bar{\Lambda} = 80$ pS is found. Following a simple Gouy-Chapman model one might have expected a considerable enhancement of the interfacial Cs^+ concentration and hence a much larger conductance increment per chan-

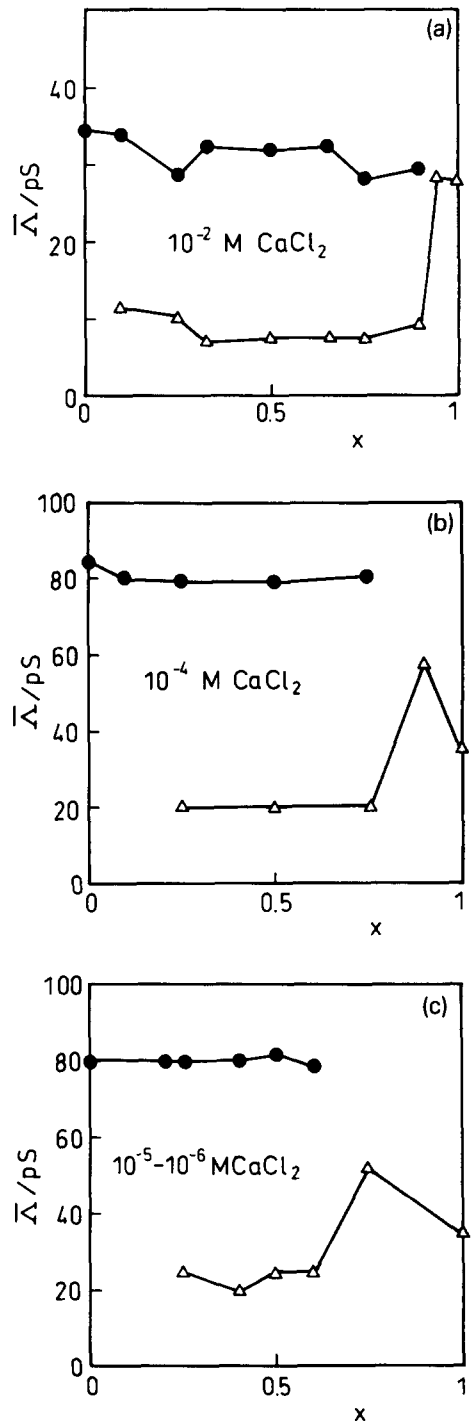

Fig. 9. Mean conductance increments, $\bar{\Lambda}$, by a single gramicidin pore in different $PG_{(1-x)}PC_x$ membranes, partially demixed in the presence of (a) 10^{-2} M $CaCl_2$, (b) 10^{-4} M $CaCl_2$, (c) 10^{-5}–10^{-6} M $CaCl_2$. See also Fig. 6.

nel for mixtures with increasing content of negatively charged PG. According to

$$c_s = c_\infty \cdot \exp(-z \cdot \psi_s \cdot F/RT) \quad (1)$$

with c_∞ = bulk ion-concentration, ψ_s = electrical potential difference between the membrane surface and the bulk solution far from the surface, z = valency of the transported ion (here $z_{Cs^+} \equiv 1$), F = Faraday constant, R = gas constant, and T = absolute temperature, the interfacial Cs^+ concentration is enhanced by a Boltzmann factor. The surface potential ψ_s, on the other hand, is related to the membrane charge density σ according to

$$\psi_s = 2\frac{RT}{F}\ln\left[\frac{\sigma}{\sigma_0} + \sqrt{\left(\frac{\sigma}{\sigma_0}\right)^2 + 1}\right] \quad (2)$$

with $\sigma_0 = \sqrt{8\epsilon_0 \epsilon RTc}$, $\epsilon_0 = 8.85 \cdot 10^{-12}$ $CV^{-1}m^{-1}$ the permittivity of free space, ϵ the dielectric constant of water. Increasing amounts of PG added to PC and mixed homogeneously therefore should have a strong non-linear enhancing effect on the interfacial Cs^+ concentration. As discussed already by Apell et al. [15] a true quantitative description of the experimental situation is complicated by various deviations from the simple picture outlined above. However, these authors, too, found for high bulk Cs^+ concentrations a very limited enhancement of Λ for (negatively charged) phosphatidylserine membranes as compared to PC (lecithin) and attributed this saturation effect to the fact "that the channel can accomodate only a limited number of ions" [15]. If we assume an average area per lipid molecule of about $A \approx 0.60$ nm^2 we obtain a maximum surface charge density for a pure PG membrane of $\sigma = 0.27$ Cm^{-2}. Any $PC_xPG_{(1-x)}$ mixed membrane has a correspondingly lower charge density. If we calculate now for the various mole fractions x the hypothetical Cs^+ concentration at the interface (i.e., at the entrance to the channel) and plot the experimentally determined conductance increments of the gramicidin pore, $\bar{\Lambda}_x$, relative to that of the (uncharged) PC membrane, $\bar{\Lambda}_{PC}$ (see Fig. 11), this saturation behavior is clearly demonstrated. (A more pronounced enhancement, of course, would have been found, had we worked at a lower Cs^+ concentration in the bulk.) It is important to note that for these considerations, we have to take into account only the Cs^+ ions because no divalent ions that could respond to the charges at the interface are present. When, however, Ca^{2+} ions are added with their intrinsically much stronger response (i.e., accumulation) to a negatively charged surface it will depend very much on the respective pre-exponential factors in Eqn. 1, i.e., the relative bulk concentrations as to whether the monovalent Cs^+ ions or the divalent Ca^{2+} ions dominate the Poisson-Boltzmann behavior at that membrane/electrolyte interface.

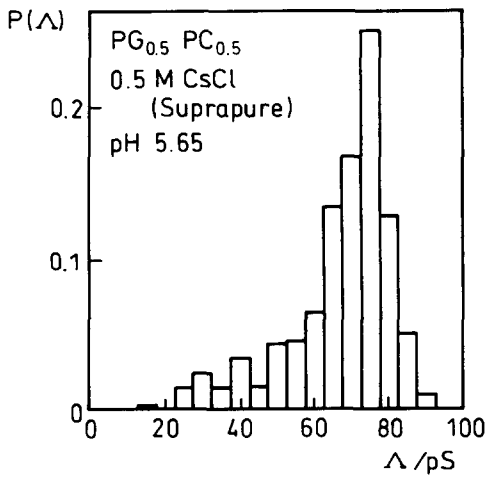

Fig. 10. Normalized conductance histogram of gramicidin current fluctuations in a 1:1 PG/PC mixed membrane with 0.5 M CsCl of suprapure quality in the electrolyte.

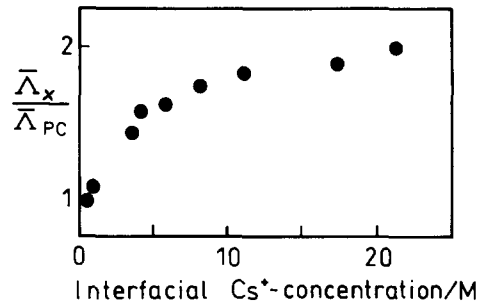

Fig. 11. Conductance increase of single gramicidin channel, $\bar{\Lambda}_x$, in various $PG_{(1-x)}PC_x$ mixed membranes (relative to that of a pure PC membrane, $\bar{\Lambda}_{PC}$ as a function of the (hypothetical) interfacial Cs^+ concentration calculated according to Eqns. 1 and 2 for a charge density varying as given by $\sigma_x = -0.27 \cdot (1-x)$ Cm^{-2} linearly with the mole fraction, x, of PC.

Finally, an interesting result obtained with homogeneously mixed membranes concerns the increased pore stability for certain mixtures (see Fig. 3b): particularly the 1:2 PC/PG system shows a significantly longer lifetime for the gramicidin pore. Little is known about the correlation between general membrane properties and the stability of incorporated pore-forming peptides. Neher and Eibl [16] had suggested that the surface tension γ might be a relevant parameter for the prediction of lifetime values. It seems to be at least conceivable that a certain (stoichiometric) complex formation between the components of special mixtures leads to a particularly low-energy surface with a low interfacial tension as has been reported for some lipid mixtures on the basis of Langmuir-monolayer studies [17]. This, in turn, would stabilize the conducting dimer state of gramicidin.

(B) Demixed systems

The induction of phase separation in binary lipid mixtures by charged species is a complex interplay of transported ions, Cs^+ in our case, the PG mole fraction dependent negative surface charge density of the membrane, and the actual demixing agent, Ca^{2+} in this study. A quantitative description would require more information than is available of the various interaction potentials. A qualitative understanding, however, is possible within the framework of the Poisson-Boltzmann theory. We will discuss this on the basis of the results given in Figs. 5, 6, and 7, respectively, obtained with 10^{-3} M $CaCl_2$. This concentration is still sufficiently low so that blocking of gramicidin channel [18] does not occur (see below).

For pure PC we find a single pore population with a mean conductivity of $\bar{\Lambda} \approx 38$ pS. Small amounts of PG introduce a negative surface potential and hence an enhancement of the interfacial Cs^+ concentration – the conductivity increases as in the absence of divalent Ca^{2+}. At a certain PG content, however, the membrane charge density has increased to a point where the larger exponential factor in Eqn. 1 for the interfacial concentration of divalent Ca^{2+} has overcompensated the larger pre-exponential factor of Cs^+ (0.5 M). The Ca^{2+} concentration at the membrane, therefore, has reached a level where blocking of the conducting channel sets in [18]. This is clearly seen in Fig. 5 for $x = 0.85$: we still find a monomodal conductance histogram though with a reduced mean conductivity. (The difference of the channel conductance in the presence or absence of EDTA for low Cs^+ concentrations ($c_\infty < 10^{-3}$ M) found by Apell et al. [15] with charged phosphatidylserine membranes can be explained along the same lines).

Now, it takes only little more PG and hence only a slightly higher Ca^{2+} concentration [19] to trigger the onset of phase separation by crossing the phase-boundary to a two-phase region, which manifests itself by a bimodal conductance histogram (see $x = 0.75 \ldots 0.80$ in Figs. 5 and 6). Once this phase coexistance is reached a further increase of the PG content does not change the electrostatics at the membrane interface anymore but changes only the relative amount of the two coexisting phases according to the lever rule (see below).

The onset of the phase separation coming from PG-rich membranes is less pronounced when observed by the analysis of single-channel current fluctuations because the pure PG membrane with its high negative surface charge density has driven the gramicidin channel completely into its current saturation. A slight dilution of these surface charges by PC molecules does not change this situation significantly. At some point the solubility limit of PC in PG is reached and a second PC-rich phase appears.

The analysis of the channel lifetimes seems to indicate more clearly the near phase boundary when adding little PC to PG membranes (cf. Fig. 7d): Above $x = 0.10$ a drastic decrease of $\bar{\tau}$ points to a destabilization of the lipid mixture as one approaches the two phase region. This behavior is significantly different from the one found for homogeneous mixtures in the absence of Ca^{2+}.

This interpretation of the blocking and demixing effect of Ca^{2+} concentration, enhanced at the interface by increasing amounts of negative surface charges, is supported by the results found for lower Ca^{2+}-bulk concentrations: at 10^{-5}–10^{-6} M $CaCl_2$ the conductance increase by the enhancement of the Cs^+ ions at

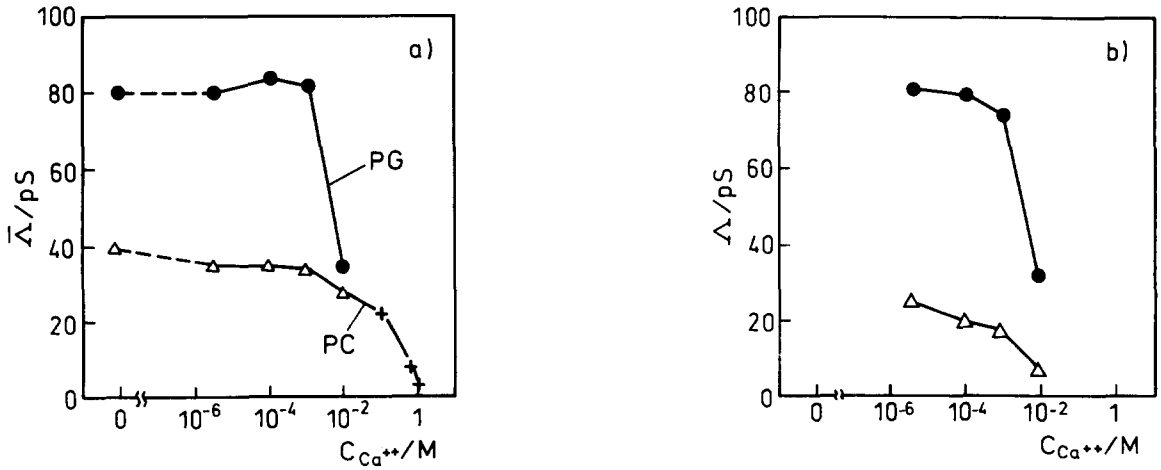

Fig. 12. (a) Blocking-effect of Ca^{2+} on the mean conductance increment, $\bar{\Lambda}$, of gramicidin single channel in pure PG (●) and pure PC (△) membranes, respectively. Given are also data points taken from Ref. 18 (+). Lines are only guides to the eye. (b) As in (a) but for the two channel population found for a 1:1 (de)mixed PG/PC membrane. $c_{Cs^+} = 0.5$ M, $T = 22$°C, pH 6, $U = 100$ mV.

increasing PG concentrations up to $x = 0.75$ in the membrane is virtually the same as in the absence of Ca^{2+} and it takes by far more negative surface charges to 'activate' the blocking and demixing influence of Ca^{2+} (see Fig. 9c). The result is a phase boundary which is located at a significantly lower PC content.

At higher Ca^{2+} concentrations, on the other hand, e.g., at 10^{-2} M $CaCl_2$, already the pure PC membrane shows some blocking of the gramicidin channel (see Fig. 9a). The addition of some 5 mol% PG, therefore, does not induce a higher conductance by the enhancement of the interfacial Cs^+ level, but brings the system close to the demixing point which is reached at $x = 0.90$, considerably higher than for lower Ca^{2+} concentrations. The blocking effect of Ca^{2+} which always precedes the demixing process is summarized in Fig. 12. Fig. 12a shows the single-channel conductance of gramicidin in pure PG (full circles) and pure PC (open triangles) as a function of the bulk Ca^{2+} concentration when added to 0.5 M CsCl. Our data can be nicely extended by values taken from the work of Bamberg et al. [18]. While the PC values show a smooth increase of the blocking influence of Ca^{2+}, the PG data with the abrupt decrease of $\bar{A}$ between 10^{-3} and 10^{-2} M $CaCl_2$ indicate the competitive, non-linear enhancement of the monovalent Cs^+ ions and the divalent Ca^{2+} ions. Fig. 12b summarizes the same dependence for channels in the two phases within the coexistance range. Their blocking behavior further supports our interpretation that the high-conductance state is in the PG-rich phase whereas the low-conductance state originates from pores in PC-rich domains.

(C) The Ca^{2+} concentration–composition phase diagram

All the above discussed assignments between channel populations and (coexisting) phases are summarized in Fig. 13 where we have plotted the Ca^{2+} concentration–composition phase diagram. At very low Ca^{2+} levels we find a homogeneous mixture of PC and PG over the full range of composition.

At surprisingly low Ca^{2+} concentrations a destabilization of the membrane can be observed which already at about 10^{-6} M Ca^{2+} leads to a demixed two-phase system. In view of the physiological concentrations of Ca^{2+} in a living cell, the above observed phase separation phenomena cannot be put aside as artefacts caused by too high Ca^{2+} concentrations in a model membrane system. The shape of the phase boundaries can be understood qualitatively on the basis of the Poisson-Boltzmann description of the electrostatic interaction of ions with a charged membrane surface. It should be pointed out that details of this phase diagram would also depend on the temperature, the pH, the ionic strength, i.e., the concentration of the transported ions and other non-transported ions, and

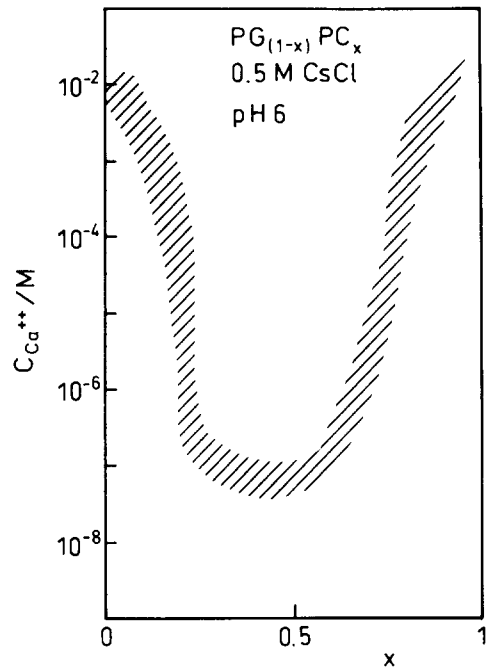

Fig. 13. Ca^{2+} concentration–compositon phase diagram for $PG_{(1-x)}PC_x$ membranes. $T = 22°C$.

on their chemical nature. Given the example of Ca^{2+} discussed in this paper in some detail it seems to be clear that phase separation in mixed membranes is an ubiquitous phenomenon also in the model system BLM which, therefore, is best suited to study the coupling of this aspect of lateral membrane organization on membrane function.

Acknowledgement

This work was financially supported by the Deutsche Forschungsgemeinschaft (KN 224/2-1).

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