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Direct evidence for Ca^{++}-induced lateral phase separation in black membranes of lipid mixtures by the analysis of gramicidin A single-channels

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Abstract. Single-channel conductance fluctuations are analysed for gramicidin A incorporated into binary-mixed black lipid membranes of charged phosphatidic acid and neutral lecithin in different molar ratios. At very low Ca^{++} concentrations in the electrolyte (i.e. in the presence of EDTA) homogeneous lipid mixtures are identified through their conductance and life time probability distributions for integral gramicidin pores. As for the pure lipid components, the conductance histograms each show a single maximum with regular width and for all channels a single mean lifetime is found.

For Ca^{++}-levels ($10^{-6}-10^{-5}M$) that are close to the critical demixing concentration ($\sim 10^{-4}M$) unusually broad conductance distributions and reduced lifetimes are found provided the PC content, x, of the membrane is close to the critical mixture ($x_{crit} \approx 0.5$). We interpret this as a first example of the coupling of a membrane function (the transport of ions) to a lipid matrix with locally fluctuating composition close to a critical demixing point.

For $c_{CA^{++}} = 10^{-2}M$ the conductance histogram of gramicidin A in an equimolar mixture of PA and PC shows two well-separated maxima. A correlation analysis between conductance and lifetime of the single pores shows that the two channel populations also differ significantly in their mean channel lifetime, τ^*. This finding is interpreted as being direct evidence for Ca^{++}-induced lateral phase separation in black lipid membranes, as has been postulated recently.

Key words: Black lipid membranes, lipid mixtures, critical concentration fluctuations, Ca^{++}-induced phase separation, gramicidin single-channel conductance

Abbreviations used: HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid; EDTA, ethylenediaminetetraacetic acid

Introduction

One of the striking effects of Ca^{++} ions on artificial membranes is the isothermal induction of lateral phase separation (Ito and Onishi 1974; Galla and Sackmann 1975). The question that remains to be answered, however, is how such structural reorganisations in membranes may influence integral functional units and hence may be of physiological relevance to biological membranes. To answer this question, we studied recently how the electrical characteristics of the ion carrier valinomycin (Schmidt et al. 1982) and the hydrophobic ion dipicrylamine (Miller et al. 1985) incorporated into bimolecular lipid membranes (BLM) of lecithin mixed with phosphatidic acid are modified by the addition of Ca^{++} ions. By the analysis of voltage jump-current relaxation experiments we could show that in these model membranes phase separation can be triggered by Ca^{++}. We could determine phase boundaries in the demixed region of the phase diagram and could investigate how the two transport systems couple to the changed lateral lipid distribution in the membrane.

These results, however, were mostly obtained under conditions where the current through one of the two coexisting phases could be neglected because of a negligable conductivity and/or a sufficiently small area fraction of that phase. This was necessary because for valinomycin, for example, the simultaneous analysis of current contributions through two coexisting phases would have required the resolution of four exponential decays of the current relaxation after a voltage jump, which is impossible with sufficient accuracy. And in the case of the relaxation processes of dipicrylamine, deviations from exponential behavior even for a homogeneous phase (Jordan and Stark 1979) set limits for the

analysis of two current contributions (Miller et al. 1985).

It was therefore desirable to study the electrical properties of a poreforming antibiotic. The possibility of resolving the characteristics of single channels in different environments should in principle allow the detection of two coexisting phases provided that the ion transport through the pores in the two membrane areas of different composition are sufficiently distinguishable from each other. The cation permeable poreformer gramicidin was chosen as an appropriate probe for this purpose since on the one hand its conducting state is characterized by a relatively narrow single peaked conductance probability distribution and an easily obtainable mean lifetime, τ^* (Hladky and Haydon 1970; Bamberg and Läuger 1973; Apell 1978), and on the other hand its single channel conductance in uncharged and charged membranes is significantly different (Apell et al. 1979).

We will show in this paper that it is indeed possible to identify two different channel populations of gramicidin A in a binary mixed membrane which is interpreted as a direct demonstration of Ca^{++}-induced lateral phase separation. Moreover, we present evidence that this destabilization of the lipid mixture is manifested by critical concentration fluctuations at very low Ca^{++}-levels, which broadens the conductance distribution and considerably reduces the lifetimes of the single channels.

Experimental

The lipids used in this study – 1,2-dipentadecyl-methylidene-glycero-3-phosphorylcholine (PC) and 1,2-dipentadecyl-methylidene-glycero-3-phosphatidic acid (PA) were chromatographically pure (Eibl and Niksch 1978). Membranes were formed by the method of Mueller et al. (1962) from 1% (wt/vol) lipid solutions in n-decane (Fluka, purum).

Membrane areas were typically 5×10^{-4} cm^2. All experiments were performed at $T = 36$ °C where all lipids are fluid (Blume and Eibl 1981). The electrolyte solutions contained 0.5 M CsCl, 10 mM HEPES (pH 6, adjusted by NaOH and HCl), for the phase separation studies it also contained 10^{-2} M $CaCl_2$. For some experiments 10^{-4} M EDTA was added to the aqueous phase.

The mean single-channel conductance, $\bar{\Lambda}$, and mean lifetime, τ^*, of gramicidin A was measured as described previously (Bamberg et al. 1976; Apell 1978). The records of conductance fluctuations were analyzed by digitizing the height and the lifetime of each single event and storing both parameters in a micro-computer (Apple II). It was thus possible to analyse the data not only with respect to the conductance and lifetime probability distribution but it also allowed us to search for correlations between the conductance and the lifetime of the pores.

Results

In a first series of experiments we determined the single channel characteristics of gramicidin A in mixed membranes of negatively charged phosphatidic acid and neutral lecithin. Figure 1 shows an example of the current fluctuations in a 1:1 mixture of the two lipids. The aqueous phase contained 10^{-4} M EDTA in addition to 0.5 M CsCl. By analysing several hundred single pores with respect to their current increment a probability distribution, $P(\Lambda)$, of the single channel conductance, Λ, is obtained, as shown in Fig. 2a ($x = 0.5$). By comparison with the histograms found for the pure lipid

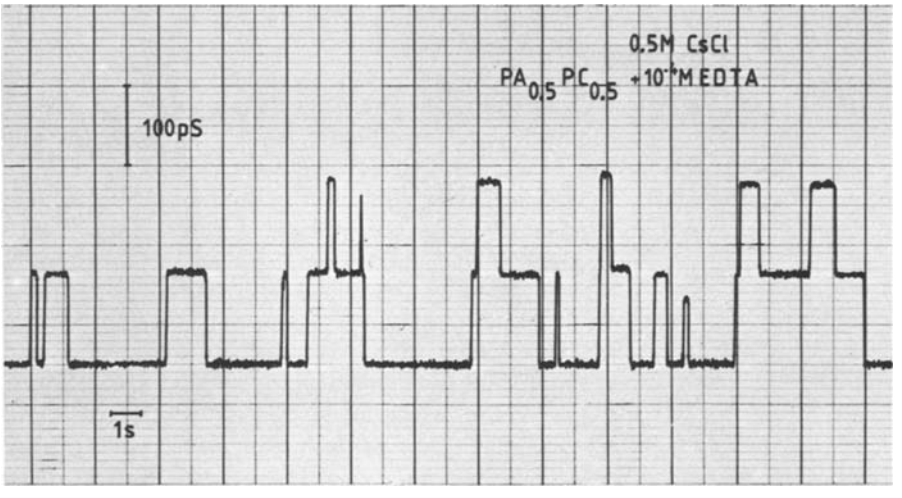

Fig. 1. Record of conductance fluctuations of gramicidin A in a 1:1 mixed membrane of PA and PC. The electrolyte contained 0.5 M CsCl and 10^{-4} M EDTA. T = 36 °C, pH 6. The full bars indicate the conductance scale of the ordinate and the time scale of the abscissa, respectively. The applied membrane voltage was $U = 100$ mV

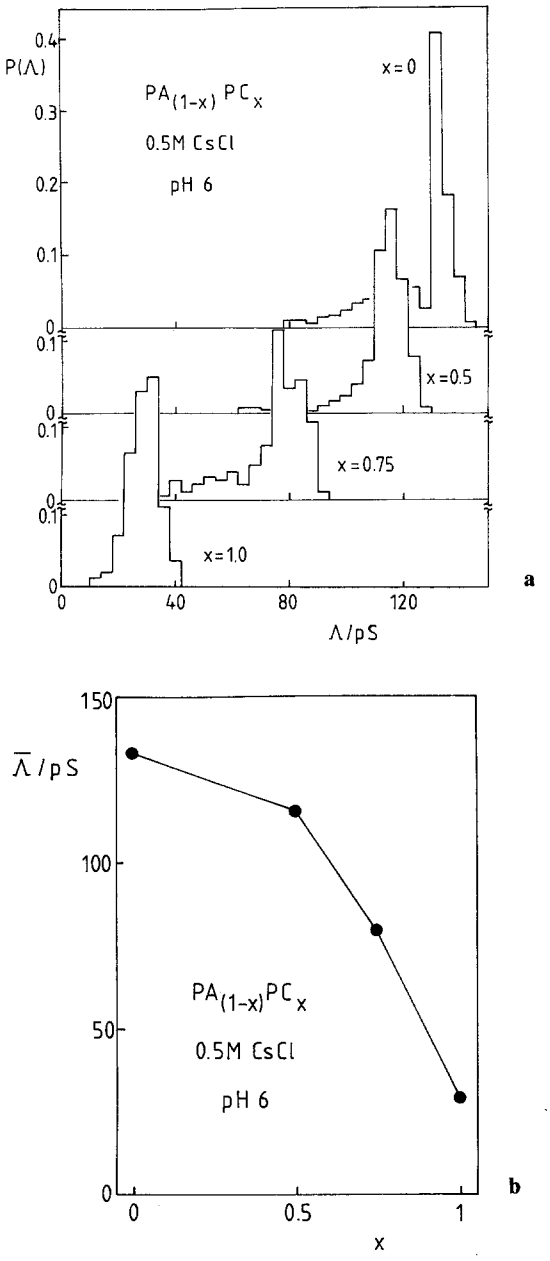

Fig. 2. **a** Normalized conductance histogram of mixed PA/PC-membranes with different mole fraction, x, of PC. 0.5 M CsCl + 10^{-4} M EDTA, 10 mM HEPES, pH 6, $T = 36$ °C, $U = 100$ mV. **b** Mean conductance increment, $\bar{\Lambda}$, by a single gramicidin pore in different mixed PA/PC-membranes. x denotes the mole fraction of PC. $\bar{\Lambda}$ is obtained from the histograms presented in **a**

components (also shown in Fig. 2a: $x = 1.0$ for pure PC membranes and $x = 0$ for pure PA) no marked difference is detectable for the width and the maximum height of the distribution. The same also holds true for other mixtures, e.g., for a 3:1 PC:PA-mixture ($x = 0.75$ in Fig. 2a).

The mean single channel conductance, $\bar{\Lambda}$, derived from the presented histograms is plotted in Fig. 2b as a function of the mole fraction, x, of PC in the membranes. The decrease of $\bar{\Lambda}$ with increasing PC content is expected for 0.5 M CsCl as the cation enrichment at negatively charged interfaces is more and more reduced as the surface charges of the PA headgroups get diluted by the PC molecules. Similar behavior has been reported for diphytanoyl-phosphatidyl-serine membranes mixed with choline (Apell et al. 1979).

Another important experimental parameter of single channels is their mean lifetime, τ^*. This quantity, which is also very sensitive to different membrane properties (Neher and Eibl 1977), can be obtained by analysing many single pores with respect to their lifetime, τ. Figure 3a shows, for a 1:3 PA:PC mixed membrane, the semilogarithmic plot of the probability, $P(\tau)$, of finding a gramicidin A channel with lifetime, τ. From the slope of the straight line a mean lifetime, $\tau^* = 900$ ms is derived.

Figure 3b shows τ^* for the mixed and the two pure membranes as a function of the PC mole fraction, x. While for PC rich membranes ($x > 0.5$) a constant mean lifetime independent of the composition is found, the channel seems to be somewhat destabilized in PA-rich environments.

Most interesting are the differences of the single channel characteristics found for mixed membranes depending on whether EDTA was added to the electrolyte or not. Figure 4a shows the effect for an equimolar mixture of PC and PA. The shaded histogram is obtained if all divalent impurities are complexed by 10^{-4} M EDTA in the aqueous phase. If the same experiment is performed without EDTA a very unusual broad distribution of different single pore conductivities is found (full line in Fig. 4a). Similar behavior was found for diphytanoyl-phosphatidyl-serine: choline mixed membranes (H. J. Apell, unpublished). Parallel to this finding a destabilization of the conducting state of the gramicidin pore is observed. This is demonstrated in Fig. 4b. While in the presence of EDTA, $\tau^* = 950$ ms is obtained (open circles), the mean channel lifetime is reduced to $\tau^* = 520$ ms (full circles) if EDTA is absent. Since for each channel, we measure its conductance and its lifetime simultaneously we can also analyse the data with respect to correlations between the two parameters. For the broad conductance distribution presented in Fig. 4a, however, all channels are characterized by the same mean lifetime, irrespective of their different conductances.

If the same experiments are performed with a 3:1 PC:PA mixed membrane no differences are found between EDTA-containing or EDTA-free electrolytes: both the mean conductance (and the width of the histogram) and the mean channel lifetime are unaffected.

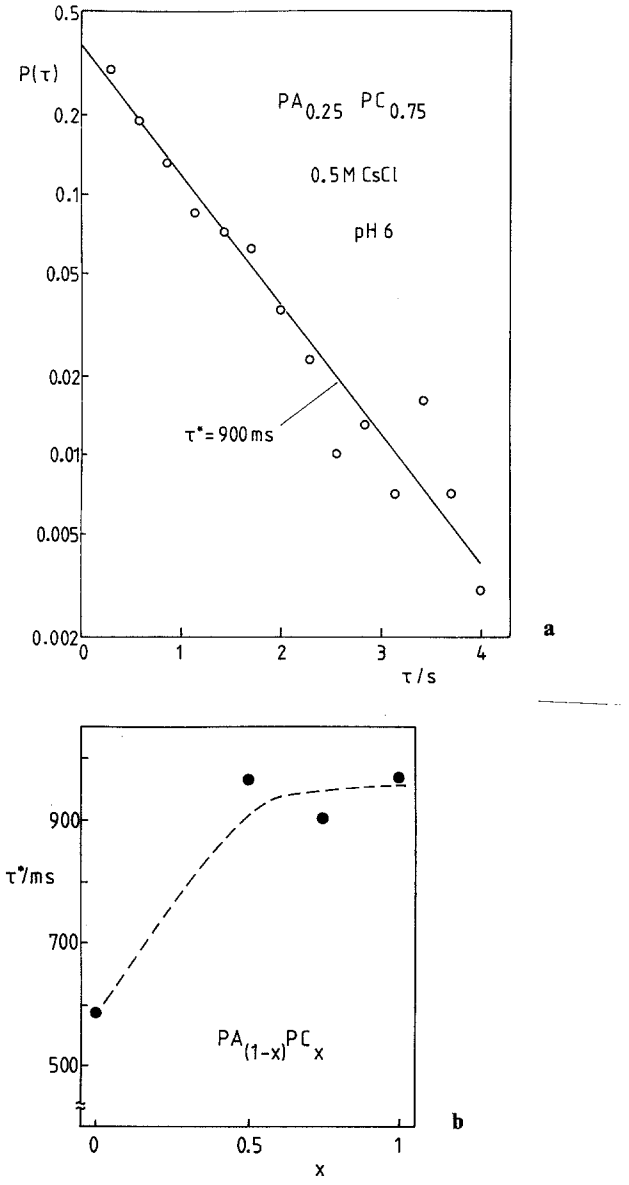

Fig. 3. **a** Semilogarithmic plot of the probability distribution, $P(\tau)$, of finding a channel of lifetime, τ, in 1:3 mixed PA/PC-membranes. From the straight line a mean channel lifetime, τ^*, is obtained: $\tau^* = 900$ ms. **b** Mean channel lifetime, τ^*, for gramicidin A in PA/PC-mixed membranes of different mole fraction, x, of PC. τ^* is obtained from plots like those presented in **a**. The line represents an eye guide. All other conditions as in Fig. 2

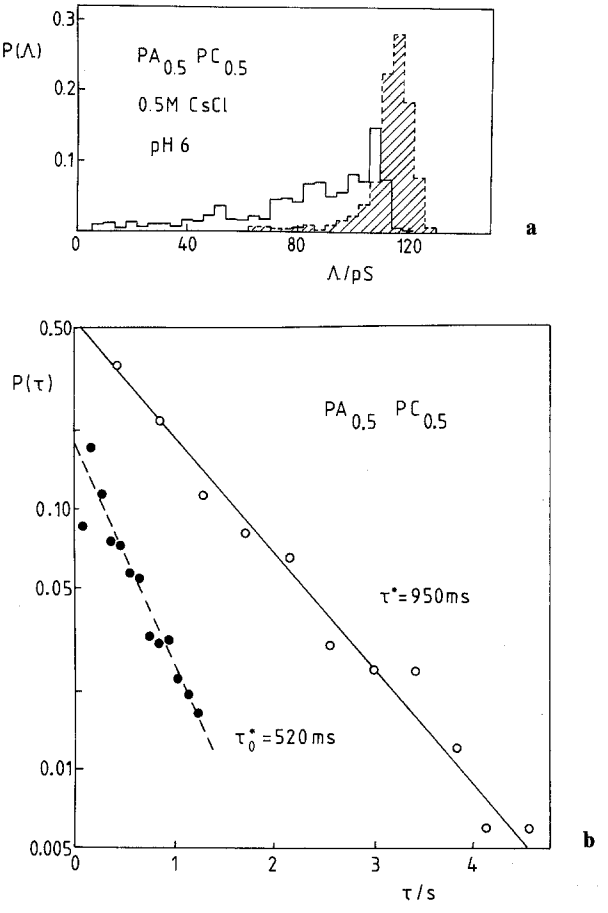

Fig. 4 a and b. Single channel properties of gramicidin A in a 1:1 mixed PA/PC membrane without and with $10^{-4}M$ EDTA. 0.5 M CsCl, 10 mM HEPES (pH 6), $T = 36\,^{\circ}$C, $U =$ 100 mV. **a** Normalized conductance histogram (shaded area: with EDTA). **b** Lifetime probability distribution (-○- $10^{-4}M$ EDTA, -●- without EDTA)

The main aim of the present study was to test whether gramicidin is capable of monitoring the Ca^{++}-induced phase separation in mixed membranes through different single channel characteristics in the two coexisting phases. For that purpose we monitored the current fluctuations in a 1:1 PC:PA membrane in the presence of $10^{-2}M$ Ca^{++}, where phase separation is expected (Miller et al. 1985). Figure 5 a shows an example. Quite obviously, two states with distinctly different conductivities coexist. The two kinds of channels open and close independently of each other and each exists separately as well as simultaneously. Figure 5 b (histogram ④) shows the histogram obtained by summing up all single events of the membrane partly presented in Fig. 5 a. Two well separated but otherwise regularly broad peaks in the conductance probability distribution can be seen. Not all membranes studied showed the two channel populations with equal frequency. In some cases the lower conducting state was found almost exclusively (histogram ②), whilst some membranes showed only the high single-channel conductance (histogram ③). The sum of all 9 membranes investigated (about 1,000 events) yields the histogram labelled ① in Fig. 5 b.

If these two channel populations are analysed with respect to their mean lifetimes, τ^*, it is seen that they also differ in this parameter, as is demonstrated in Fig. 5 c. The mean lifetime of all channels with conductivities between 0 and 16 pS is $\tau^* =$

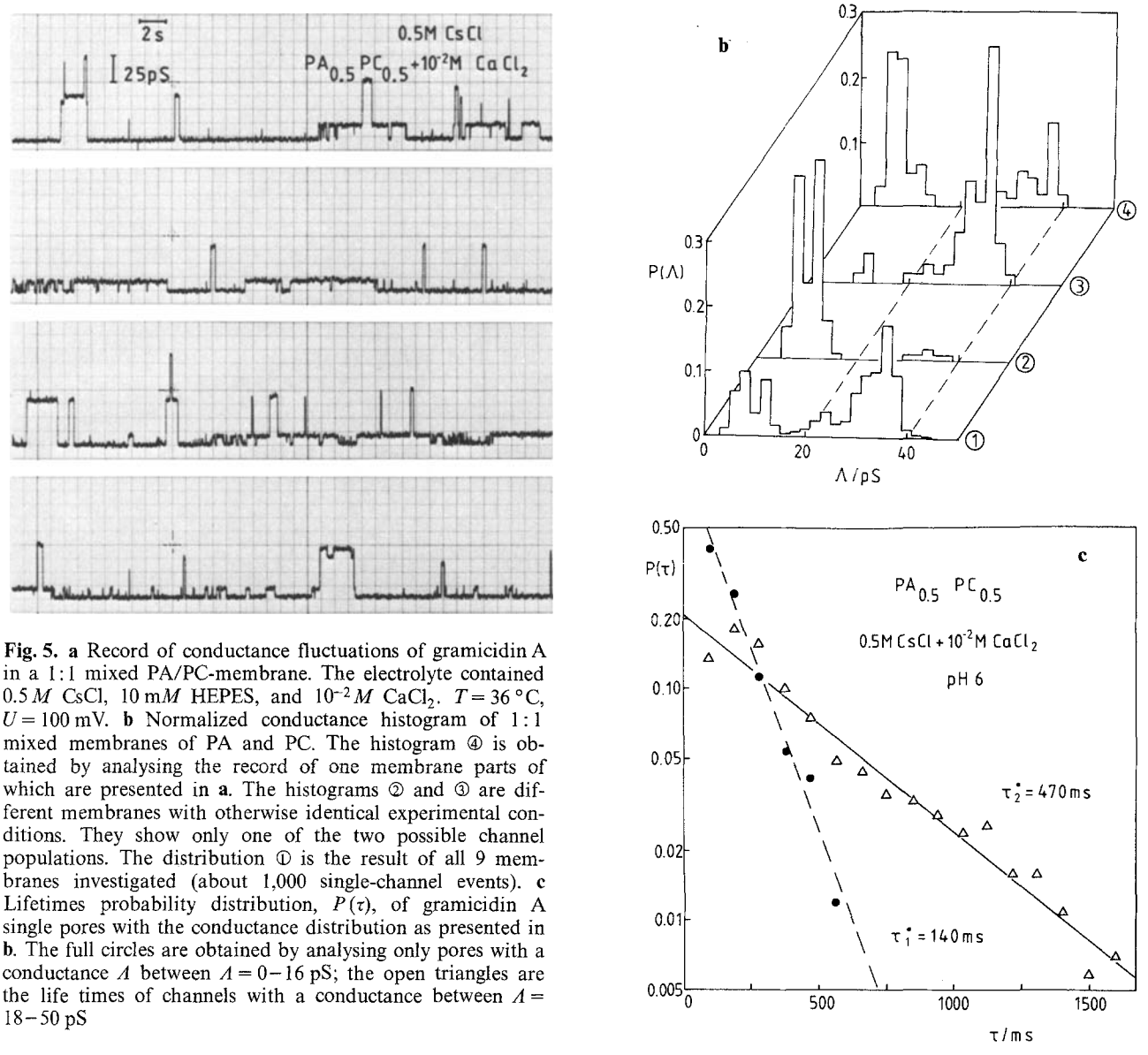

Fig. 5. **a** Record of conductance fluctuations of gramicidin A in a 1:1 mixed PA/PC-membrane. The electrolyte contained 0.5 *M* CsCl, 10 m*M* HEPES, and 10^{-2} *M* $CaCl_2$. $T = 36\,°C$, $U = 100$ mV. **b** Normalized conductance histogram of 1:1 mixed membranes of PA and PC. The histogram ④ is obtained by analysing the record of one membrane parts of which are presented in **a**. The histograms ② and ③ are different membranes with otherwise identical experimental conditions. They show only one of the two possible channel populations. The distribution ① is the result of all 9 membranes investigated (about 1,000 single-channel events). **c** Lifetimes probability distribution, $P(\tau)$, of gramicidin A single pores with the conductance distribution as presented in **b**. The full circles are obtained by analysing only pores with a conductance Λ between $\Lambda = 0-16$ pS; the open triangles are the life times of channels with a conductance between $\Lambda = 18-50$ pS

140 ms (full circles), while those between 18 pS and 50 pS have $\tau^* = 470$ ms in the conducting state (open triangles).

Discussion

From the analysis of voltage-jump, current-relaxation studies of the ion-carrier, valinomycin, and the hydrophobic ion, dipicrylamine, the phase behavior of PA/PC mixtures as a function of the Ca^{++}-ion concentration was deduced to be as depicted in Fig. 6. With no Ca^{++} ions in the electrolyte, a homogeneous mixture of the two lipids was found whereas, at higher Ca^{++} concentrations – starting at about 10^{-4} *M* at pH 6 – a demixing into two phases of different composition was observed (see the shaded area in Fig. 6). The two coexisting phases were identified through their different influence on the kinetic and stationary electrical properties of incorporated ionophores. Although the phase-separating action of Ca^{++} ions is well established for vesicular systems (Galla and Sackmann 1975; Hartmann et al. 1977), these results were the first evidence that such phenomena can also be studied in black lipid films as model membranes. Moreover, the observation of the response of integral ion transport systems to the induction of phase separation allowed, for the first time, a detailed analysis of an example of the important structure – function relationship in membranes.

The observation of two populations of gramicidin A single-channels characterized by distinctly different conductances and mean lifetimes as shown in

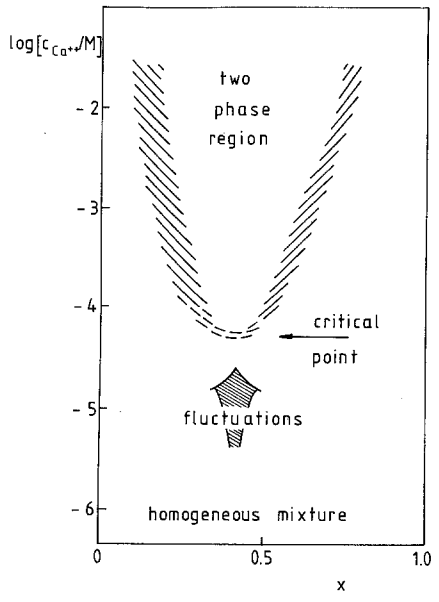

Fig. 6. Phase diagram for PA/PC binary mixed membranes at different Ca^{++}-concentrations in the aqueous phase. The homogeneous mixture at $c_{Ca^{++}} = 0$ and the two-phase region for $c_{Ca^{++}} > 10^{-4} M$ (shaded boundaries) were also obtained from voltage-jump, current-relaxation studies of valinomycin and dipicrylamine. The critical point that closes the coexistence region induces lipid concentration fluctuations at very low Ca^{++} concentrations that are peaked at the critical concentration

this paper provide further evidence for the induction of phase separation by Ca^{++}. The simultaneous recording of current fluctuations with two different characteristics is interpreted as being caused by gramicidin in two different membrane areas – the two coexisting phases in the demixed black membrane.

Since one observes on the average only a few molecules, it is also reasonable that some membranes show only the characteristics of gramicidin in one of the two phases (see Fig. 5 b). In this case the pool out of which the conducting channel is formed is only large enough in one phase. Because of the fact that this phenomenon occurs in both phases and with a high formation probability, an aggregate formation of gramicidin could explain this observation, as is claimed by Stark et al. (1984, submitted).

The fact that single-channel measurements in demixed membranes have not been reported so far may be because of the choice of the lipids used. Other reasons may be a preference of gramicidin for one of the coexisting phases, or an insufficient conductance difference between the two populations, which is amplified by a stronger Gouy-Chapman effect on Ca^{++} ions in the charged lipid phase of the bilayer, or a too low mean conductance of one distribution that prevents its detection.

The transition from the homogeneous mixture to the demixed region is expected to be of second-order (Landau and Lifschitz 1966). For the critical mixture, x_{crit}, strong concentration fluctuations should be observed approaching the critical point. This is schematically indicated in Fig. 6 by the thick arrow. For incorporated ionophores a strongly fluctuating composition of the lipid matrix would result in varying electrical properties of the single channels. In our opionion, our data present strong evidence that these phenomena can, indeed, be observed by single-pore analysis. At very low Ca^{++} concentrations, i.e. in the presence of EDTA, all mixtures show regularly narrow probability distributions of the single-channel conductance of incorporated gramicidin. For the 1:1 mixture, however, in the absence of EDTA and hence in the in presence of divalent impurities in the concentration range between 10^{-6} and $10^{-5} M$, which might be sufficiently close to the critical concentration, the conductance distribution is drastically broadened and the mean channel lifetime is considerably reduced. We interpret this as a direct consequence of the fluctuating lipid membrane, which represents a very heterogeneous matrix for integral transport systems. This would be expected on the basis of the proposed phase diagram which suggests the critical mixture to be near $x = 0.5$. For other mixtures far away from x_{crit}, e.g., $x = 0.75$, no difference should exist between the conductance distribution and mean lifetime with and without EDTA in the aqueous phase. This is exactly what is found experimentally.

It is conceivable that the fluctuation phenomena of PA/PC membranes described can only be detected by single-pore experiments because a multi-ionophore experiment, such as studies with valinomycin and dipicrylamine, sum up all different 'sites' for the single molecules and are hence analysed on the basis of a homogeneous lipid matrix.

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