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PHASE SEPARATION IN BIMOLECULAR MIXED LIPID MEMBRANES INDUCED BY POLYLYSINE

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SUMMARY. We demonstrate, for the first time, polylysine–induced phase separation in a bimolecular lipid membrane of a lecithin/phosphalidylglycerol–mixture by analysing the single channel current fluctuations of gramicidin. The bimodal conductance histograms are direct evidence for the incorporation of the transport system into the two coexisting phases of different composition.

INTRODUCTION. Bimolecular lipid membranes (BLM's) are well–established model systems for the investigation of details of the molecular mechanisms of ion–translocation mediated by different ionophores like carriers or pores (1). However, it was only recently that it was shown that these "black films" also allow for the study of phase separation phenomena in binary mixed membranes (2) and that it is thus possible to elucidate how an altered lateral organization of two (or more) lipid components can influence the performance of integrated model proteins (2–4). These studies were therefore a first step towards a better understanding of the important order–function–relationship in membranes which complements more established investigations of the structure–function relation. Much is known about the thermotropic and lyotropic polymorphism of lipids and lipid alloys (5), the implications of this phase– and miscibility behavior of the lipid matrix for physiological processes like the transport of ions, however, is still highly speculative (6). Particularly important, in this context, are isothermally triggered phase separation processes by changing (eventually locally and/or transiently) the concentration of ions or peptides in the aqueous phase (6). Thus, numerous studies on divalent ion (7,8) and polypeptide–induced domain formation (9,10) in mixed membranes with one component being charged have been performed with liposomes and vesicles as membrane models. But so far, only Ca^{++} as a demixing agent has been investigated with BLM. However, if the concept of lipid matrix phase separation as a mechanism for the control of the distribution

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and the performance of integral functional units is more generally valid, other systems that have been shown to undergo phase separation must be observable in experiments with black films, too.

We show in the following that polylysine, under certain conditions, can induce the formation of two coexisting phases in BLM's. This phase separation is identified by analysing the single channel conductance fluctuations of incorporated gramicidin, a well–characterized pore–forming polypeptide (11–13).

MATERIALS AND METHODS. The lipids used in this study – 1,2–dioleoyl–sn–glycero-3–phosphatidylcholine (PC) and 1,2–dioleoyl–sn–glycero–3–phosphatidylglycerol (PG) – were from AVANTI (Birmingham, Alabama, USA) and used without further treatment because they were found to be chromatographically pure. All measurements were performed with membranes prepared according to the procedure given by Mueller et al (14) from 1% (wt/vol) decane (Fluka, purum) solutions of the lipids or lipid mixtures as described before (4). Mixtures are characterized in the following by the mole fraction, x, of their PC content. Membrane areas were typically $5 \cdot 10^{-4}$ cm^2. All experiments were performed at room temperature unless otherwise stated. The electrolyte solutions contained 0.5 M CsCl (adjusted to pH6 or pH9 by NaOH and HCl) and various amounts of polylysine (Sigma) of different molecular weight. Gramicidin (a commercially available mixture of A,B, and C) was added from methanolic stock solution as needed. For some experiments also 10^{-4} M ethylenediaminetetraacetate (EDTA) was added in order to remove traces of divalent ions. The mean single–channel conductance, $\bar{\Lambda}$, and the mean life time, $\bar{\tau}$, of gramicidin were measured as described (4) by digitizing the height and the lifetime of each single event and storing both parameters in a micro–computer (Apple II). Thus is was possible to analyse the data not only with respect to the conductance and life time probability distributions, $P(\Lambda)$ and $P(t)$, respectively, but it allowed also to look for correlations between conductance and lifetime of different pore populations (4).

RESULTS

Binary mixtures of dipentadecyl–phosphatidylglycerol and dimyistoyllecithin have been shown recently to mix homogeneously in the liquid–crystalline state at any mole fraction, x, if dispersed as multilamellar liposomes in divalent–ion free electrolyte solution (15). This monotectic behavior is also found for BLM in CsCl–solution if either ultrapure salts are used for the electrolyte preparations or if EDTA removes all divalent impureties of regular salt solutions. We have checked this for the full composition range ($0 < x < 1$, (16)) but present here only the conductance histogram found for $x = 0.80$ (Fig. 1a) which shows the typical width found for homogeneously mixed membranes (4). The applied voltage was 100 mV, the other parameters are indicated in Fig. 1a). For comparison we show in Fig. 1b) the result obtained with regular CsCl (p.A. quality) without EDTA. The conductance histogram is noticably broadened because we are under these conditions close to the phase boundary ($x \approx 0.7$) of a miscibility gap which is opened in $PC_{(1-x)}PG_x$ mixtures ($0.3 < x < 0.7$) already by the usual $10^{-6} - 10^{-5}$ M Ca^{++}–impurities present in 0.5

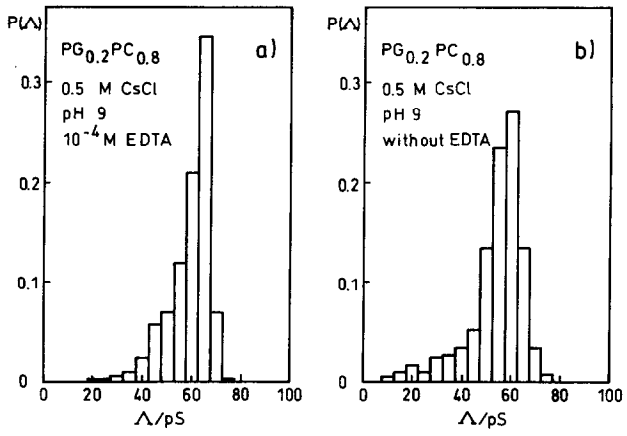

Figure 1: Normalized conductance histogram of gramicidin single–channel fluctuations in mixed $PC_{(1-x)}PG_x$–membranes with x = 0.8; 0.5 M CsCl; pH9; T = 22° C; U = 100 mV. a) 10^{-4} M EDTA, b) no EDTA added.

M p.A.–salt solutions. However, one still would consider this to be a mixed membrane (16).

The mean channel conductance is $\overline{\Lambda}$ = (60 ± 5)pS which is significantly higher than the value for pure PC ($\overline{\Lambda}$ ≈ 40 pS). This is due to a Cs^+ enhancement at the slightly negatively charged membrane surface (17). The high Cs^+ concentration in the bulk solution, 0.5 M, prevents Ca^{++} from being substantially enhanced (18) to interfacial concentrations that would cause a blocking of the gramicidin channel (19).

A significantly different behavior is found if the same experiments are performed in the presence of polylysine (PL, molecular mass M_r = 5100 equivalent to an average degree of polymerization, N= 24). The employed concentration corresponds to 5 · 10^{-4} M monomer units. Irrespective of whether EDTA was added (10^{-4} M, Fig 2a)) or not (Fig. 2b)) we find a bimodal channel conductance histogram which is interpreted as being the result of a PL–induced phase separation: Gramicidin partitiones into the two coexisting phases of different composition which then gives rise to a different single channel conductance. We find $\overline{\Lambda}_1$ = (16 ±2)pS and $\overline{\Lambda}_2$ = (30 ± 3)pS for the two channel populations. These mean conductance increments are significantly lower than the reference value in the absence of PL and indicates a blocking of the conducting channel by PL in both phases.

Other than in the case of the Ca^{++}–induced phase separation in mixed membranes of lecithin and phosphatidic acid (4), here the mean channel life time, $\overline{\tau}$, does not give any

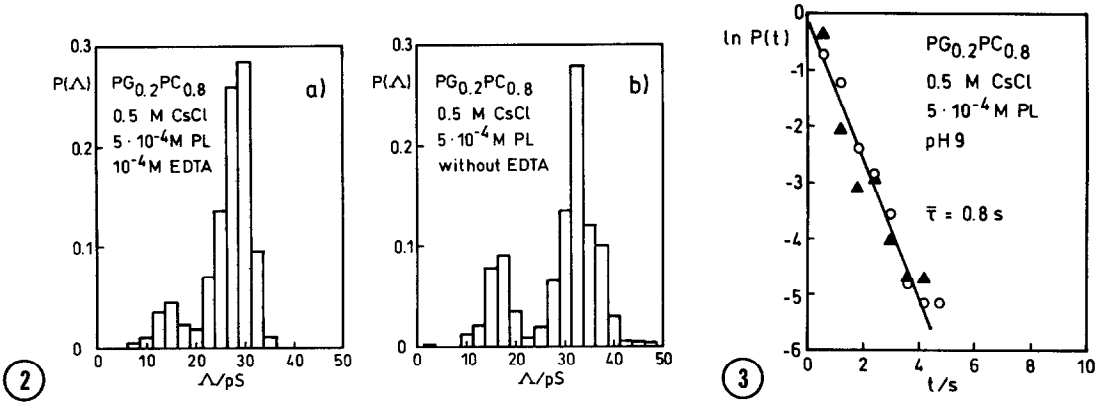

Figure 2: Normalized conductance histogram as in Figure 1, but in the presence of $5 \cdot 10^{-4}$ M polylysine (PL). a) 10^{-4} M EDTA, b) no EDTA. T = 32° C.

Figure 3: Semilogarithmic plot of the probability distribution, $P(t)$, of finding a gramicidin–channel of lifetime, t, in a $PG_{0.2}$ $PC_{0.8}$–mixed membrane in the presence of PL. Full triangles are obtained by analysing pores with a conductance $\Lambda = 0 - 22$ pS; open circles are lifetimes of channels with $\Lambda = 23 - 80$pS. Both populations have a mean lifetime $\bar{\tau} = 0.8$ s.

additional evidence for two different populations: both channels, even when analysed separately, give a mean lifetime $\bar{\tau} = 0.8$ s (Fig. 3).

In a first attempt to localize the phase boundaries of the PL–induced miscibility gap we performed the corresponding experiment with an equimolar mixture of PC and PG, i.e. x = 0.5. Interestingly enough, we found only <u>one</u> channel population with a mean conductance of $\bar{\Lambda} = 33$ pS although this finding, of course, does not yet fully exclude the presence of a second electrically inactive or unobservable phase.

Finally, test experiments were performed at pH6 with mixtures of different compostion, with different PL concentrations and different molecular masses, but in no case we found more than one channel population though with different properties depending on the various experimental conditions (20).

DISCUSSION

The interaction of the basic polypeptide polylysine with negatively charged membranes is well established for liposomal membranes (9, 21–25) although details of this interaction are highly complicated and not too well understood. The chemical nature of the negative headgroup (22,25) and the concentrations (22) and molecular mass of PL (23) seem to play an important role mainly through their influence on the conformation of the

interacting (binding) polypeptides. PL of $M_r \approx 4000$ was shown to only shift slightly the main phase transition temperature of dipalmitoylphosphatidylglycerol with no indication of domain formation when added to binary lecithin/phosphatidic acid mixed membranes (25).

Our results, however, clearly indicate that even short PL interact with PC/PG mixed bilayers thereby changing the whole phase diagram of this mixture. It is interesting to note that it is just the concentration range ($0.6 \lesssim x \lesssim 0.9$) where we find the demixing influence of PL where one observes peculiarities in the otherwise regular cigar–like phase diagram of a similar lipid alloy (15,26). This may point to some intrinsic tendency to phase separate for those molar ratios because of a plat thermo–dynamic potential i.e. $\delta G/\delta x \approx 0$, which then leads to a destabilization with domain formation already under the influence of minor modifications e.g. by peptide binding.

We currently don't understand the different behavior found for pH6 and pH9 because neither the degree of dissociation of PG (27) or of PL nor the conformation and structure of both interacting partners should have changed for these pH variations.

In conclusion, we have demonstrated the first polypeptide–induced phase separation in bimolecular lipid membranes. We could show that gramicidin as a model–transport–system couples to this changed lateral organization of the lipid matrix. The general concept of functional control of proteins by lipid phase separation has been demonstrated thereby to be more widely operational and therefore of more physiological importance than argued on lipid phase behavior studies alone.

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