Biochimica et Biophysica Acta, 643 (1981) 626—635
Elsevier/North-Holland Biomedical Press

BBA 79198

POLYMYXIN INTERACTION WITH NEGATIVELY CHARGED LIPID BILAYER MEMBRANES AND THE COMPETITIVE EFFECT OF CA^{2+}

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(Received December 30th, 1980)

Key words: Polymyxin; Ca^{2+}; Fluorescence polarization; Membrane-antibiotic interaction

Summary

The binding of cationic polymyxin-B to negatively charged phosphatidic acid and phosphatidylglycerol membranes has been investigated by fluorescence polarization study. Competition experiments with Ca^{2+} were performed.

1. Binding of polymyxin-B to mixed dipalmitoylphosphatidic acid/distearoylphosphatidylcholine membranes leads to a phase separation. Domains of polymyxin-bound phosphatidic acid are formed.

2. Ca^{2+} is found to be a strong competitor in displacing polymyxin from the complex in the mixed membrane system. Complete displacement is obtained at pH 9.0. With decreasing pH value, Ca^{2+} becomes a less strong competitor and is ineffective at pH 5.0.

3. Binding of polymyxin to dipalmitoylphosphatidylglycerol membranes is observed. Incorporation of polymyxin lowers the lipid phase transition by 10°C. One polymyxin is found to bind five phosphatidylglycerol molecules. The binding curve is determined and in contrast to phosphatidic acid membranes, a noncooperative binding could be established.

4. Addition of Ca^{2+} decreases the amount of phosphatidylglycerol bound to polymyxin by about 20%. No complete displacement is achieved even at 10-fold excess of Ca^{2+} with respect to phosphatidylglycerol.

Introduction

The peptide antibiotic polymyxin-B exhibits activity against Gram-negative bacilli. The primary site of action of polymyxin is the bacterial membrane [1—3]. The mode of action may be an irreversible breakdown of the permeability barrier of the membrane [4]. Such an effect could be caused by

an interaction with acidic lipids leading to a disorganization of the bacterial membrane and/or to changes in membrane fluidity [5].

A strong interaction between polymyxin-B and negatively charged phosphatidic acid was reported in model membranes [6]. The outstanding result was the determination of a cooperative binding phenomenon. The cooperativity of the binding process was controlled by pH and ion concentration of the buffer solution [7]. More evidence for a cooperative binding to phosphatidic acid bilayer membranes was obtained from the investigation of the kinetics of polymyxin incorporation [8]. A nucleation step in the formation of the lipid-peptide complex was reported. The formation of a lipid-peptide domain was established as a consequence of the antibiotic molecular structure. The molecule contains a fatty acid residue, which is attached with a tripeptide to a heptapeptide ring and inserts into the hydrophobic part of the lipid membrane. Five positive diaminobutyric acid residues attached to the peptide part can bind to the negative membrane surface. Neutral lipid bilayer membranes do not interact with polymyxin.

In a monolayer study, El Mashak and Tocanne [9] reported a rapid and strong interaction of polymyxin with negatively charged phosphatidylglycerol, whereas zwitterionic phosphatidylcholines were ineffective. The phosphatidylglycerol monolayer was expanded by 250 $Å^2$ per polymyxin molecule bound to the lipid layer. The authors suggested the incorporation of the whole polymyxin molecule into the monolayer forming a 1 : 5 polymyxin-phosphatidylglycerol complex. According to the monolayer study, there was no evidence for a cooperative binding process in phosphatidylglycerol membranes.

The main advantage of the investigation of polymyxin-lipid interaction comes from the structure of this peptide antibiotic. One goal is to obtain information on the biophysical mechanism of the antibiotic action. Moreover, polymyxin can be regarded as a small model peptide which incorporates into lipid membranes and is well suited for the investigation of the basic aspects of lipid-protein interaction.

We have now investigated the competitive binding of Ca^{2+} and polymyxin to negatively charged mixed phosphatidic acid and pure phosphatidylglycerol bilayer membranes. A complete displacement of polymyxin was obtained in phosphatidic acid membranes, whereas much less competition was observed in phosphatidylglycerol membranes. Consistent with the report on phosphatidylglycerol monolayers [9], we observed a noncooperative binding process in phosphatidylglycerol membranes.

Materials and Methods

Materials. Dipalmitoylphosphatidylglycerol was a generous gift from Dr. Eibl, Max-Planck-Institute for Biophysical Chemistry, Göttingen, F.R.G. Distearylphosphatidylcholine and dipalmitoylphosphatidic acid-disodium salt (Fluka, F.R.G.) were checked by thin-layer chromatography (TLC) and used without further purification. Polymyxin-B sulfate was kindly supplied by Pfizer GmbH (Karlsruhe, F.R.G.). Samples were prepared in a boric acid/borate buffer at pH 9.0 comprising of 18.4 ml 0.2 M boric acid solution and 86 ml 0.05 M sodium borate solution. The ion concentration mentioned in the

experimental results was brought to each particular value by addition of NaCl.

Membrane preparations. The lipids together with the fluorescence polarization probe diphenylhexatriene were dissolved in chloroform and evaporated by a nitrogen stream to form a film in a suitable glass vessel. The samples were dried under reduced pressure for 1 h. Then, the buffer solution was added and the lipids were dispersed by ultra sonication for 2 min at 20 W using a Branson Ultra Sonifier Model W 140 equipped with a microtip. The samples were resonicated for 1 min after the addition of polymyxin or Ca^{2+}. The temperature during the sonication step was kept above the lipid phase transition temperature. The final lipid concentration was 0.1 mg/ml.

Method. The lipid dispersion contained 1 mol% diphenylhexatriene as an optical probe. Fluorescence polarization measurements were performed with a Schoeffel instrument RRS 1000. We observed, simultaneously, the fluorescence intensity parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the polarization of the excitation light with two sets of polarizer, monochromator and photomultiplier. A schematic description of the arrangement is given elsewhere [10]. The relative fluoresence polarization $p = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$ was calculated continuously by an analogue computer as a function of temperature to obtain the phase transition curves. The temperature was controlled by a thermocouple.

Results

Interaction of polymyxin with mixed membranes containing dipalmitoylphosphatidic acid

Phase transition curves of lipid mixtures containing dipalmitoylphosphatidic acid and distearylphosphatidylcholine in a 1 : 1 molar ratio were obtained by

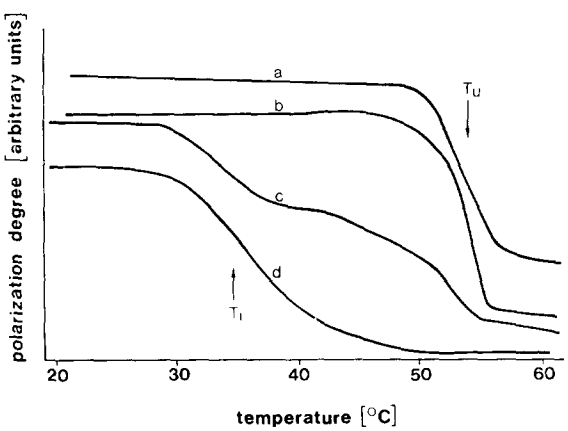


Fig. 1. Effect of polymyxin incorporation into mixed phosphatidylcholine/phosphatidic acid membranes in a 1 : 1 molar ratio at pH 9.0. Fluorescence polarization measurements are shown to determine the lipid phase transition by the use of diphenylhexatriene as an optical probe. For clarity, the curves are shifted along the *y*-axis and therefore only an arbitrary value of the fluorescence polarization is given. (a) Pure dipalmitoylphosphatidic acid; (b) pure distearylphosphatidylcholine; (c) mixed membranes containing distearylphosphatidylcholine and dipalmitoylphosphatidic acid in a 1 : 1 molar ratio after addition of 50 mol% polymyxin with respect to phosphatidic acid; (d) phosphatidic acid membranes after addition of 50 mol% polymyxin. The lower phase transition temperature (T_l) of the antibiotic-phosphatidic acid domains and the upper phase transition temperature of the surrounding lipid matrix (T_u) are indicated.

measuring the temperature dependence of the fluorescence polarization of diphenylhexatriene incorporated into the membranes. The results are shown in Fig. 1. The phase transition temperature, T_t = 53°C, of dipalmitoylphosphatidic acid in a buffer solution of pH 9.0 and an ion concentration of I = 0.1 M is comparable to T_t = 54°C of distearylphosphatidylcholine. A mixture of both lipids exhibits the same type of phase transition curve. Addition of polymyxin to pure phosphatidic acid membranes in a 1 : 2 molar ratio causes a downward shift in the phase transition temperature by about 20°C to T_t = 35°C (curve d, Fig. 1). A similar result is obtained for the lipid mixture (curve c, Fig. 1). A second phase transition step between 30 and 40°C appears after addition of polymyxin to a membrane containing dipalmitoylphosphatidic acid and distearylphosphatidylcholine in a 1 : 1 molar ratio. The polymyxin concentration is again 50% with respect to the phosphatidic acid.

According to our earlier papers [6,7], the lower phase transition has to be attributed to a lipid-peptide domain. Insertion of the polymyxin molecules into the negatively charged membrane expands the lipid matrix and lowers the phase transition temperature by about 20°C. The phase transition temperature of pure phosphatidylcholine membranes is not changed by the addition of polymyxin. Therefore, the appearance of the lower phase transition in the mixed membrane (curve d, Fig. 1) provides evidence for a phase separation phenomenon induced by the binding of the phosphatidic acid molecules to the inserted polymyxin. The given polymyxin concentration in this preparation exceeds the maximum amount of polymyxin that can be bound to a phosphatidic acid membrane [7].

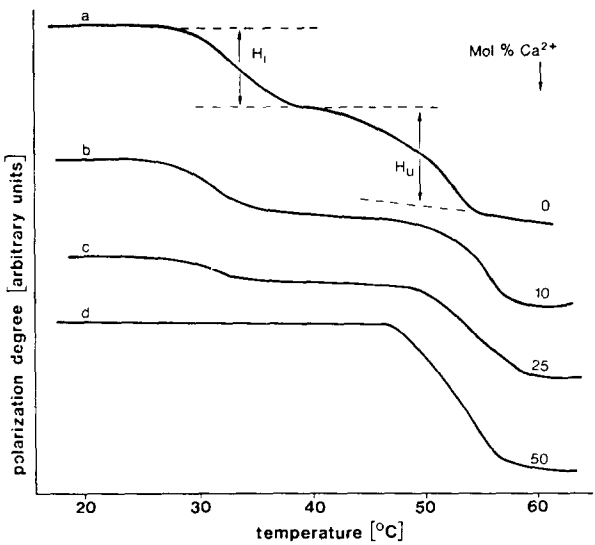


Fig. 2. Effect of Ca^{2+} on the phase transition curve of the mixed lipid system after the polymyxin addition shown in Fig. 1c. The heights of the phase transition steps are indicated by H_l and H_u for the lower and the upper phase transition, respectively. The lower phase transition disappears at 50 mol% Ca^{2+} relative to the phosphatidic acid.

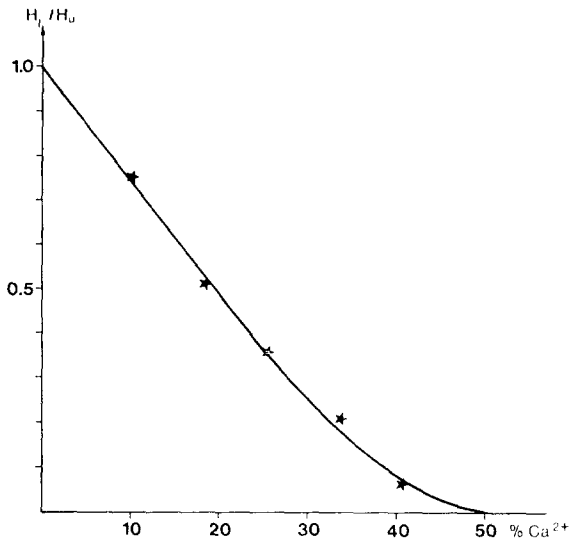


Fig. 3. Changes in the ratio H_l/H_u taken from measurements shown in Fig. 2 as a function of Ca^{2+} concentration for a lipid mixture containing equimolar amounts of dipalmitoylphosphatidic acid and distearylphosphatidylcholine. The polymyxin content is 50 mol% with respect to the phosphatidic acid, the pH is 9.0 and the ion concentration I = 0.1 M. The Ca^{2+} concentration is given as mol% relative to phosphatidic acid.

The effect of Ca^{2+} on the binding of polymyxin to mixed membranes containing phosphatidic acid

Fig. 2 shows the effect of Ca^{2+} on a mixed dipalmitoylphosphatidic acid/distearylphosphatidylcholine membrane in the presence of polymyxin, as already shown in Fig. 1, curve c. The step heights H_u and H_l shown in Fig. 2, curve a are taken as the amount of lipid in the peptide-bound phosphatidic acid domain (H_l) and in the unchanged phospholipid phase (H_u) which contains mainly phosphatidylcholine. Addition of Ca^{2+} decreases the amount of phosphatidic acid in the lipid-peptide domain shown by a relative decrease in the ratio H_l/H_u. The lower phase transition step of the phosphatidic acid/polymyxin domain disappears if the Ca^{2+} concentration reaches 50% of the phosphatidic acid molecules. This Ca^{2+} concentration is then equivalent to the polymyxin concentration. The Ca^{2+} concentration-dependence of the H_l/H_u ratio is given in Fig. 3. Increasing amounts of Ca^{2+} reduce continuously the binding between phosphatidic acid and polymyxin and finally inhibit completely the formation of the lipid-peptide complex.

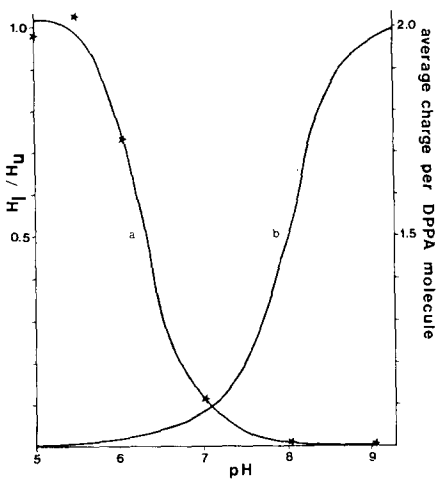
Competition of Ca^{2+} at different pH values

The pH dependence of the competition of Ca^{2+} with polymyxin in a 1 : 1 phosphatidylcholine/phosphatidic acid membrane in the presence of an equimolar concentration of Ca^{2+} relative to the phosphatidic acid is shown in Fig. 4. At pH 5, Ca^{2+} does not affect the binding properties of polymyxin to phosphatidic acid-containing membranes, whereas at pH 8.0 we observe the complete inhibition of the formation of the polymyxin-phosphatidic acid

complex. The amount of lipid in the lipid-peptide complex represented by H_l in Fig. 2, curve a approaches zero. For comparison, the calculated change in the average charge per phosphatidic acid molecule is also given in Fig. 4.

Polymyxin binding to phosphatidylglycerol bilayers

Dipalmitoylphosphatidylglycerol bilayer vesicles exhibit a lipid phase transition at 45°C in a buffer solution with an ion concentration of $I = 0.03$ M and at pH 9.0. The phase transition curve measured by the fluorescence polarization technique is shown in Fig. 5. Addition of polymyxin-B to these negatively charged lipid membranes lowers the lipid phase transition by about 10°C. Two characteristic phase transition curves for membrane preparations containing 10 and 18% polymyxin are also shown in Fig. 5. In the presence of polymyxin, we observe the appearance of the second phase transition at $T_l = 35$°C. Nevertheless, the upper phase transition of the undisturbed phosphatidylglycerol membrane with a transition temperature $T_u = 45$°C is clearly visible up to 15 mol% polymyxin. According to our earlier results with phosphatidic acid membranes [6,7], the lower phase transition T_l is interpreted as the melting of the polymyxin-lipid domains, whereas the upper phase transition temperature T_u characterizes the melting of the non-bound lipid. At 18 mol% polymyxin almost the total phosphatidylglycerol is in the bound state. Again, we used the step heights of each transition in the phase transition curves as a measure for the amount of lipid in the corresponding state. Fig. 5 shows how to determine the step heights H_l and H_u of the lower and upper phase



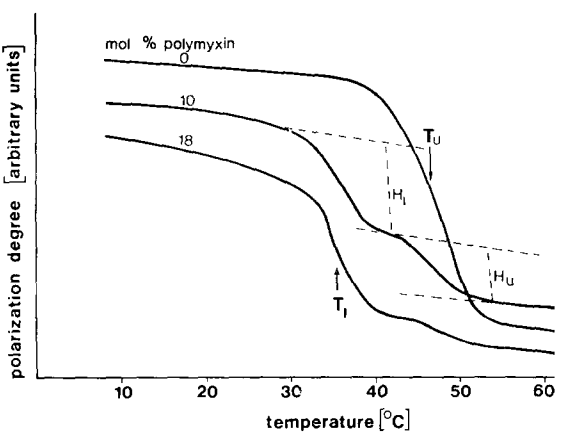


Fig. 4. Decrease in the ratio H_l/H_u with increasing pH value (curve a). The polymyxin content is 50 mol% and the Ca^{2+} concentration is equimolar with respect to the phosphatidic acid. For comparison, the increase in the average charge per phosphatidic acid molecule with increasing pH in the absence of external ions is included (curve b). DPPA, dipalmitoylphosphatidic acid.

Fig. 5. Phase transition curves obtained from fluorescence polarization measurements of diphenylhexatriene incorporated into dipalmitoylphosphatidylglycerol membranes in the absence and presence of polymyxin-B. Measurements were performed at pH 9.0 and an ion concentration (I) = 0.03 M. The phase transition temperatures T_l and T_u as well as the heights of the phase transition steps H_l and H_u are indicated.

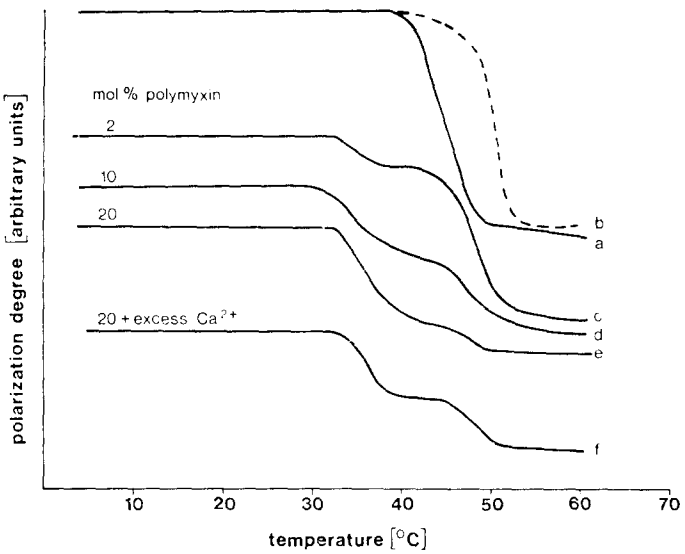


Fig. 6. Phase transition curves of dipalmitoylphosphatidylglycerol membranes containing different amounts of polymyxin in the presence of Ca^{2+} equimolar to phosphatidylglycerol at pH 9.0 and $I = 0.03$ M. (a) Pure phosphatidylglycerol membranes; (b) phosphatidylglycerol membranes after addition of equimolar Ca^{2+}; (c), (d), (e) 2, 10 and 20 mol% polymyxin added to the preparation shown in b; (f) 10-fold excess of Ca^{2+} added to the preparation containing 20 mol% polymyxin.

transition steps in the phosphatidylglycerol membranes. The ratio $H_l/(H_l + H_u) = \rho$ gives the mole fraction of lipids in the polymyxin-bound state.

The effect of Ca^{2+} on polymyxin-containing phosphatidylglycerol membranes

Addition of Ca^{2+} in equimolar concentration to dipalmitoylphosphatidylglycerol membranes increases the phase transition temperature of this lipid

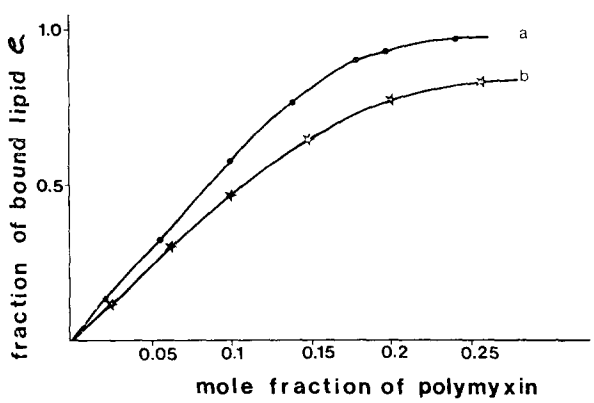


Fig. 7. Binding curves of polymyxin to dipalmitoylphosphatidylglycerol membranes in the presence and absence of Ca^{2+} at pH 9.0 and $I = 0.03$ M. The mole fraction, ρ, of lipid bound to the antibiotic is defined as $\rho = H_u/(H_l + H_u)$ and is plotted versus the mole fraction of antibiotic added to the lipid dispersion.

from 45 to 53°C (curves a and b, Fig. 6). Addition of polymyxin in the presence of Ca^{2+} leads again to the appearance of the lower phase transition of the polymyxin/phosphatidylglycerol complex. The transition of the surrounding phosphatidylglycerol matrix (T_u), is lowered from 53 to 47°C with increasing amounts of polymyxin in the presence of Ca^{2+} in a concentration equimolar to the phosphatidic acid (curves c, d, and e, Fig. 6). In contrast to the result obtained with phosphatidic acid membranes, Ca^{2+} has only a small competitive effect on the binding of polymyxin to phosphatidylglycerol membranes. A 10-fold excess of Ca^{2+} leads to only a small decrease in the fraction of the polymyxin-bound phosphatidylglycerol, and the phase transition curves (curve f, Fig. 6) still exhibit the lower phase transition step at T_l.

Binding curves of polymyxin to phosphatidylglycerol membranes

The binding curves for the formation of a polymyxin-phosphatidylglycerol complex in the presence and absence of Ca^{2+} are shown in Fig. 7. In striking contrast to our earlier results with phosphatidic acid membranes, there is no cooperative binding. The mole fraction ρ of polymyxin-bound phosphatidylglycerol increases linearly with the polymyxin concentration added to the lipid dispersion. At a 1 : 5 molar ratio of polymyxin to phosphatidylglycerol, the lipid is completely bound to polymyxin. Ca^{2+} does not change the shape of the binding curve. A decrease in the fraction of bound lipid by about 20% is observed at all polymyxin concentrations.

Discussion

Polymyxin interaction with mixed phosphatidic acid bilayer membranes

Our results show clearly that polymyxin-B binds strongly to negatively charged phospholipids. No interaction was observed with phosphatidylcholine membranes. In a mixture containing these two types of lipid, addition of polymyxin causes a phase separation between polymyxin-bound phosphatidic acid and non-bound phosphatidylcholine. The phase transition of the bound phosphatidic acid is shifted to a lower temperature.

Hartmann and Galla [11] have shown that the step height of a phase transition in a mixed lipid membrane exhibiting phase separation phenomena may be used as being equivalent to the amount of lipid in the corresponding phase. This holds for fluorescence polarization measurements. EPR experiments measuring the partition of the spinprobe 2,2,6,6-tetramethylpiperidine-1-oxide between the water and the lipid phase were shown to yield similar results [12].

Ca^{2+} is known to interact strongly with negatively charged membranes [13,14]. After addition of Ca^{2+} to phosphatidic acid, the phase transition of the lipid is increased to a temperature which is not observable in our experimental temperature range up to 80°C. Galla and Sackmann [14] have shown that Ca^{2+} segregates dipalmitoylphosphatidic acid in a mixture containing also dipalmitoylphosphatidylcholine and induces a phase separation.

A complete phase separation between phosphatidic acid and phosphatidylcholine induced by Ca^{2+} requires, at pH 9.0, an equimolar amount of Ca^{2+}

with respect to phosphatidic acid [13]. One calcium ion is reported to chelate one phosphatidic acid molecule. In our present study, we found that only half the amount of Ca^{2+} is necessary to displace polymyxin from binding to phosphatidic acid.

The competitive effect between Ca^{2+} and polymyxin is strongly dependent on the pH value of the buffer solution as is shown in Fig. 4. Polymyxin with a pK value of 9.6 for its free amino groups carries on the average four positive charges at pH 9.0 and five at pH 8. Phosphatidic acid changes its average charge between pH 5 and pH 9.0 from one to two. Addition of Ca^{2+} did not disturb the lipid-antibiotic interaction at pH 5.0. We observed a strong competitive effect to the antibiotic binding only if the average charge per phosphatidic acid molecule reaches a value 1.5 or higher.

Polymyxin interaction with phosphatidylglycerol membranes

So far we have used phosphatidic acid as a model substance for charged lipids that occur in natural membranes. An approach to more physiological conditions may be the use of phosphatidylglycerols. Synthetic dipalmitoylphosphatidylglycerol exhibits a lipid phase transition at $T = 45°C$. Again, our experiments show clearly a strong interaction with this type of negatively charged lipid. A lowered phase transition appears after incorporation of polymyxin into the membrane.

Ca^{2+} is observed to bind to phosphatidylglycerol membranes. An upward shift in the phase transition temperature by about 8°C is observed after Ca^{2+} addition. Compared to phosphatidic acid, this already symbolizes a weaker binding of Ca^{2+} to phosphatidylglycerol membranes.

This predicts a smaller competitive effect on the polymyxin binding to phosphatidylglycerol membrane. Even excess Ca^{2+} is not able to destroy completely the lipid-antibiotic domain. The result is in agreement with the results obtained with phosphatidic acid membranes at low pH where phosphatidic acid is only singly charged as is phosphatidylglycerol at pH 9.0.

Binding of polymyxin to phosphatidylglycerol membranes occurs even in the presence of Ca^{2+}. Moreover, increasing the amount of polymyxin decreases the lipid phase transition temperature (T_u) of Ca^{2+}-bound phosphatidylglycerol to about the value of phosphatidylglycerol membranes in the absence of any agents. Again, this result shows that there is an antagonism between the polymyxin and the Ca^{2+} binding to charged lipid bilayer membranes.

Acknowledgements

We would like to thank the Deutsche Forschungsgemeinschaft for financial support under contract Ga 233/2.

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