DIRECT EVIDENCE OF CHARGE-INDUCED LIPID DOMAIN STRUCTURE IN MODEL MEMBRANES

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Received 7 April 1977

1. Introduction

The phenomenological membrane properties are expected to be primarily dependent on the lateral and transversal organization of the membrane building units (lipids and proteins). In view of this question lipids with a net charge at the polar headgroups are of outstanding interest. Spin-label studies of model membranes containing phosphatidic acid and labelled lecithin showed that external charges (poly-lysine or Ca^{2+}) may trigger a phase separation leading to a lateral domain structure [1,2]. Each monolayer exhibits a mosaic like pattern of domains of poly-lysine (or Ca^{2+}) bound charged lipid embedded in mixed lecithin–phosphatidic acid regions. In the present paper we provide direct electron microscopic evidence for this charge-induced domain formation in lipid alloys containing dioleyl phosphatidic acid. Charged poly-peptides (poly-lysine) or two valent cations (Ca^{2+}) are used as domain inducing external charges. Phosphatidic acid may be considered as representative of charged natural lipids (e.g., phosphatidylserine in erythrocytes, cardiolipin in mitochondria).

2. Materials and methods

Cardiolipin, dioleyl phosphatidic acid and dioleyl lecithin from Serdary (London, Canada) and dipalmitoyl lecithin and dipalmitoyl phosphatidic acid from Fluka are checked for purity by thin-layer chromatography. Bromide salt of poly-lysine, purchased from Miles Biochemicals, has an average mol. wt 30 000. Pyrene decanoic acid is synthesized in our own laboratory. For the electron microscopy study giant bilayer vesicles are used. The method has been described elsewhere [7,3]. The final lipid concentration is 3×10^{-1} M in aqueous solution (0.01 M Na-borate buffer adjusted to pH 9). The external charges (poly-lysine or Ca^{2+}) are added after the vesicle preparation. Therefore they can only attach to the outer monolayer. The vesicle preparations are freeze etched [3] and platin shadowed under an oblique angle of 45°C. Fluorescence experiments using excimer forming pyrene decanoic acid as probe molecule are performed with sonicated vesicles.

3. Results and discussion

3.1. *Fluorescence method [4]*

Excimer forming probes may detect small changes in membrane fluidity and (or) changes in the lipid organisation [5]. The ratio of the fluorescence intensities I'/I of the excimer to the monomer emission is proportional to the collision rate of the probe molecules [6]. Therefore I'/I is a direct measure for the relative areas of regions with different microviscosity.

Figure 1 demonstrates the charge-induced phase separation in lipid lamellae of pure dipalmitoyl phosphatidic acid upon addition of poly-lysine. Consider the curve in the absence of poly-lysine (– – – –). The sharp increase in I'/I between 47°C and 45°C indicates the phase transition from a fluid to a rigid state of the lipid. Addition of poly-lysine leads to a splitting of the transition region into two steps. Part of the lipid exhibits a chain melting at $T_2 = 62$°C.

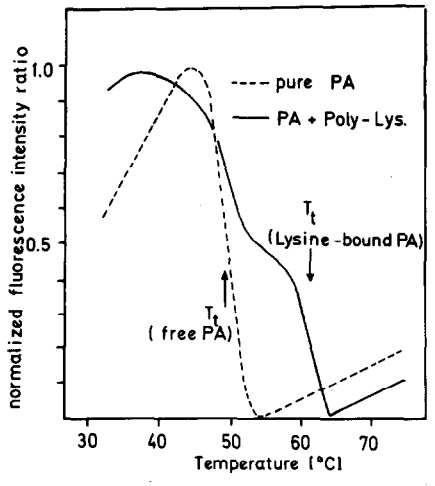

Fig.1. Demonstration of poly-lysine (charge)-induced phase separation in vesicle preparation of dipalmitoyl phosphatidic acid. The intensity ratio, I'/I, of the excimer to the monomer emission is plotted as a function of temperature both in the absence (————) and in the presence (– – – –) of poly-lysine.

while the transition temperature of the other part is unshifted. The high melting lipid is considered to be bound by poly-lysine. The vesicle preparation was slightly sonicated after poly-lysine addition. Thus both the inner and the outer monolayers were accessible to the poly-peptide. The equal height of the two steps in the transition curve of fig.1 means that about 50% of the lipid is attached to poly-lysine. Since a 1:1 molar ratio of lipid to lysine groups was adjusted, about 50% of the side groups of poly-lysine are involved in the binding.

3.2. *Electron microscopy*

This domain structure is verified in a direct way by freeze etching electron microscopy (fig.2–4).

3.2.1. Figure 2

(a) Pure lecithin/phosphatidic acid bilayers (1:1 mixture) (fig.2a);

The electron micrograph shows a giant vesicle of about 1 μm diameter. The bilayer has been broken along the plane separating the monolayers. At the rims of the vesicle, part of the outer monolayer is also exhibited. The line of cleavage is indicated by thin arrows. From this part the water has been removed during the etching process. A completely smooth membrane surface is obtained. This is characteristic for bilayers in the fluid (smectic A or C) state (cf. ref. [3]).

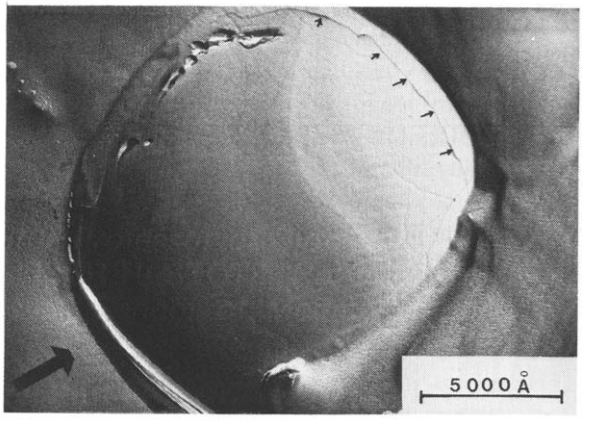

Fig.2a. Electron micrograph of giant vesicle of a 1:1 mixture of dioleyl lecithin and dioleyl phosphatidic acid embedded in water. The arrow gives direction of shadowing.

(b) Effect of poly-lysine on 1:1 mixture of dioleyl lecithin and dioleyl phosphatidic acid (fig.2b): Again the membrane has been cut through the average symmetry plane. The surface of both the

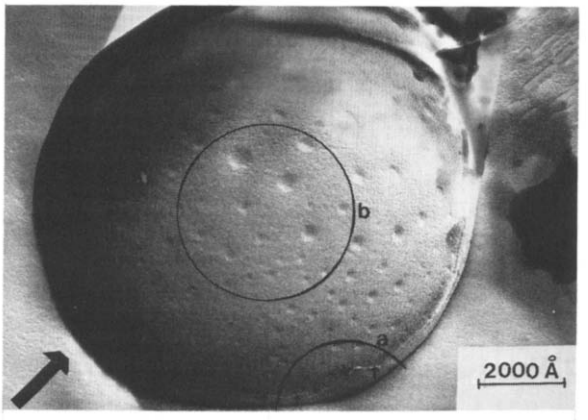

Fig.2b. Demonstration of domain structure caused by addition of poly-lysine to vesicle described in fig.2a. The variation in the average size of the circular domains from bottom to top is due to the curvature of the vesicle. The thin arrows indicate the line of cleavage where the outer monolayer has been broken off.

inner and the outer monolayers is covered by a large number of circular, plate-like, domains.

The replica obtained with the freeze etching technique represent essentially copies of the surface curvature of the preparation. The technique is thus only sensitive to changes in the surface curvature of the lipid bilayer. Therefore, the circular domains observed in fig.2b represent shallow bumps within the lipid bilayer surface. The direction of curvature is directly obtained from the direction of the shadow. Inspection of fig.2 shows that in the inner monolayer the domains are curved towards the inside of the vesicle. In the outer monolayer the domains are curved towards the outside. Since the poly-peptide could only attach to the outer monolayer of the vesicle, the formation of a domain in the outer monolayer must also affect the curvature of the inner monolayer. Inspection of those regions where the bilayer has been broken shows indeed that the domains in the outer and in the inner monolayer are coupled together (circle a).

The average size of the domains varies continuously along the direction of shadowing. Such a behavior is expected for the curved surface of the vesicle. Therefore the size of the domains was determined from the region about the top of the vesicle as indicated by circle b. In total twelve electron micrographs of different vesicle preparations are analysed. This analysis showed that the domain size increases in a stepwise manner. Characteristic diameters of about 100 Å, 200 Å, 350 Å, 450 Å and 650 Å are found. The domains cover about 15% of the total surface.

3.2.2. Effect of poly-lysine on 1:1 mixture of cardiolipin and lecithin (fig.3)

The phosphatidic acid was used as model component representing the charged natural phospholipid. Figure 3 demonstrates that charged natural lipids exhibit the same behavior. A domain-like modulation of the surface curvature is again induced by addition of the charged poly-peptide. The size of the domains is of the order of 1000 Å.

3.2.3. Effect of Ca^{2+} (fig.4)

In analogy to charged poly-peptides, two-valent positive ions, such as Ca^{2+}, are expected to trigger lateral phase separation in membranes containing a negatively charged lipid component. Figure 4 shows the effect of Ca^{2+}-addition to giant vesicles of a 1:1

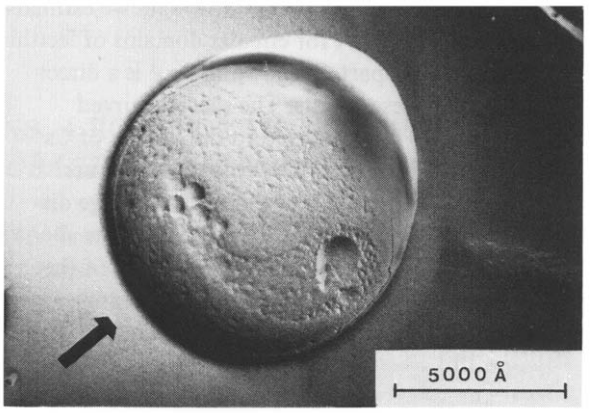

Fig.3. Domain structure in a mixed membrane of dioleyl lecithin and cardiolipin induced by addition of poly-lysine.

dioleyl lecithin/dioleyl phosphatidic acid mixture. The surface is covered by a large number of slightly elongated domains and also by some large plates. The domains are again due to sharp changes in the membrane surface curvature. Quantitative analysis of a previous spin-label study [2] led us to the following conclusion:

Ca^{2+}-Addition causes a mosaic-like lateral distribution pattern of a fluid phase composed of a lecithin/phosphatidic acid mixture and of a rigid phase of

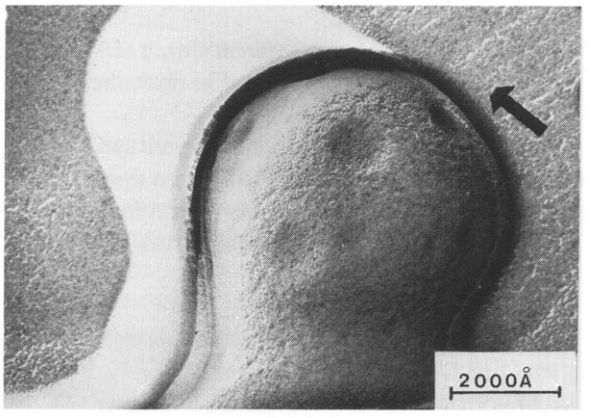

Fig.4. Effect of Ca^{2+} on giant bilayer vesicle of 1:1 mixture of dioleyl lecithin and dioleyl phosphatidic acid. The molar ratio of Ca^{2+} to the charged lipid was 10:1. Total lipid concentration as in fig.2. Arrow: Direction of shadowing.

Ca^{2+}-bound charged lipid. From ESR-data we estimated a diameter of 100 Å for circular domains of lecithin-label. The domain pattern shown in fig.4 is a direct verification of these results. The sharply curved domains have to be attributed to the regions of Ca^{2+}-bound phosphatidic acid. These rigid patches are embedded in fluid lecithin regions. The average distance between adjacent Ca^{2+}-bound domains is about 200 Å. Taking into account the uncertainty of the circular cluster model used for the EPR-measurements, these values are in reasonable agreement.

Acknowledgements

The authors are most thankful to Professor R. Martin, Mr R. Kulka and Mr W. Fritz of the Section for Electron Microscopy at the University of Ulm for their most generous help. We are indepted to Professor H. Gruler for helpful discussions. The technical assistance of Mr U. Theilen with the optical measurements is gratefully acknowledged. This work was supported by Deutsche Forschungsgemeinschaft under contract No. Sa 246.

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