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DIVALENT CATIONS AND CHLORPROMAZINE CAN INDUCE NON-BILAYER STRUCTURES IN PHOSPHATIDIC ACID-CONTAINING MODEL MEMBRANES

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(1) The structural organization of aqueous dispersions of 1,2-dioleoylphosphatidic acid has been investigated by freeze-fracture electron microscopy in relation to variations in pH, divalent cations and the local anaesthetic chlorpromazine. (2) In the pH range 4–8 in the presence of 100 mM Na^+, dioleoylphosphatidic acid is organized in bilayers. (3) At pH 6 and not at pH 4 and 8.5 addition of Ca^{2+}, Mg^{2+}, Mn^{2+} and chlorpromazine results in the formation of the hexagonal H_{II} phase. (4) Ca^{2+} and chlorpromazine addition to mixed phosphatidylcholine-dioleoylphosphatidic acid bilayers at pH 6 results in the formation of lipidic particles.

Introduction

The abundant occurrence of non-bilayer lipids in biological membranes has in recent years initiated intensive studies on the structural and functional aspects of these lipids (for review, see Ref. 1). It has been shown that aqueous dispersions of such lipids under physiological conditions show polymorphism and that dependent upon the conditions both bilayer and non-bilayer structures can be formed. Among the factors which can modulate bilayer → non-bilayer transitions are temperature, lipid composition, local anaesthetics and divalent cations. An example of a lipid which shows this latter behaviour is beef heart cardiolipin. Transitions from a lamellar to a hexagonal H_{II} phase can be induced by the addition of divalent cations [2,3] and local anaesthetics [3]. In cardiolipin containing mixed systems, Ca^{2+} can induce the formation of lipidic particles. We have suggested that such lipidic particles represent inverted micelles formed either within one single bilayer or at the nexus of intersecting bilayers intermediary in fusion [4–7]. Alternative interpretations of these lipidic particles have been presented as well, in that they represent intermembrane attachment sites [8,9] of bilayer deformation associated with interbilayer contact and fusion [10]. Is has been further suggested [1] and extrapolated [11,12] that these structures may be involved in transbilayer movements of lipids and can facilitate the transport of divalent cations across the membrane.

Since in higher organisms cardiolipin only is found in significant amounts in the inner mitochondrial membrane, it is important to establish whether other negatively charged phospholipids show a similar behaviour. The other major negatively charged membrane phospholipids, e.g., phosphatidylserine [13], phosphatidylglycerol [14] and phosphatidylinositol [15] prefer under physiological conditions bilayer organizations both in the absence and presence of divalent cations. Another

Abbreviation: Mes, 2-(*N*-morpholino)ethanesulphonic acid.

intriguing negatively charged phospholipid is phosphatidic acid. This key intermediate in phospholipid biosynthesis occurs in small but significant amounts in many membranes, it has a very high turnover rate and appears to be involved in many important membrane processes [16]. Strong evidence has been presented that in biological [17,18] and model membranes [19] this molecule can act as a Ca^{2+} ionophore. In addition it has been observed [18,20] that this lipid can form an inverted micellar organic solvent soluble complex with Ca^{2+}, which is suggested [17] to be a prerequisite for a molecule to be an effective Ca^{2+} ionophore. With these observations in mind we investigated by freeze-fracturing the structures formed by 1,2-dioleoylphosphatidic acid, in particular in relation to its charge and the presence of divalent cations and the local anaesthetic chlorpromazine. It will be shown that dependent on the pH, both divalent cations and chlorpromazine can induce non-bilayer structures in dioleoylphosphatidic acid-containing model membranes (Part of these results were presented during the Fourth Conference on Surface and Colloid Science in Jerusalem in July 1981.). In addition, some of these findings are compared and related to those observed in cardiolipin containing systems.

Materials and Methods

Egg phosphatidylcholine was isolated from hen eggs. Dioleoylphosphatidic acid was prepared from dioleoylphosphatidylcholine with phospholipase D as described before [21] and was converted to its Na^+ salt as reported earlier [22]. Na^+ salt of cardiolipin (diphosphatidylglycerol) was purchased from Sigma (St. Louis, MO, U.S.A.). All lipids were chromatographically pure. Chlorpromazine (largactil) was purchased from S.P.E.C.I.A. (Paris, France).

Phospholipid samples were prepared by dispersing 5 μmol of lipid dried under N_2 in 1 ml of buffer containing 10 mM Mes or 10 mM Tris, 100 mM NaCl at the desired pH. Divalent cations were added as aliquots of a 100 mM solution dissolved in the same buffer at the desired pH. In all experiments with chlorpromazine, dissolved in ethanol at different concentrations, 100 μl of the ethanol solution was added to 1.9 ml of liposomal dispersion in buffer at the desired pH. Ethanol at this concentration (5%) does not induce changes in the lipid structure.

After 30 min of incubation at room temperature, the pH of the lipid dispersions was checked and was found to be identical to the starting value.

Small vesicles of egg phosphatidylcholine/dioleoylphosphatidic acid were obtained by sonication for 2 min at 0°C with a Branson-tip-sonicator.

Freeze-fracture electron microscopy was performed according to established procedures [23]. Samples were quenched conventionally in a slush of solid and liquid nitrogen or/and with the jet-freezing technique [24], which features an ultrarapid cooling rate. Before quenching with the conventional procedure glycerol was added to prevent freeze-damage.

The repeat distance of the tubes of the hexagonal H_{II} phase was determined as described before [25].

Results

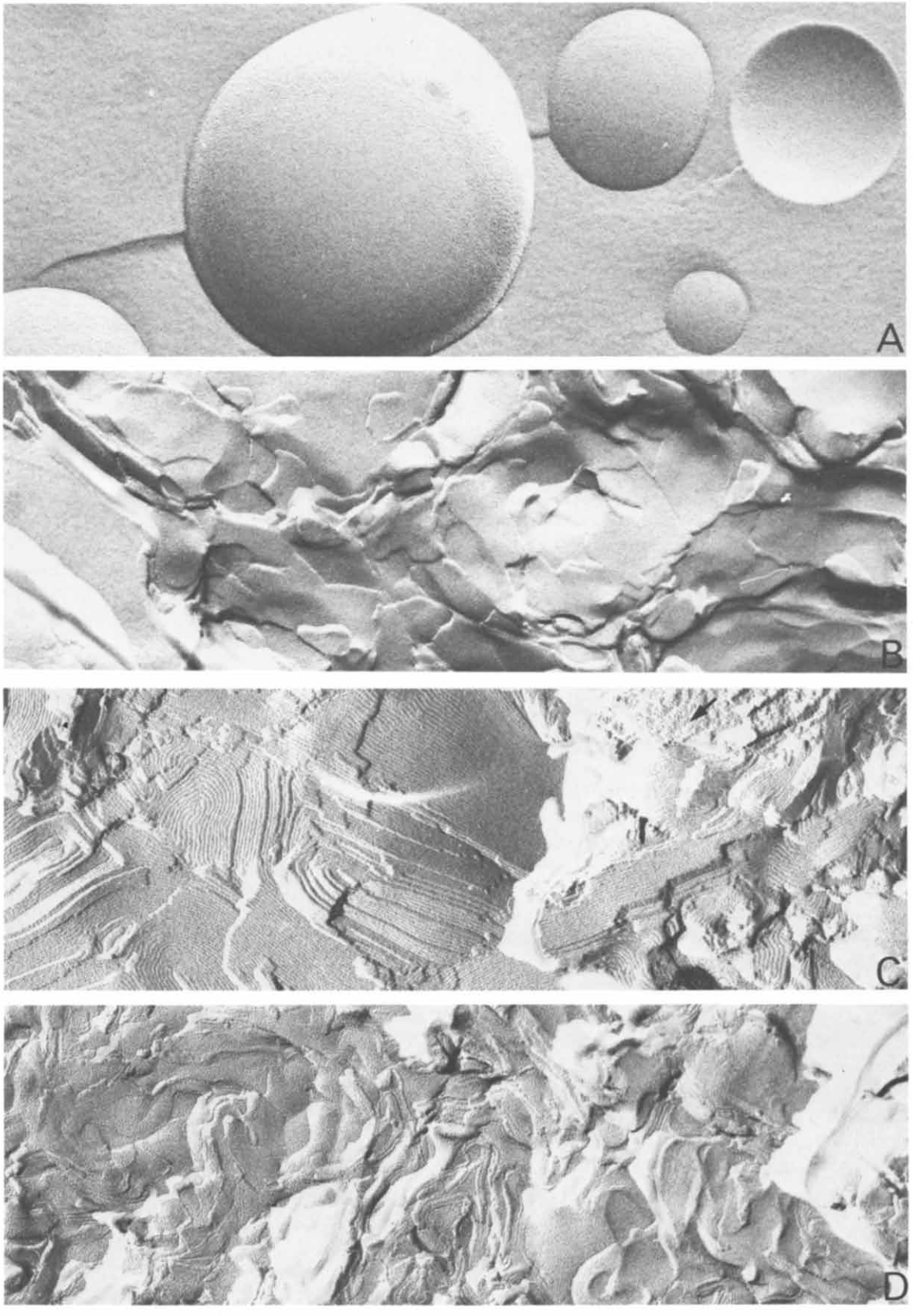
Dioleoylphosphatidic acid Na^+ salt

As the charge of phosphatidic acid is strongly dependent on the pH, pK_1 and pK_2 of the phosphate group are 3.5 and about 8.5, respectively [18,22], the effect of divalent cations on the structural properties of dioleoylphosphatidic acid were investigated at several pH values. Dioleoylphosphatidic acid dispersed in buffer between pH 4 and 9 forms liposomal structures, as indicated by freeze-fracturing. Smooth fracture faces of bilayers are apparent (Fig. 1A).

Dioleoylphosphatidic acid Me^{2+} salt

Addition of excess divalent cations Ca^{2+}, Mg^{2+} and Mn^{2+} (10 mM Me^{2+}, ratio Me^{2+}/dioleoylphosphatidic acid 2:1) results at pH values between pH 4 and 9 in a visual flocculation of the lipid. Fig. 1 shows the morphology of Ca^{2+}/di-

Fig. 1. Freeze-fracture micrographs of dioleoylphosphatidic acid dispersed in buffer containing 10 mM Mes or 10 mM Tris, 100 mM NaCl, in the absence of divalent cations at pH 6.0 (A); in the presence of 10 mM $CaCl_2$ ratio Ca^{2+}/dioleoylphosphatidic acid 2:1 at pH 4.0 (B), at pH 6.0 (C) and pH 8.0 (D). Magnification: ×100000.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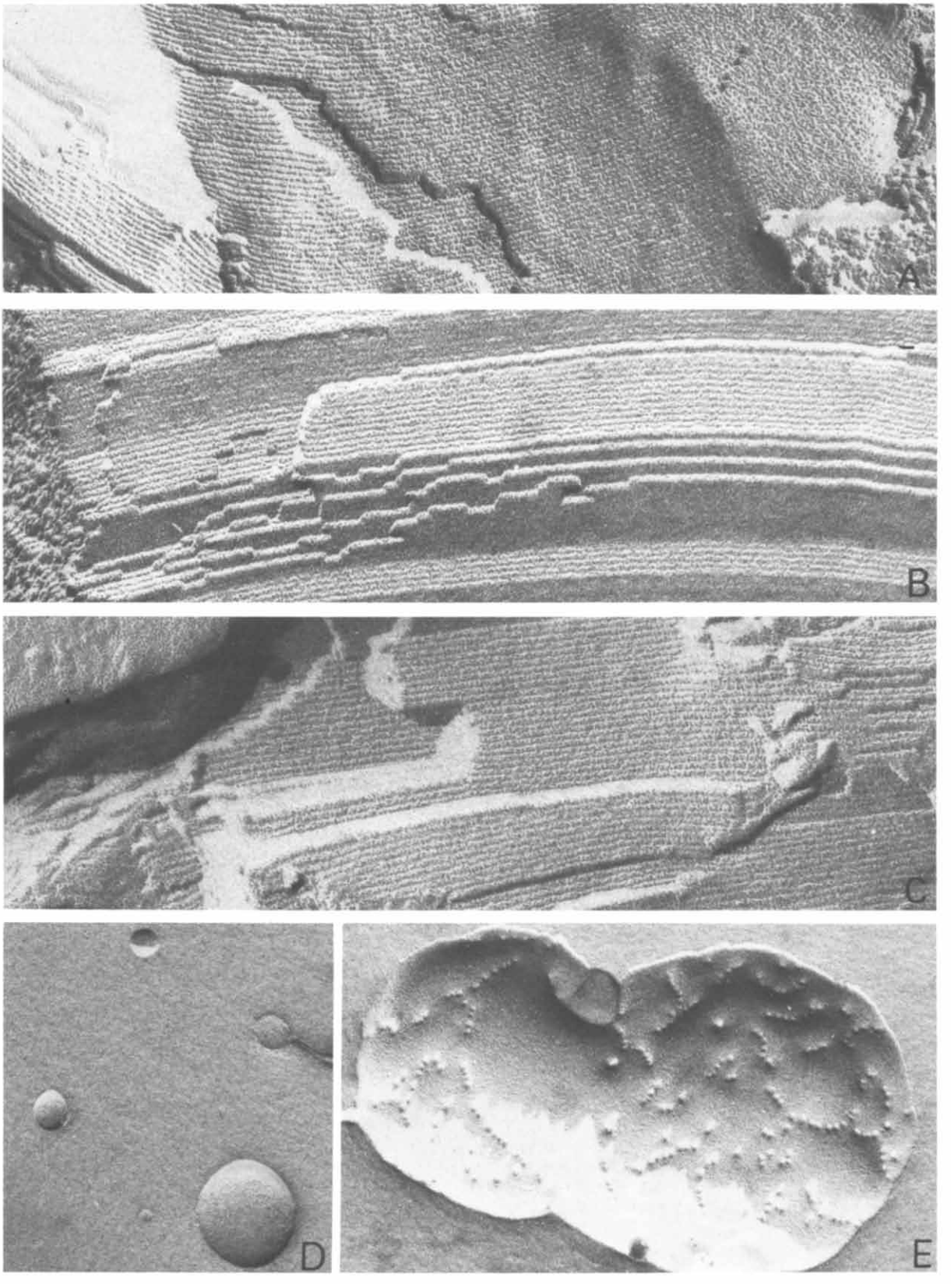
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oleoylphosphatidic acid at pH 4, 6 and 8.5. At pH 6 (Fig. 1C), where phosphatidic acid exposed one negative charge, freeze-fracturing clearly demonstrates that dioleoylphosphatidic acid adopts the hexagonal H_{II} phase, exhibiting the characteristic ribbed appearance at different angles of fracturing and cross-fractured cylinders (arrow). Next to hexagonal H_{II} phase, smooth lamellae and transitions from bilayer to H_{II} as described before [21] were also found at this pH. Moreover, the cylinders of the H_{II} phase were curved. At lower pH (pH 5.5) less lamellar phase was apparent and the H_{II} cylinders were less curved. The H_{II} phase could be observed at pH 6.0 also in the absence of glycerol when samples were frozen by jet-freezing. At pH 4 and pH 8.5 only stacked lamellar liposomal structures were found (Fig. 1B, D).

A similar structural behaviour was found for Mg^{2+} and Mn^{2+}. Also with these divalent cations the H_{II} phase was observed at pH 6.0. Figs. 2A, B and C show the H_{II} phase of Ca^{2+}, Mg^{2+} and Mn^{2+}/dioleoylphosphatidic acid at pH 6.0. The diameter of the tubes of the different Me^{2+}/dioleoylphosphatidic acid complexes are 5.2, 5.7 and 7.4 nm for Ca^{2+}, Mg^{2+} and Mn^{2+}, respectively. At pH 4 and 8.5 also only stacked lamellar liposomal structures were found.

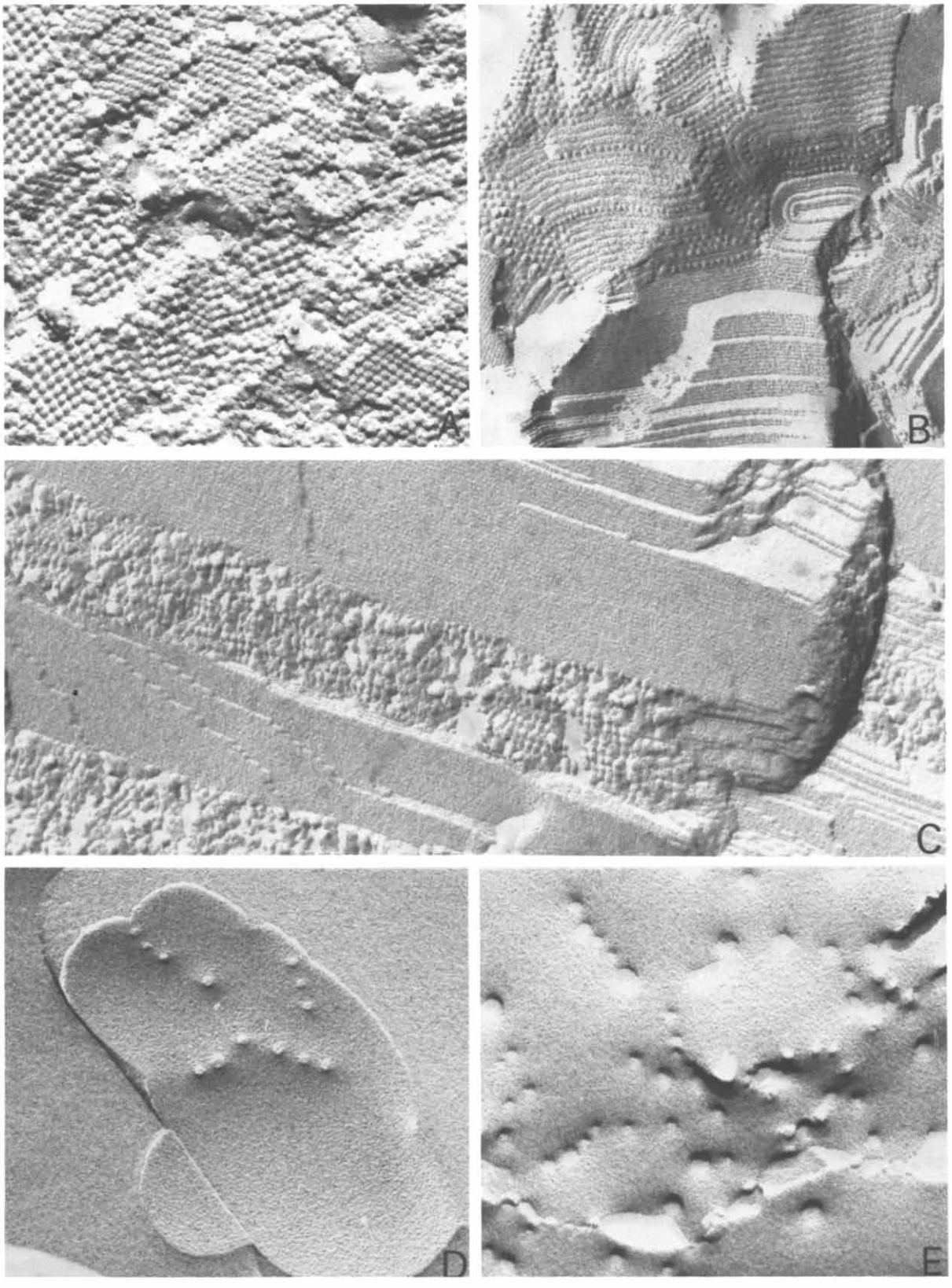
We further investigated the effect of Ca^{2+} on dioleoylphosphatidic acid in a mixture with egg phosphatidylcholine at pH 6.0, at which pH this negatively charged phospholipid adopts the H_{II} phase. For this study we chose a mixture of dioleoylphosphatidic acid/egg phosphatidylcholine which when sonicated yields small vesicles (Fig. 2D). After addition of Ca^{2+} up to a molar ratio of Ca^{2+}/dioleoylphosphatidic acid of 2, it was observed that the vesicles fuse. This fusion is associated with the presence of lipidic particles at the fracture faces (Fig. 2E).

Similar results were obtained for Mg^{2+}. It has to be noted that no H_{II} phase and agglomerates of inverted micelles were found in an excess of divalent cations. These results are in contradiction to cardiolipin/phosphatidylcholine mixtures in the presence of excess Ca^{2+}, Mg^{2+} and Mn^{2+} [25]. The reason for this discrepancy is not understood.

Dioleoylphosphatidic acid/chlorpromazine

Chlorpromazine, added in a constant amount of ethanol to a liposomal dispersion of dioleoylphosphatidic acid at pH 6.0, results in a precipitation of the lipid at a molar ratio of chlorpromazine/dioleoylphosphatidic acid of 1 : 1 and 2 : 1. At higher concentrations of the local anaesthetic the solution becomes increasingly clear, probably because chlorpromazine starts to act as a detergent at these conditions. At a molar ratio chlorpromazine/dioleoylphosphatidic acid of 1 : 1, conventional freeze-fracturing in the presence of glycerol reveals droplets of lipid completely filled with lipidic particles (Fig. 3A). Different fracture phases at different angles are relevant. One can observe particles in a hexagonal arrangement, and also an arrangement which is compatible with a cubic arrangement of the particles. In addition to that droplets containing H_{II} cylinders and lipidic particles are found (Fig. 3B). It has to be noted that one can recognize cylinders with two distinctly different diameters, which has been observed in several H_{II} containing systems [25]. Jet-freezing in the presence and absence of glycerol reveals similar results, exhibiting H_{II} phase and lipidic particles in a cubic and hexagonal lattice. Similar results were also obtained for chlorpromazine/dioleoylphosphatidic acid (1 : 1) at pH 6.0 if chlorpromazine was added in buffer. Addition of chlorpromazine to dioleoylphosphatidic acid/egg phosphatidylcholine mixtures resulted in the appearance of lipidic particles which are not well-defined. These particles and pits can better be defined as deformations of undefined size and shape (Fig. 3D and E) as described by Miller [8] and Hui et al. [9] which likely are intermediate stages during transitions from bilayer → nonbilayer phases or may resemble intermembrane attachment sites.

Fig. 2. Freeze-fracture micrographs of dioleoylphosphatidic acid dispersed in buffer containing 10 mM Mes, 100 mM NaCl at pH 6.0 and 10 mM divalent cations (ratio Me^{2+}/dioleoylphosphatidic acid 2 : 1); $CaCl_2$ (A), $MgCl_2$ (B) and $MnCl_2$ (C); and freeze-fracture micrographs of a mixture dioleoylphosphatidic acid/egg phosphatidylcholine (molar ratio 1 : 2) dispersed in buffer containing 10 mM Mes, 100 mM NaCl at pH 6.0 and sonicated for 2 min at 0°C (D) and upon addition of $CaCl_2$ to Ca^{2+}/dioleoylphosphatidic acid ratio of 2 : 1 (E). Magnification: A, B and C ×180000; D and E ×100000.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Cardiolipin/chlorpromazine

To relate these findings to those observed with cardiolipin we investigated the effect of chlorpromazine on the morphology of cardiolipin containing model containing model membranes. As described earlier chlorpromazine can also induce H_{II} phase with cardiolipin [2]. Fig. 3C shows the complex of chlorpromazine/cardiolipin at a molar ratio 1:1, quenched by jet-freezing in the absence of glycerol. Similar to the results found with dioleoylphosphatidic acid one observes the H_{II} phase next to an agglomerate of lipidic particles in a cubic and hexagonal lattice.

Discussion

This study clearly demonstrates that Ca^{2+}, Mg^{2+} and Mn^{2+} can induce the H_{II} phase in pure dioleoylphosphatidic acid and lipidic particles in mixed dioleoylphosphatidic acid/phosphatidylcholine systems. The formation of hexagonal H_{II} phase of egg phosphatidic acid, by Ca^{2+} has been reported before [26]. This non-bilayer organization of the divalent cation dioleoylphosphatidic acid complexes is only found at pH values where the lipid molecule exposes one net negative charge. Therefore, it can be suggested that these inverted non-bilayer structures are formed by the Me(dioleoylphosphatidic acid)$_2$ complex similarly to that observed for the Ca^{2+}/cardiolipin system. In the latter case it is proposed that two phosphates are pulled together by the divalent cation, thereby decreasing the cross-sectional area of the head group resulting in a cone shape of the complex.

In analysing the morphology of the H_{II} phase, it is clear that the thickness of the cylinder of dioleoylphosphatidic acid/Me^{2+} is dependent on the type of divalent cation: Ca^{2+}/dioleoylphosphatidic acid having the smallest and Mn^{2+}/dioleoylphosphatidic acid having the largest diameter. A similar dependency has been found for cardiolipin [25]. These differences in tube thickness indicate different head group areas or head group hydrations of the various Me^{2+}/dioleoylphosphatidic acid complexes.

In analogy with other mixed systems [6,7], fusion induced in dioleoylphosphatidic acid/phosphatidylcholine mixture by the addition of Ca^{2+} is associated with the presence of lipidic particles. This strongly supports the notion that a non-bilayer organization, likely the inverted micelles, could be intermediary in fusion.

The observation that divalent cations only around pH 6.0 can induce non-bilayer structure in dioleoylphosphatidic acid containing model membranes suggests intriguing structural and functional possibilities of phosphatidic acid in biological membranes. Variations in pH in the physiological range for instance could trigger bilayer → non-bilayer structures in phosphatidic acid containing membranes. Further, our experiments show that phosphatidic acid can form inverted structures upon interaction with Ca^{2+}, which fulfill the requirement postulated for the ionophoric action of phosphatidic acid [7,27].

The experiments with chlorpromazine show that this type of local anaesthetic bearing one positive charge, can interact with dioleoylphosphatidic acid in such a way that this phospholipid adopt the H_{II} phase and an organization of the lipid in particles which very likely represent an agglomerate of inverted micelles. This latter type of organization has also been reported for other lipid mixtures [25]. It is difficult to deduce the three-dimensional organization of these lipid droplets from a two-dimensional picture. However, it is clear that different arrangements of the particles can be distinguished at different fracture faces, i.e. planes at different angles through the lipid droplet. One arrangement is of hexagonal organization of particles and another one which is compatible with a cubic arrangement of the particles. From this we suggest that this structure reflects spheres likely inverted micelles, in a closed packed organization.

Fig. 3. Freeze-fracture micrographs of dioleoylphosphatidic acid dispersed in a buffer containing 10 mM Mes, 100 mM NaCl at pH 6.0 upon addition of chlorpromazine at a ratio chlorpromazine/dioleoylphosphatidic acid of 1:1 (A and B); cardiolipin dispersed in same buffer at pH 6.0 and upon addition of chlorpromazine at a similar ratio (C); and of a mixture dioleoylphosphatidic acid/egg phosphatidylcholine (molar ratio 1:1) and cardiolipin/egg phosphatidylcholine dispersed in the same buffer at pH 6.0 upon addition of chlorpromazine at chlorpromazine/dioleoylphosphatidic acid and chlorpromazine/cardiolipin ratio of 1:1 (D and E, respectively). Magnification: ×100000.

These results further indicate that the action of these positively charged local anaesthetics next to the alternative mechanisms proposed for these drugs [28] may work by modulating bilayer – non-bilayer confirmation as has been suggested by Cullis et al. [29].

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