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Ca^{2+} AND pH INDUCED FUSION OF SMALL UNILAMELLAR VESICLES CONSISTING OF PHOSPHATIDYLETHANOLAMINE AND NEGATIVELY CHARGED PHOSPHOLIPIDS: A FREEZE FRACTURE STUDY

M.J. Hope[a], D.C. Walker[b] and P.R. Cullis[a]

(a) Department of Biochemistry, University of British Columbia Vancouver, B.C. V6T 1W5 Canada

(b) Pulmonary Research Laboratory, Department of Pathology St. Paul's Hospital, Vancouver, B.C. Canada

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Fusion processes in small (sonicated) unilamellar vesicle (SUV) systems composed of 80 mol % unsaturated phosphatidylethanolamine and 20 mol % of phosphatidylserine, phosphatidylinositol, phosphatidic acid or cardiolipin have been examined by freeze-fracture techniques. All these mixtures have the property that Ca^{2+} and, in some cases low pH, can trigger bilayer to hexagonal (H_{II}) phase transitions in large multilamellar dispersions of these lipid mixtures. It is shown that when the SUV systems are subjected to similar protocols, the vesicles first fuse to form larger systems prior to H_{II} phase formation and this fusion is accompanied by the appearance of lipidic particle structures, often localized to the fusion interface. We conclude that factors promoting H_{II} phase structure initially result in fusion of unilamellar systems and that this fusion proceeds via intermediary formation of non-lamellar, possibly inverted micellar, structure.

A number of studies have indicated that factors which enhance the ability of lipids to adopt hexagonal (H_{II}) phase structure will promote fusion in small unilamellar vesicle (SUV) systems (1-3). For example, Ca^{2+} which is generally required for fusion events in vivo (4) has been shown to induce fusion of cardiolipin (CL)-phosphatidylcholine (PC) vesicles accompanied by the formation of "lipidic particles" (2). The fact that CL adopts the hexagonal (H_{II}) phase in the presence of Ca^{2+} has led to the proposal that these lipidic particles represent inverted micellar structures which are an intermediate structure between the bilayer and hexagonal (H_{II}) phases (3).

Ca^{2+} and pH changes can also trigger the bilayer - H_{II} transition in multilamellar mixtures of various acidic phosopholipids with unsaturated phosphatidylethanolamine (PE) (4, 5). In the work presented here we examine the ability of SUV systems with similar lipid compositions to

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undergo fusion when subjected to such protocols, with particular emphasis on the mechanisms involved. Such features are of interest as the mechanism by which Ca^{2+} triggers bilayer-H_{II} transitions in multilamellar PE-acidic phospholipid systems depends on the nature of the negatively charged species (for review see ref. 6). In the case of mixtures containing CL and phosphatidic acid (PA) for example, Ca^{2+} can convert the CL and PA to H_{II} preferring species, thus allowing the entire mixture to adopt the H_{II} phase (see refs. 7-9). Alternatively, in the case of phosphatidylserine (PS), Ca^{2+} appears to induce a lateral segregation of PS into "cochleate" domains [10-12], allowing the PE to adopt the H_{II} configuration it prefers in isolation. This contrasts with the behaviour of systems stabilized by phosphatidylglycerol (PG), where the presence of Ca^{2+} appears to reduce the bilayer stabilizing capacity of PG and both PG and PE enter the H_{II} configuration [13]. For phosphatidylinositol (PI), a Ca^{2+} induced lateral segregation of PI into liquid crystalline lamellar domains is indicated, (although not yet proven) leaving the PE to revert to the H_{II} phase [14]. Finally bilayer-H_{II} transitions are observed for PS-PE and PA-PE systems as the pH is reduced below 5 [11, 15] as such pH values convert the PA and PS to H_{II} preferring lipid species. Given this variety of mechanism and effector, it is of interest to determine whether SUV systems with these lipid compositions undergo fusion under appropriate protocols, and whether the mechanism involved is similar. We show that SUV's composed of mixtures of PE with PS, PG, PI, CL and PA all undergo fusion in the presence of Ca^{2+}, as do mixtures of PE with PS and PA at reduced pH values.

MATERIALS AND METHODS

Soya PE was prepared from soya PC and ethanolamine employing the base exchange capacity of phospholipase D [16], and was purified by preparative liquid chromatography employing a Waters Prep 500 apparatus. PS, PA and PG were prepared from egg phosphatidylcholine by similar methods and purified as described previously [9, 12, 13]. PI (Sigma, St. Louis) was purified by preparative liquid chromatography using chloroform/methanol/water/25% ammonium hydroxide (60:40:2:1 v/v) as eluting solvent, and this material was further purified employing carboxymethyl cellulose chromatography (14). The negatively charged phospholipids were converted to their sodium salt form, and demonstrated to be more than 99% pure by two dimensional

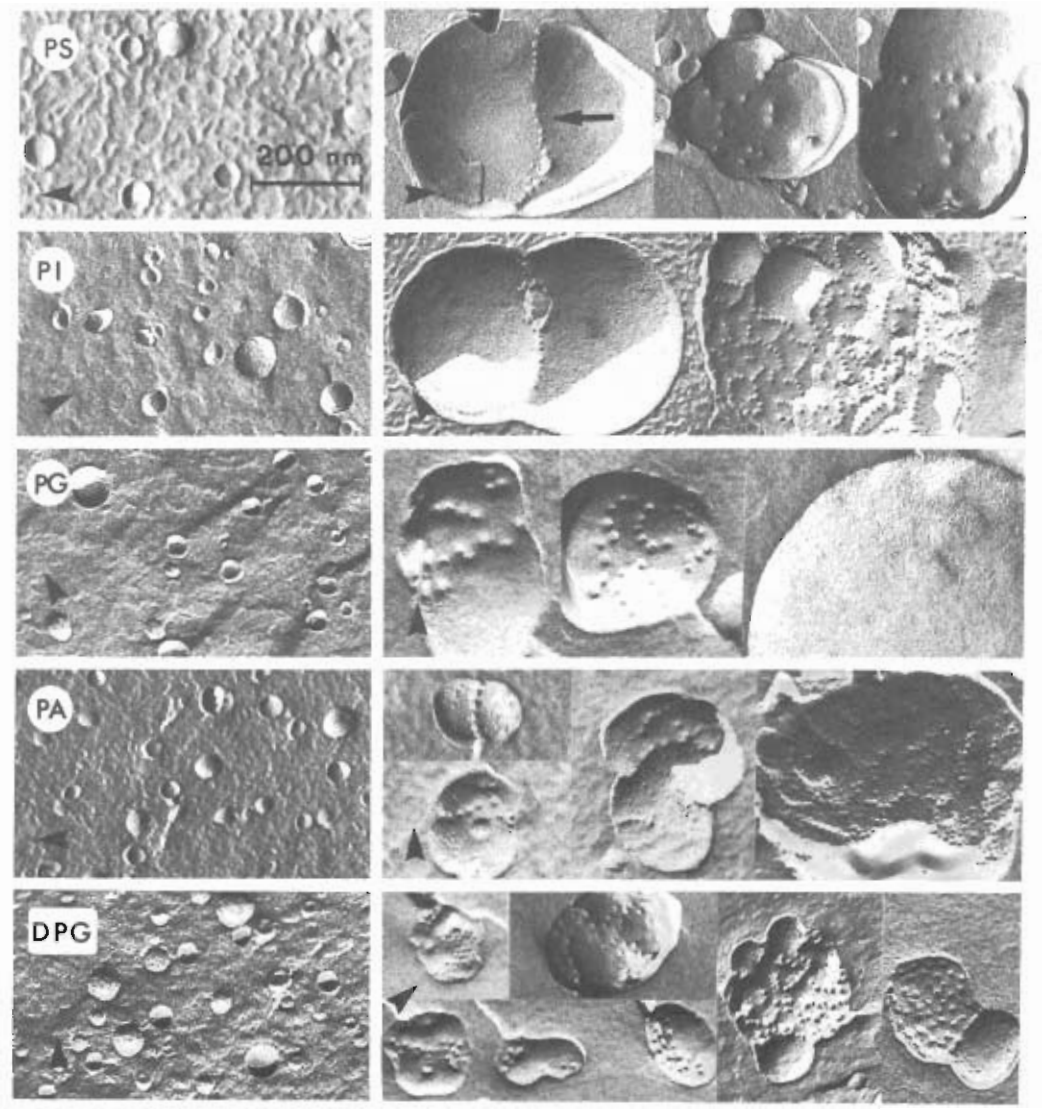

Fig. 1. Freeze-fracture micrographs of small (sonicated) unilamellar vesicles composed of 80 mol % soya phosphatidylethanolamine (PE) and 20 mol % of the following acidic phospholipids: egg phosphatidylserine (PS); soya phosphatidylinositol (PI); egg phosphatidylglycerol (PG); egg phosphatidic acid (PA); beef heart cardiolipin (DPG); before (left hand side) and after (right hand side) dialysis at 20°C against a Ca^{2+} containing buffer. The various Ca^{2+} concentrations and the dialysis times were: PS-PE:3 mM Ca^{2+} (40 min); PI-PE: 10 mM Ca^{2+} (20 min); PG-PE: 10 mM Ca^{2+} (20 min); PA-PE: 10 mM Ca^{2+} (20 min); DPG-PE: 10 mM Ca^{2+} (20 min). Samples were removed, mixed with glycerol (25% v/v) and quenched from 20°C. The arrow in the PS-PE-Ca^{2+} mictograph indicates an array of lipidic particles in a region corresponding to a fusion interface and similar localizations can be observed for the PI-PE-Ca^{2+} system, the PA-PE-Ca^{2+} system and the DPG-PE-Ca^{2+} system. The arrowheads in each micrograph indicate the direction of shadowing.

thin layer chromatography. Beef heart CL (U.S.B.C., Cleveland) was used without further purification.

Mixtures of soya PE and the negatively charged phospholipids were mixed in the molar ratio 4:1 in chloroform. The chloroform was evaporated under a stream of N_2 and by subsequent exposure to high vacuum for 2 hr. The resulting lipid film was hydrated in 10 mM TRIS-HCl at pH 7.0 containing 100 mM NaCl and dispersed by sonication (Branson tip sonicator) for 3-10 min at 4°C. The resulting dispersion (50 - 100 μmoles phospholipid/ml) was transferred to 18 mm diameter dialysis tubing (Spectrapore, molecular weight cut off 3500) and dialyzed at room temperature against the dispersion buffer containing 3-10 mM $CaCl_2$. In the case of the PE-PS and PA-PS systems the vesicles were also dialyzed against Ca^{2+} free buffer (10 mM glycine, 100 mM NaCl) at pH 3.0. Aliquots were removed for freeze-fracture analysis at various time intervals and 25% (v/v) glycerol was added to prevent freeze damage. Samples were quenched from room temperature and replicas prepared according to standard freeze-fracture procedures employing a Balzers BAF 301 apparatus. Micrographs were obtained employing a Phillips 400 electron microscope.

RESULTS AND DISCUSSION

As shown in Fig. 1, SUV systems composed of egg PS - soya PE (1:4, mol/mol), soya PI - soya PE (1:4), egg PG-soya PE (1:4), egg PA - soya PE (1:4) and beef heart CL-soya PE (1:4) all undergo fusion, as evidenced by the appearance of much larger structures, on dialysis against various concentrations of Ca^{2+}. Further, in all cases this fusion is accompanied by the appearance of lipidic particle [17] structures which are often localized to regions corresponding to the fusion interface. Similar behaviour is also observed for SUV systems composed of egg PS-soya PE (1:4) and egg PA-soya PE (1:4) on dialysis against a pH 3.0 buffer, as illustrated in Fig. 2. As indicated in the Introduction all these systems have the property that multilamellar dispersions with equivalent lipid composition undergo bilayer to H_{II} transitions when subjected to similar protocols. The results presented here therefore establish that fusion, accompanied by lipidic particle formation, is a feature common to SUV systems incubated under conditions which eventually lead to formation of H_{II} phase structure. This conclusion is reinforced by earlier observations that beef heart CL-egg PC (1:1) SUV systems fuse in association with lipidic particle formation in the presence of Ca^{2+} [2] as do $18{:}1_c/18{:}1_c$ PE-$18{:}1_c/18{:}1_c$ PC-cholesterol (3:1:2) SUV systems incubated at 50°C [3] (for details on the ability of PE-PC-cholesterol multilamellar dispersions to adopt the H_{II} phase see ref. 18).

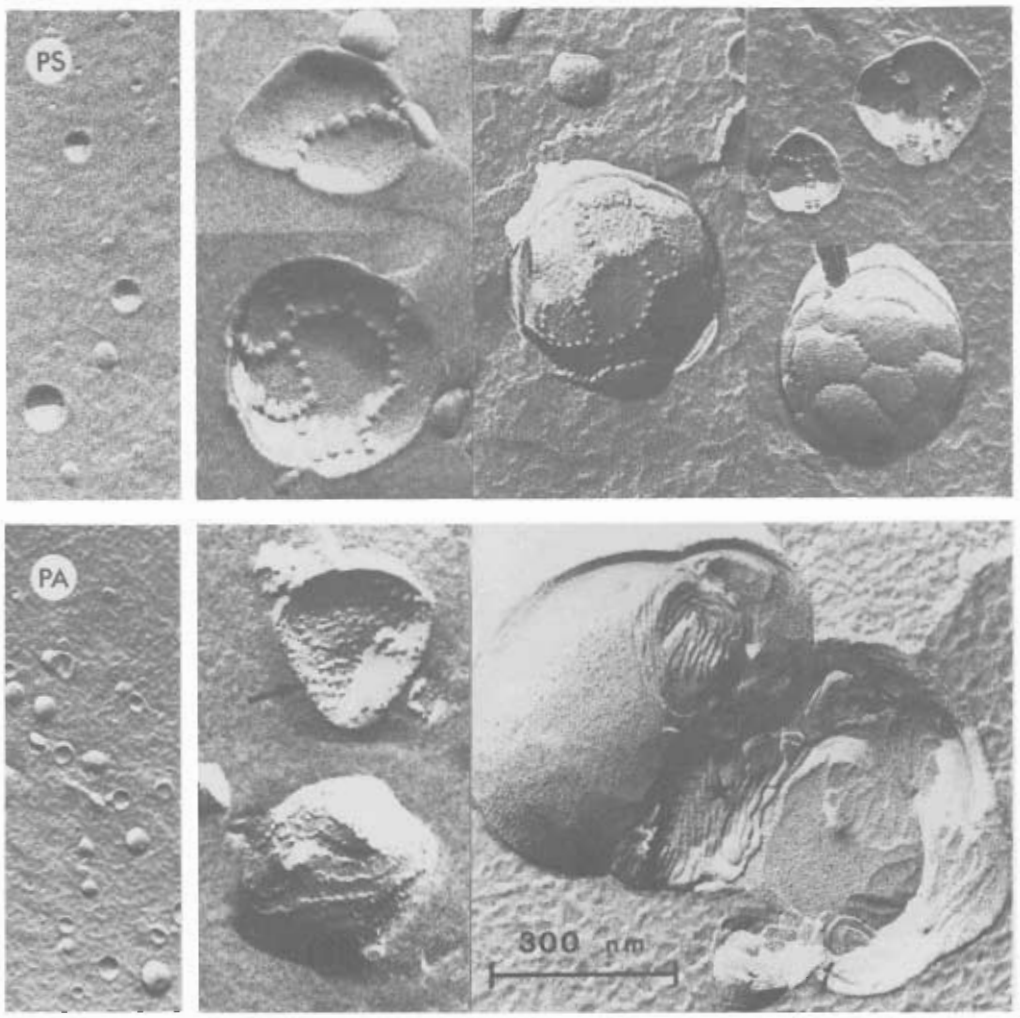

Fig. 2. Freeze-fracture micrographs of small (sonicated) unilamellar vesicles composed of 80 mol % soya phosphatidylethanolamine (PE) and 20 mol % of egg phosphatidylserine (PS) (top section) or 20 mol % egg phosphatidic acid (PA) (bottom section) before (left hand side) and after (right hand side) dialysis at 20°C against a pH = 3.0 buffer. The length of dialysis time was 40 min. Samples were mixed with glycerol (25% vol/vol) and quenched from 20°C. It may be noted that large systems formed for the PE-PS systems after dialysis closely resemble the honeycomb type of structure proposed elsewhere [21]. The lipidic particles observed for the PE-PA systems are indicated by an arrow, and are less prominent than are those in the PE-PS systems. The PE-PA structure on the far right is suggestive of a region of hexagonal (H_{II}) phase tubes expanding into stacked layers (see ref. 8). The shadowing direction is from the bottom to the top of the micrographs.

As noted earlier (see Introduction) the mechanism whereby the H_{II} phase structure is induced in the multilamellar analogues of the SUV systems investigated here differs. However, the results of Fig. 1 and 2 suggest that the mechanisms of fusion are similar. It is instructive to note the progression involved. In particular, it is only after large lipid aggregates have formed, via the initial fusion processes, that other structures such as H_{II} phase organization or (in the case of PS containing systems in the presence of Ca^{2+}) cochleate [10] PS-Ca^{2+} aggregates (see ref. 19, for example) are manifest. A second point concerns the similarity between the Ca^{2+} induced fusion processes depicted in Fig. 1 and the pH induced fusion processes of Fig. 2, suggesting a common mechanism. Other authors [20] have suggested that cation induced vesicle fusion involves formation of a dehydrated divalent cation "trans" complex. The results presented here imply that for the PE-acidic phospholipid mixtures at least, the ability of Ca^{2+} to provide conditions conducive to formation of H_{II} phase structures is of more basic importance.

A remaining question concerns whether it is the hydration characteristics of lipids in fusing systems, or the ability to assume non-bilayer structure (or both) which are essential to fusion events in general. In particular, vesicular systems composed of pure PS can be induced to fuse by Ca^{2+} [20] but Ca^{2+} does not induce H_{II} phase structure in multilamellar PS dispersions. It may be noted that H_{II} phase lipids (such as PE) hydrate poorly in comparison to normal lamellar phospholipids, as does the Ca^{2+}-PS complex. Thus it might be suggested that factors which lead to reduced hydration and which therefore encourage close contact between membranes (e.g. Ca^{2+}-PS complexes, or lipids which prefer H_{II} phase organization) are both necessary and sufficient for fusion to proceeed, and that the subsequent ability to assume non-lamellar structure is not essential. We believe that such dehydration is indeed necessary to obtain the close contact required. However, preliminary studies suggest

that the availability of non-lamellar intermediates such as the inverted micelle result in less leakage during the fusion process. Such structures may also allow formation of stable interconnected semi-fused membrane systems resulting in possibilities of compartmentalization within a continuous membrane matrix (see ref. 21), possibly including "tight-junction" systems (25).

The results presented here have direct potential relationships to fusion processes in biological membranes, as the lipid composition of the inner or outer leaflets of certain plasma or organelle membranes is similar to mixtures employed here. These include the inner monolayer of the erythrocyte [22] (predominantly PE and PS) and the inner monolayer of the inner mitochondrial membrane [23] (predominantly CL and PE). Such asymmetric lipid distributions may play important roles in the Ca^{2+} stimulated fusion events involved in exocytosis [19].

In summary, factors which promote formation of H_{II} phase structure in various multilamellar systems initially result in fusion of unilamellar sonicated vesicle systems with the same lipid compositions. The observation that this fusion is accompanied by formation of lipidic particle structure may be employed to suggest that lipid particles, interpreted as inverted micelles [3, 24], play intermediary roles in the fusion event. However, recent experiments (A.J. Verkleij, unpublished) employing fast-freezing techniques suggest that lipidc particles appear subsequent to the fusion process. Although it may be that such particles represent stable versions of transitory intermediate structures occurring in fusion, we take the more general view that the presence of lipids which prefer H_{II} phase organization satisfies two fundamental requirements for fusion to proceed, irrespective of the detailed nature of the intermediate structure. These are first a condition of low hydration allowing close apposition, and second an ability to undergo the transitory departure from lamellar organization clearly required for fusion to proceed.

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