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Phospholipase C activity-induced fusion of pure lipid model membranes. A freeze fracture study

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The structural effects of *in situ* production of diacylglycerol by phospholipase C in pure lipid model membranes have been examined by freeze fracture electron microscopy. Phospholipase C-activity induces massive aggregation and fusion of large unilamellar lipid vesicles and leads to the formation of a 'sealed' lipid aggregate; the outer membrane of this aggregate appears to be continuous. In some areas lipid arranges into a honeycomb structure; this structure is probably a precursor of a discontinuous inverted (type II) cubic phase. Similarly, enzyme treatment of multilamellar vesicles leads to extensive membrane fusion and vesiculation. Thus morphological evidence is obtained showing the ability of phospholipase C to induce bilayer destabilization and fusion. It is speculated that phospholipase C-induced membrane fusion involves a type II fusion intermediate induced by diacylglycerol produced locally.

Introduction

Activation of phospholipase C in cells has been associated with signal transduction via diacylglycerol (DAG) production [1,2]. In particular, phospholipase C activity appears to be involved in platelet aggregation and secretion [3] and it may also be involved in the control of membrane curvature in erythrocytes [4,5]. Under exogenous treatment with phospholipase C, cell membranes become permeable to macromolecules [6], and when the enzyme is injected into fibroblasts morphological alterations and transformation are induced [7]. Moreover, recent studies have shown the ability of phospholipase C to promote liposome fusion [8], and the hypothesis of its involvement in cell membrane fusion has been put forward [5,9–11]. It is believed that membrane effects of phospholipase C are mediated by its lipidic reaction product, DAG, particularly since this molecule, when added to pure lipid model membranes, induces bilayer destabilization and the formation of inverted, or type II, non-bilayer lipid structures [9,10,12,13].

The morphology of various model membrane systems containing DAG has been described (see, for example, Ref.14), however, ultrastructural data on the effects of *in situ* production of DAG in model membranes, during phospholipase C treatment, have been lacking. We studied the phospholipase C activity-induced fusion of liposomes composed of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cholesterol (Chol); both conventional, and fast-freeze, freeze fracture electron microscopy were used. In this experimental system, phospholipase C has been shown to induce fast, efficient and non-leaky membrane fusion [8]. The morphological data presented in the current report confirm the ability of the enzyme to induce bilayer destabilization; vesicle aggregation and fusion are observed. Moreover, lipids are found to organize in a honeycomb structure. This structure is very similar to that described for a PC-DAG mixture [14] which has

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Abbreviations: Chol, cholesterol; DAG, diacylglycerol; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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been suggested to represent a novel discontinuous inverted cubic phase [15].

Materials and Methods

Large unilamellar vesicles (LUVs) and multilamellar vesicles (MLVs), consisting of egg PC, egg PE and Chol in a 2:1:1 molar ratio, were prepared and enzyme assays performed on LUVs as described in Ref. 8. Samples were processed for either conventional freeze-fracture or fast-freeze freeze fracture electron microscopy. In each case phospholipid breakdown was assayed according to Ortiz et al. [13] in an independent experiment performed under the same conditions as those used in the electron microscopy experiments. For conventional freeze fracture (see later), LUVs were prepared in the presence of 30% (v/v) glycerol. Enzyme treatments of MLVs (made of various PC, PE, Chol mixtures) were as follows: the enzyme was added to a vesicle suspension at 4°C and the mixture frozen in liquid nitrogen. It was then freeze-thawed five times, the temperature during thawing not exceeding 4°C. Under these conditions no appreciable phospholipid breakdown could be detected. The enzyme was activated by raising the temperature to 37°C.

For conventional freeze fracture, experiments were performed in the presence of 30% (v/v) glycerol. Small aliquots were transferred to gold platelets and frozen from roomtemperature by dipping the samples either into a mixture of solid and liquid nitrogen or into liquid propane cooled to its melting point. For fast-freeze freeze fracture, samples were transferred to a sandwich composed of two hat-shaped copper covers and a copper spacer (see Ref. 16). The sandwiched samples were fast-frozen from room temperature by plunging them into liquid propane cooled to its melting point, using a plunge-freezing device (KF80; Reichert Jung, Wien, Austria); no cryoprotectants were used.

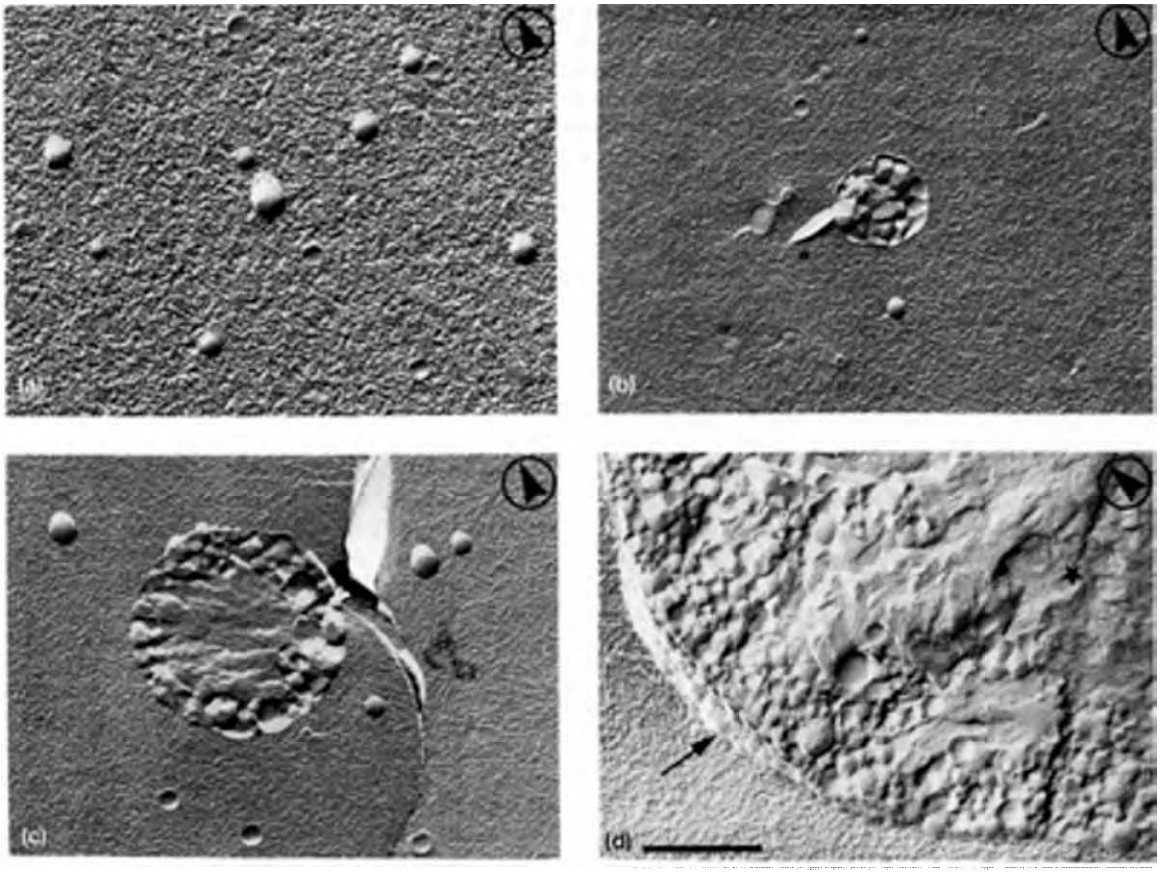

Fig. 1. Phospholipase C-induced aggregation and fusion of LUVs as visualized by conventional freeze fracturing. Frozen (a) before, (b) 30 s after, (c) 90 s after, and (d) 300 s after enzyme addition. The outer membrane of the aggregates appears to be continuous (e.g. arrow in d, inner fracture face). The core of the aggregate has an amorphous appearance (star in d). Lipid concentration: 10 mM; enzyme concentration: 400 IU/ml. Printed at the same final magnification; bar 500 nm. Direction of Pt/C-shadowing indicated by encircled arrowhead.

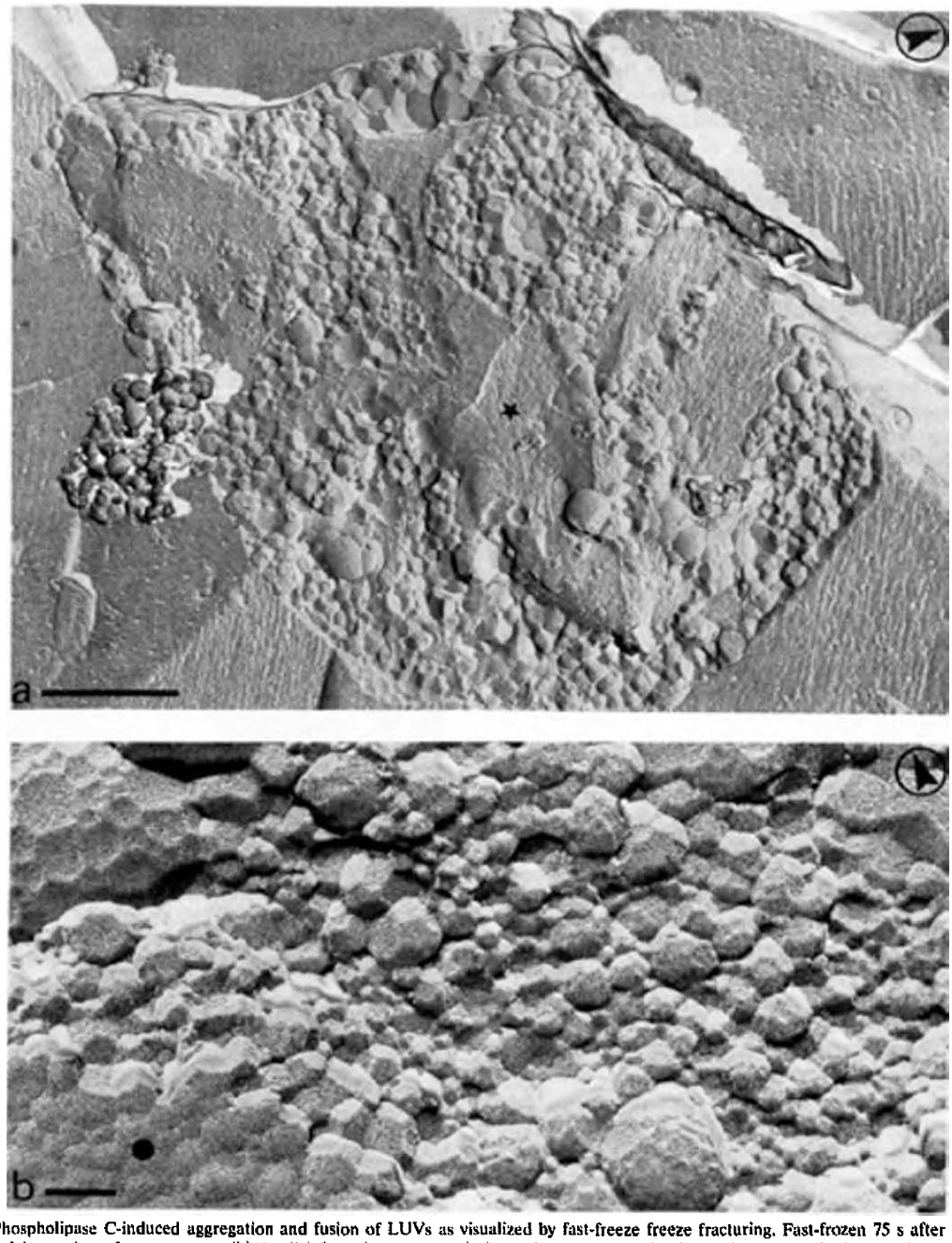

Fig. 2. Phospholipase C-induced aggregation and fusion of LUVs as visualized by fast-freeze freeze fracturing. Fast-frozen 75 s after enzyme addition; (a) overview of an aggregate; (b) detail (of another aggregate). Amorphous core (star in a). Continuous enveloping membrane (closed circle in b, inner fracture face). Lipid concentration: 10 mM; enzyme concentration: 40 IU/ml. Bars, 500 nm (a), and 100 nm (b). Direction of Pt/C-shadowing indicated by encircled arrowhead.

Freeze fracture and replication were carried out following standard procedures. The replicas were stripped off on water and cleaned with commercial bleach and distilled water (conventional freeze fracture); or the replicas were stripped off and cleaned with dilute chromic/sulphuric acid and distilled water according

to Ref. 17 (fast-freeze freeze fracture). The replicas were examined with a Philips CM10 electron microscope at 100 kV.

Results and Discussion

Fusion experiments were performed as described before [8]. By using a cryoprotectant (glycerol), the conventional freeze fracture method avoids the (technical) problems connected with the fast-freezing of specimens; therefore experiments were first performed in the presence of 30% (v/v) glycerol. Independent measurements showed that glycerol slowed down the kinetics of the fusion process, though it hardly affected phospholipase C activity (data not shown). Samples were taken from a suspension of LUVs and frozen, before, and 30, 90 or 300 s after adding phospholipase C. At these stages DAG contents, arising from phospholipid cleavage, were about 0, 15, 35 and 60 mol% (of total lipid), respectively. The corresponding freeze fracture results are shown in Fig. 1; vesicle aggregates are seen to form and to increase in size with time. The outer membrane of these aggregates appears to be continuous (Figs. 1b–d; also see Fig. 2b) and this enveloping membrane must have resulted from fusion processes. At an early timepoint (30 s, Fig. 1b) other signs of bilayer destabilization are not observed. It should be noted that the vesicular compartments of the aggregate have a clear polygonal appearance. At longer incubation times (90 and 300 s, Figs. 1c and d, respectively) the vesicular compartments decrease in size and, in addition, the aggregate core progressively looses its bilayer structure becoming almost amorphous in appearance (marked by a star in Fig. 1d). The freeze fracture images show no sign of an intermembrane space separating neighbouring vesicular compartments; instead the images suggest that neighbouring compartments share one bilayer.

The images presented in Fig. 1 suggest that aggregates grow peripherally via fast aggregation and non-leaky (see Ref. 8) fusion, for which only small amounts of DAG are required, while a slower process of extensive phospholipid hydrolysis and leaky fusion could be responsible for the changes observed in core structure; the amorphous core may well contain (segregated) DAG and Chol almost exclusively. The fact that aggregates always appear to be surrounded by a continuous membrane may explain the absence of (detectable) vesicle leakage even when substantial phospholipid hydrolysis has occurred (see Ref. 8). This finding constitutes an important warning to those relying on biochemical (dilution) assays to determine whether vesicle fusion does or does not involve leakage of internal contents; these assays will fail to detect leakage if it occurs within a ('sealed') aggregate of vesicles.

Inclusion of glycerol leads to an increase in buffer viscosity and probably results in a steep decrease in the rate of vesicle aggregation; glycerol slows down the fusion kinetics and decreases the efficiency of fusion relative to the amount of DAG formed. Moreover, the use of glycerol as a cryoprotectant may affect sample ultrastructure. Therefore in a parallel series of experiments glycerol was omitted and samples were studied using fast-freeze freeze fracture electron microscopy. Again vesicle aggregates were seen to form and to increase in size with time. Fig. 2 illustrates the ultrastructure of a sample fast-frozen 75 s after enzyme addition, when approximately 20 mol% DAG is present. In the absence of glycerol, the fusion process as detected by lipid mixing and leakage-free mixing of vesicle contents, is completed in about 30 s [8]. Thus the images in Fig. 2 probably represent end products of vesicle fusion. Lipid structures of variable size, the

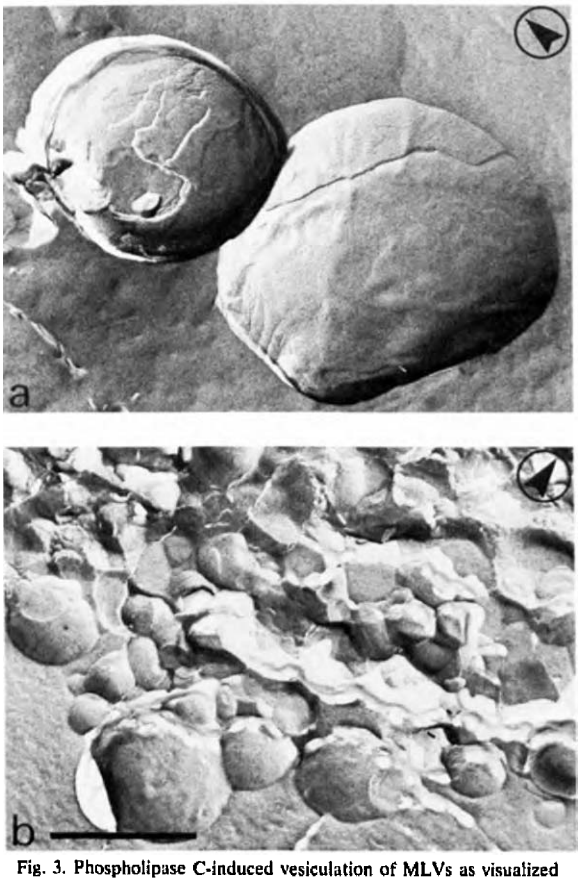

Fig. 3. Phospholipase C-induced vesiculation of MLVs as visualized by fast-freeze freeze fracturing. Fast-frozen (a) before, and (b) 75 s after activation of the enzyme. Lipid concentration: 10 mM; encapsulated enzyme, concentration: 1 IU/ml. Printed at the same final magnification; bar 500 nm. Direction of Pt/C-shadowing indicated by encircled arrowhead.

smallest structures being approximately 14 nm in diameter (Fig. 2b), are seen to assemble into a honeycomb structure. The ultrastructure is complex and highly unusual and its molecular interpretation is not straightforward. DAG is known to promote bilayer to inverted non-bilayer lipid structure transitions [9,10,12,13,18], and the lipid organization illustrated in Fig. 2 closely resembles that described by Cunningham et al. [14] for PC-DAG dispersions containing 80 mol% DAG which has been suggested to represent a novel discontinuous inverted cubic phase [15]. The current study shows that a similar structure forms upon treating LUVs with phospholipase C, in the absence of glycerol and at a relatively low DAG content (cf. Ref. 14). The fact that this honeycomb structure is observed at much lower DAG contents, especially in fast-frozen samples (Fig. 2, 20 mol% DAG), is probably due to the presence of PE and Chol which should favour a type II lipid organization (see Refs. 19 and 20). Contemporary theory (see, for example, Ref. 21) predicts the involvement of inverted, or type II, lipid structures in membrane fusion as well as in the formation of inverted cubic lipid phases. Phospholipase C-induced LUV fusion may very well involve a DAG-induced bilayer to inverted non-bilayer lipid structure transition. However, it should be noted that in the current study direct morphological evidence showing the involvement of these type II fusion intermediates in membrane fusion was not obtained (see also Ref. 11), and that an interpretation even of the smallest structures found at equilibrium (Fig. 2b) as being inverted lipid structures would be highly speculative.

We also explored the effects of phospholipase C on multilamellar vesicles in which it had been encapsulated; fast-freezing and freeze fracture electron microscopy were used. The results obtained for a mixture of PC, PE and Chol in an 1:2:1 molar ratio are shown in Fig. 3; similar results were obtained for the 2:1:1 mixture. Phospholipase C was activated by raising the temperature (see Materials and Methods) and a sample taken before and 75 s after activation (Figs. 3a and b, respectively); at the latter timepoint the sample contained 13 mol % DAG. Activation of phospholipase C leads to extensive membrane fusion and vesiculation (Fig. 3b), changes in bilayer curvature being probably due to the transbilayer diffusion of DAG [4].

In conclusion, we have provided morphological evidence showing that phospholipase C is able to induce aggregation and fusion of LUVs and vesiculation of MLVs. In addition lipids were found to arrange in structures that have been related to an inverted cubic phase. The induction of inverted lipid fusion intermediates may well represent a crucial step in phospholipase C-induced membrane fusion. In this context, it is interesting to note that externally added DAG does not induce fusion of LUVs (Nieva et al., unpublished observation). This could be due to an inefficient incorporation of externally added DAG into the lipid bilayer. Alternatively, only a local production of DAG by the action of phospholipase C may be able to trigger a (local) bilayer to inverted non-bilayer lipid structure transition, leading to membrane fusion. Whether phospholipase C plays a similar role in the control of membrane fusion *in vivo*, e.g. during secretion, remains a matter for future research.

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