Origin of the Lag Period in the Phospholipase C Cleavage of Phospholipids in Membranes. Concomitant Vesicle Aggregation and Enzyme Activation†

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ABSTRACT: When phospholipase C is added to a suspension of large unilamellar vesicles of egg phosphatidylcholine, maximal rates of hydrolysis occur only after a latency period. No lag period is seen when the substrate is in the form of small (sonicated) vesicles, or of short-chain phosphatidylcholine monomers. For a given vesicle concentration, the lag time may vary as a function of Ca^{2+}, enzyme concentration, or temperature, but activation occurs at a fixed molar fraction of diacylglycerol produced. Lag times decrease gradually with vesicle size, and also with the amount of diacylglycerol present in the bilayers when it is mixed with phospholipid prior to enzyme addition. Parallel recordings of enzyme activity and suspension turbidity reveal that in all cases the latency period ends concomitantly with the start of a process of vesicle aggregation. Both the lag time and the amount of diacylglycerol formed before activation decrease with vesicle concentration, suggesting that enzyme activation is somehow related to vesicle aggregation. The latency period of phospholipase C may be explained in terms of a hypothesis according to which (a) full enzyme activity requires the presence of membrane surface irregularities or *defects*, (b) the diacylglycerol generated in the lag phase produces some kind of phase separation, with the formation of diacylglycerol-rich "patches" or domains, (c) vesicles aggregate through contacts between those patches, and (d) aggregation causes (and/or increases, and/or stabilizes) the surface inhomogeneities that allow fast enzyme activity. These data and suggestions may be relevant to the process of model membrane fusion promoted by phospholipase C.

Among the multiplicity of enzymes found in the main metabolic routes, phospholipases are particularly interesting for two reasons: they are involved in the poorly understood processes of surface catalysis, and they appear to be instrumental in cell signal transduction. Of the various phospholipid phosphohydrolases, phospholipase A_2 has received particular interest, and much attention has been devoted to its mechanism of action [for a review, see Burack and Biltonen (1994)]. Phospholipases C have been comparatively less studied, in spite of their essential role in several aspects of metabolic regulation (Berridge *et al.*, 1987; Exton, 1990; Haines *et al.*, 1992). Phospholipases C may equally be involved in membrane fusion events (Roldan & Harris, 1989; Liu *et al.*, 1993; Spungin *et al.*, 1995). Previous investigations from this laboratory have revealed that the catalytic activity of phospholipase C may promote phospholipid vesicle fusion (Nieva *et al*., 1989, 1993, 1995).

One interesting feature in the action of many phospholipases is that, unlike most enzymes with soluble substrates, the initial rates are not the maximal rates of activity, instead mixing enzyme and substrate is often followed by a period of hardly detectable activity (the so-called lag or latency period), and only after such a period a "burst" of activity ensues, and the enzyme works at its maximal rate until a significant fraction of the substrate is consumed. Such a pattern of behavior has been recognized since the early days of phospholipase research (Bangham & Dawson, 1959). More recently, lag periods in phospholipase C kinetics have also been shown (Dawson *et al*., 1984; Kimura, 1987; Nieva *et al*., 1989, 1993).

The investigations reported in this paper are aimed at defining some of the factors responsible for the lag period of phospholipase C when attacking phospholipid bilayers. A bacterial source of enzyme, *Bacillus cereus*, was chosen because of its availability, extensive use in similar studies from other laboratories, and apparently similar mechanism as mammalian phospholipases C (Clark *et al.*, 1986; Larrodera *et al.*, 1990; Graziani *et al.*, 1991). The substrate in the present study are large unilamellar vesicles of egg phosphatidylcholine, obtained by extrusion. These vesicles are widely accepted to represent adequately many physical properties of cell membranes. The choice of a natural source of phospholipid is due to a desire of avoiding effects linked to gel−fluid phase transitions in our study, to the fact that cell membranes appear to exist usually in the fluid state, and to an intention of applying the present findings to our previous obervations of lag times in vesicle fusion (Nieva *et al*., 1989, 1993), in which egg lipids were mainly used.

Our results show that a certain fixed fraction of the substrate is consumed during the lag period, and that the accumulation of products leads to a change in the substrate that marks the onset of the "burst", in a process resembling, but not identical to, phospholipase A_2 activation.

MATERIALS AND METHODS

Phospholipase C (EC 3.1.4.1) from *Bacillus cereus* was supplied by Boehringer-Mannheim. Egg phosphatidylcholine

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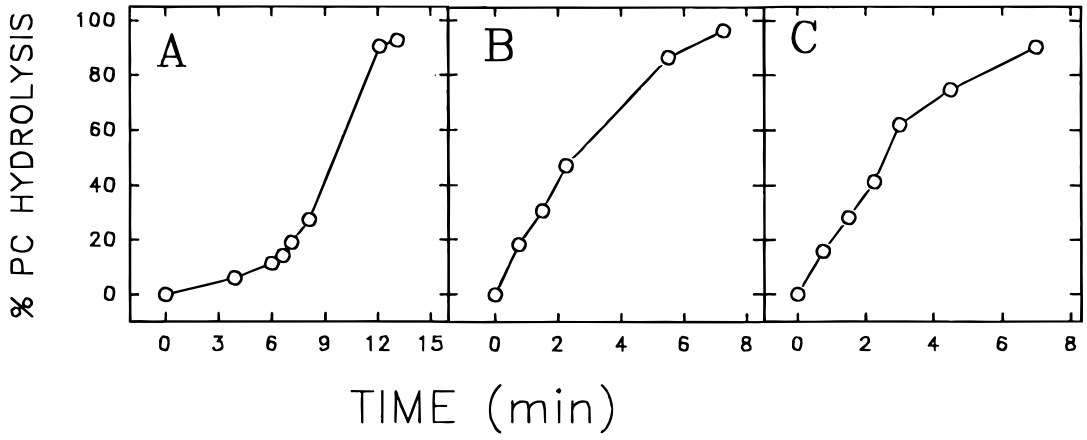

FIGURE 1: Phospholipase C hydrolysis of PC in different aggregation states. (A) LUV of egg PC; (B) SUV of egg PC; (C) monomeric DHPC. Lipid concentration was 0.3 mM and enzyme concentration was 0.16 units/mL in all cases.

(PC) and 1,2-diacylglycerol (DG) derived from egg PC were grade I from Lipid Products (South Nutfield, U.K.). Dihexanoylphosphatidylcholine (DHPC) and Triton X-100 were from Sigma (St. Louis, MO).

Substrate Preparation and Size Measurements. Phospholipid dispersions were prepared by rehydrating lipid films dried from organic solvents under high vacuum. Large unilamellar vesicles (LUVs) were prepared by the extrusion method of Mayer *et al*. (1986). Unless otherwise stated, LUV diameter was of ~100 nm. Small unilamellar vesicles were made by sonication (Alonso *et al*., 1982). Average vesicle diameters were measured by quasi-elastic light scattering (QELS) using a Malvern Zeta-Sizer instrument.

Enzyme Assays and Turbidity Measurements. Unless otherwise stated all the measurements were carried out in 100 mM NaCl, 10 mM $CaCl_2$, 10 mM Hepes, with continuous stirring. In a typical experiment PC concentration was 0.3 mM and enzyme concentration was 0.16 units/mL (~3.4 nM). Enzyme activity was assayed by determination of phosphorous contents in the aqueous phase of an extraction mixture (chloroform/methanol 2:1) after addition of aliquots from the reaction mixture at different times. Phosphorous contents of the samples were determined by the method of Bartlett (1959). Vesicle aggregation was monitored continuously as turbidity (absorbance at 405 nm) in an Uvikon 860 (Kontron Instruments).

RESULTS

In a preliminary experiment phospholipase C was assayed in the presence of 0.3 mM PC in different aggregation states, namely egg PC in large unilamellar vesicles (LUV), egg PC in small unilamellar vesicles (SUV), and monomeric dihexanoyl PC. Enzyme concentration was the same (0.16 units/mL) in all cases. The results show (Figure 1) that a lag period is only found when the substrate is in the form of LUV, although the maximum rate appears to be similar in all three cases. This suggests that the existence of a period of latency in phospholipase C activity is directly linked to the state of aggregation of the substrate.

An important clue on the mechanism of phospholipase C activation came from an experiment in which the extent of hydrolysis of PC (in the form of LUV) was studied in parallel with the change in turbidity of the LUV suspension (Figure 2). The turbidity increases, after a lag phase, when the rate of phospholipid hydrolysis displays a burst in activity. This

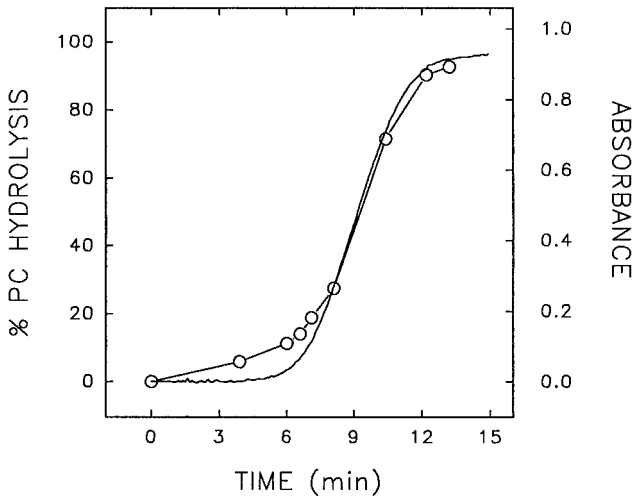

FIGURE 2: Parallel study of enzyme activity and changes in suspension turbidity after treatment of PC LUV with phospholipase C. Turbidity (A_{405}) was continuously recorded; the experimental points correspond to PC hydrolysis.

increase in turbidity was interpreted as an indication of vesicle aggregation. In fact, aggregation has been detected in our system by cryotransmission electron microscopy (K. Edwards and G. Basáñez, unpublished data). Measurements of aggregate sizes in our system, by means of QELS, have revealed increases of 1 order of magnitude 15 min after enzyme addition under the conditions in Figure 2. We can thus conclude that vesicle aggregation marks the beginning of the burst period of enzyme activity.

A series of experiments were then designed in order to establish a correlation between the length of the lag time, the extent of substrate hydrolysis, and other parameters. In all these measurements, substrate concentration was kept constant at 0.3 mM PC in the form of LUV. The results are shown in Figure 3. The lag time can be decreased by increasing enzyme concentration, by increasing temperature, by increasing Ca^{2+} concentration, or by decreasing vesicle size. Only in the latter case, when the size becomes that of SUV (~30 nm) does the lag time become zero, in agreement with the data in Figure 1. Note as well that the effect of Ca^{2+} on aggregation is the same as its effect on hydrolysis (Figure 3C). Unlike phospholipase A_2 (Roberts *et al*., 1978), phospholipase C may work in the absence of Ca^{2+}, although with long lag times. Most interestingly, as long as vesicle size (curvature) does not change (Figure 3A−C) a fixed

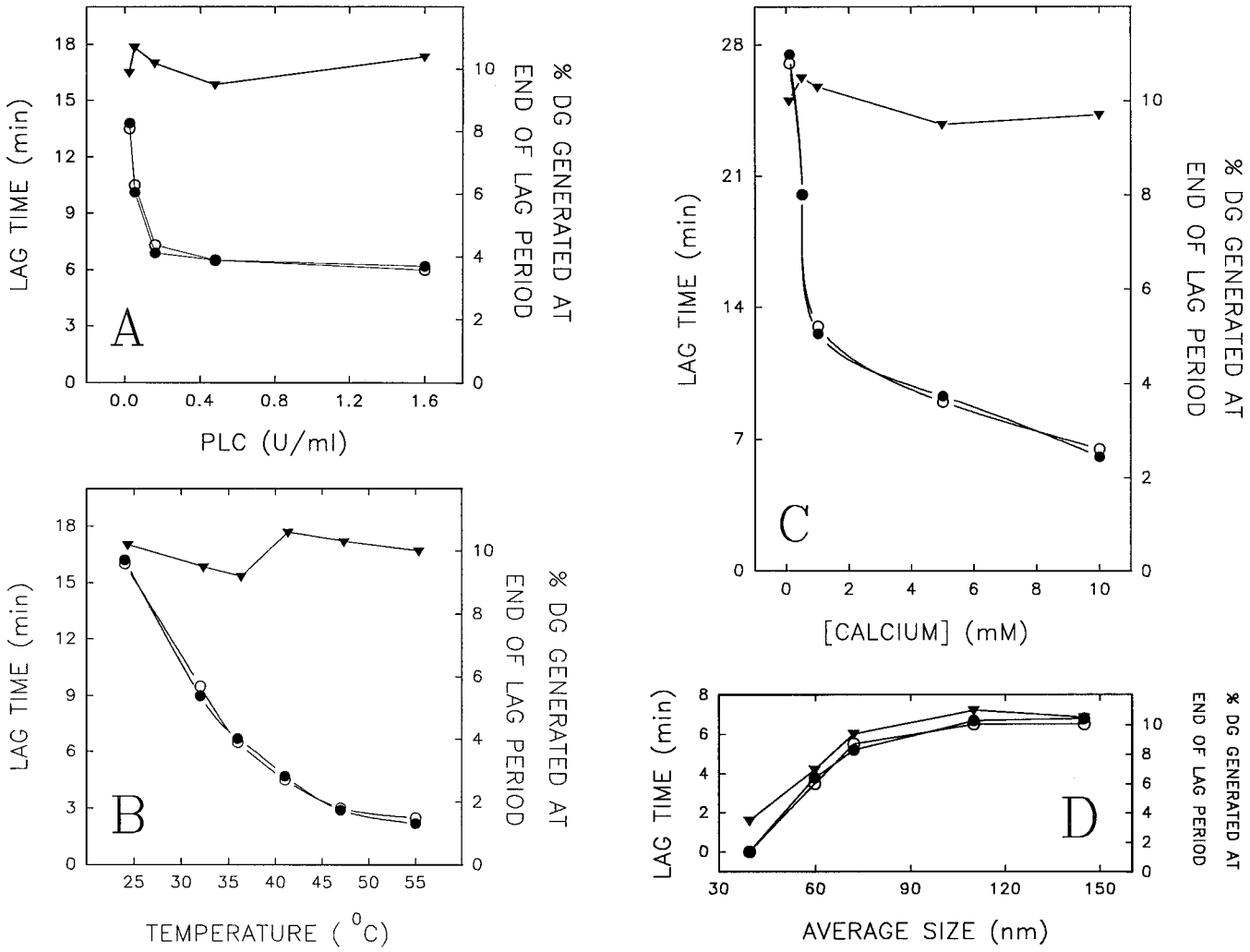

FIGURE 3: Effect on the lagtime of (A) phospholipase concentration; (B) temperature; (C) calcium concentration; (D) vesicle size. Lag time measured (○) from lipid hydrolysis measurements (●) from turbidimetric measurements; (▼) proportion of diacylglycerol generated at the end of the lag period.

proportion of substrate (~10%) is consumed at the end of the lag period, irrespective of its length. When vesicle diameter decreases, however, the proportion of diacylglycerol formed in the bilayers decreases in parallel with the extent of the latency period (Figure 3D). This identifies two different factors, namely, vesicle size and diacylglycerol production, that are important in determining the duration of the lag phase.

If a definite proportion of diacylglycerol is required to put an end to the lag period, one would expect that adding that compound from the beginning, i.e., preparing extrusion LUV of a given size with PC-diacylglycerol mixtures would decrease and even abolish the lag period. In fact, the presence of diacylglycerol in the original liposomal composition does indeed reduce the lag time, as described in a different system by Nieva *et al.* (1993), but it does not abolish the latency period until added diacylglycerol is ~20% (Figure 4). This is not in contrast with the figure of enzymically-produced 10% diacylglycerol that marked the end of the lag period under virtually any conditions (Figure 3), because 20% total diacylglycerol corresponds to ~10% in each monolayer, and most of the enzyme-generated 10% diacylglycerol is expected to remain in the outer monolayer, enzyme activity being faster than flip-flop rate. The situation is somewhat similar to the activation of phospholipase A_2 by lysolecithin (Sheffield *et al.*, 1995).

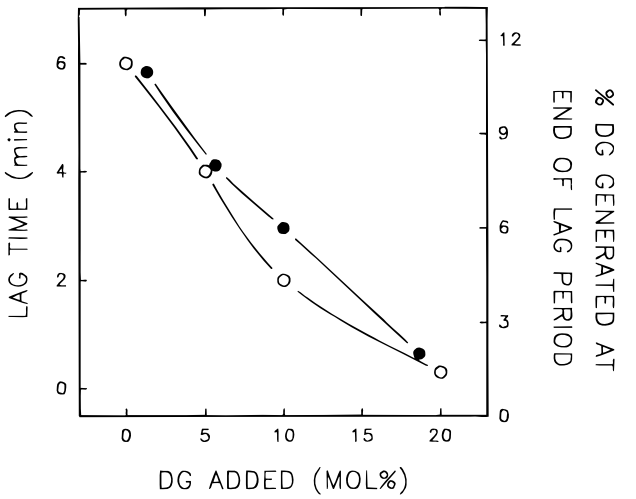

FIGURE 4: Effect of added diacylglycerol on the lag time (○) and on the proportion of diacylglycerol generated at the end of the lag period (●). Different diacylglycerol amounts were cosolubilized with the phospholipid prior to liposome formation.

An important parameter in unraveling the mechanism of phospholipase C activation is substrate concentration. The effect of changing egg PC concentration on the lag time and amount of diacylglycerol generated during that time is shown in Figure 5. The lag time and the amount of diacylglycerol

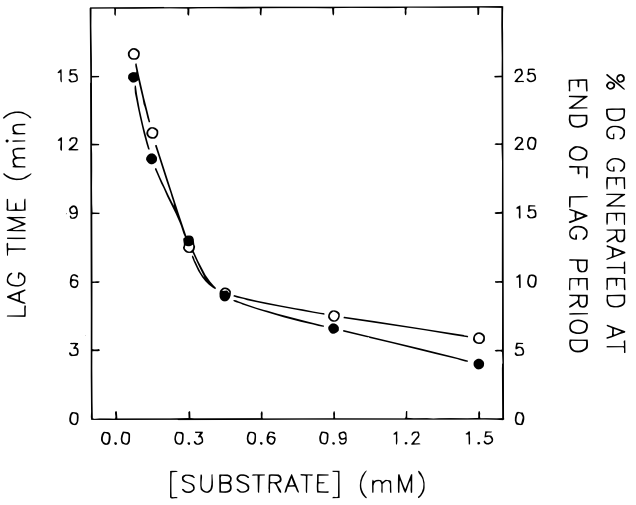

FIGURE 5: Effect of substrate (egg PC) concentration on the lag time (○) and proportion of diacylglycerol produced in the lag period (●).

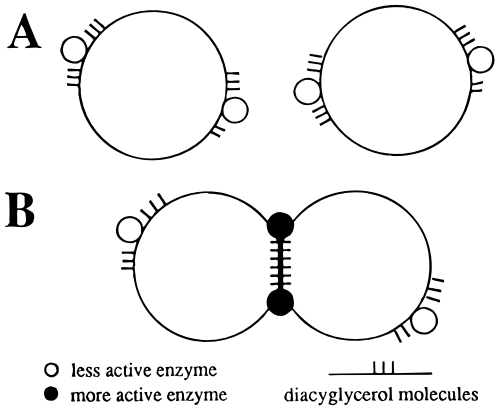

FIGURE 6: Simplified scheme of our hypothesis for phospholipase C activation through vesicle aggregation. (A) The enzyme hydrolyzes the substrate at low rates (lag period) until a certain amount of diacylglycerol is accumulated in the membrane. (B) The formation of diacylglycerol-enriched domains leads to vesicle aggregation and this is in turn creates or stabilizes the membrane defects allowing full enzyme activity.

required for the burst period decrease in parallel with increasing substrate concentrations. This inverse relationship, observed in spite of the reduction in enzyme/lipid ratio in this experiment conducted at a constant phospholipase C concentration, is a clear indication that enzyme activation is enhanced by vesicle aggregation.

DISCUSSION

The experimental results presented in this paper can be interpreted in terms of a simple two-proposition hypothesis, namely that (a) full enzyme activity requires some kind of membrane surface irregularities or *defects*, and (b) vesicle aggregation (induced by the diacylglycerol generated in the lag phase) causes or increases the surface inhomogeneities that allow fast enzyme activity. Let us examine separately these suggestions. It is well-known that synthetic lipid vesicles show a series of structural defects or fluctuations. These are minimal in large vesicles consisting of a single phospholipid in the fluid state, but increase notoriously in the presence of amphiphils, including diacylglycerols (Zidovetzki *et al*., 1992; Bell *et al*., 1996). The defects are frequent in sonicated liposomes because of their high curvature (Wilschut *et al*., 1978; Lawaczek *et al*., 1979). In turn, phospholipases A_2 are widely accepted to be activated by membrane defects (op den Kamp *et al*., 1975; Romero *et al*., 1987; Jain & Vaz, 1987; Sen *et al*., 1991; Zidovetzki *et al*., 1992; Burack & Biltonen, 1994, 1995; Bell *et al*., 1995). Thus the available literature supports our contention that phospholipase C may be activated by bilayer irregularities and explains why maximum rates are seen from the beginning when the substrate is in the form of sonicated vesicles (Figure 1).

The second part of our hypothesis, namely that vesicle aggregation causes the defects that lead to enzyme activation, requires a previous comment on diacylglycerol-induced vesicle aggregation. Activation of phospholipase A_2 and C by diacylglycerols has been reported (Dawson *et al*., 1984; Cunningham *et al*., 1989; Zidovetzki *et al*., 1992). However, a direct relation between diacylglycerol formation and vesicle aggregation was only shown by Nieva *et al*. (1989) in the context of phospholipase C-induced liposomal fusion. The idea has been explored in detail in more recent publications (Nieva *et al*., 1993, 1995). It is now clear that *in situ* enzymically generated, but not externally added diacylglycerol leads to vesicle aggregation, although the presence of diacylglycerol prior to enzyme addition does reduce the lag time of vesicle aggregation. The asymmetric activity of phospholipase C with respect to the lipid bilayer is believed to be essential in the requirement of enzymatically-generated diacylglycerol-rich patches or domains for aggregation. In this paper (Figure 2) we have shown that phospholipase C activation and vesicle aggregation are virtually simultaneous phenomena. It should be noted that diacylglycerol induces aggregation not only of vesicles but also of lipoproteins (Liu *et al*., 1993). Vesicle aggregation and enzyme activation may be related as follows. The stress of hydration repulsion and even weak van der Waals attraction is such that virtually any curved bilayer surface must deform to some extent when in adhesive contact (Evans & Parsegian, 1983). In the adhesion zone the lateral tension of the bilayer increases (Rand & Parsegian, 1989) and the lipid is induced to adopt a stressed disposition (Helm *et al*., 1992). As a consequence, membrane defects should appear. In addition, aggregation would certainly exclude the enzyme molecules from the contact area, leaving them at the ridge of that area, the ridge being a prime example of a large and long-lived structural defect in the bilayer surface (Figure 6). Note also that aggregates are three-dimensional, what may provide the enzyme molecules with extra access to substrates.

Since diacylglycerols are potent destabilizers of the lamellar disposition of phospholipids the possible role of non-lamellar phases in phospholipase activation has been suggested (Hui *et al*., 1981; Bentz *et al*., 1985; Cheng *et al*., 1986). Sen *et al*. (1991) suggested that the activation is not due to the actual presence of a non-bilayer phase, but is rather a consequence of the pretransitional packing stress and related packing defects. If this were the case for phospholipase C, as the lamellar to non-lamellar phase transition is temperature-dependent, one should expect a decrease in the required diacylglycerol proportion at the end of the lag period with increasing temperatures. However, this is not supported by the experimental evidence (Figure 3B). In addition, ^{31}P-NMR studies of mixtures, containing egg PC/egg diacylglycerol (80/20 mol ratio), that allowed full phospholipase C activity, still showed spectra compatible with fully lamellar

structures in the 20−50 °C temperature range (G. Basáñez, unpublished). Thus it appears that non-lamellar phase formation is not necessarily related to phospholipase C activation.

The idea that activation consists of, or is accompanied by, an increased binding of the phospholipase to the bilayer is an old one in the field (Bangham & Dawson, 1959). In the case of phospholipase C we have been unable to detect any change in enzyme binding to the vesicles before and after activation, neither looking at the intrinsic protein fluorescence nor measuring resonance energy transfer between the enzyme Trp residues and NBD-PE (data not shown). As stated above, our hypothesis for phospholipase C activation does not require changes in the amount of bilayer-bound protein. However, phospholipase C appears to have special affinity for diacylglycerol, and its activity increases when the product remains linked to the phospholipid aggregate (El-Sayed & Roberts, 1995; Soltys *et al*., 1993).

In summary, the available data on phospholipase C, from this and other laboratories, may be rationalized as follows: the enzyme, during the low-activity or lag period generates products that are at least transiently accumulated in the region near or around the enzyme; when a certain mole fraction of products is reached, lateral phase separation occurs, and the activity "burst" ensues, presumably related to structural defect formation. Both lateral phase separation and vesicle aggregation may induce the kind of surface irregularities or defects that allow phospholipase activation. Whether one or the other phenomenon is the primary agent of phospholipase C activation cannot be ascertained at present, mainly because, under our experimental conditions, both appear to occur simultaneously.

In our previous studies (Nieva *et al*., 1989, 1993, 1995, and references therein) we have described that, for certain bilayer compositions (e.g., egg phosphatidylcholine:egg phosphatidylethanolamine:cholesterol) phospholipase C induces aggregation and then fusion of lipidic vesicles. This was the first report of a model system in which fusion was induced by a catalytic agent. Other authors have pointed out the putative role of phospholipase C in physiological cell fusion events (Roldan & Harris, 1989; Liu *et al.*, 1993; Spungin *et al.*,1995). While the degree of implication of phospholipase C in cell membrane fusion remains to be established, the enzyme-driven liposome fusion process shows nevertheless remarkable regulatory properties. Although in the studies by Nieva and co-workers the effect of vesicle composition on the enzyme phosphohydrolase activity was not specifically analyzed, a re-appraisal of those data [in particular Nieva *et al.* (1989, 1993)] shows that substitution of some of the PC for PE or cholesterol in the bilayers leads to a significant decrease in the lag time of enzyme activity and of vesicle aggregation. In all cases these two phenomena appear to occur in parallel, confirming that they are directly related.

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