Liposome Fusion Catalytically Induced by Phospholipase C†

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ABSTRACT: Large unilamellar vesicles composed of phosphatidylcholine/phosphatidylethanolamine/cholesterol (50:25:25 mole ratio) were treated with phospholipase C. The early stages of phospholipid cleavage are accompanied by mixing of bilayer lipids (monitored by dequenching of octadecylrhodamine fluorescence) and leakage-free mixing of vesicle contents [measured by using 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylylenebis(pyridinium bromide) (DPX)]. These results are interpreted in terms of vesicle fusion induced by the catalytic activity of phospholipase C. The use of sonicated unilamellar vesicles decreases the lag time, but does not modify the amplitude, of the fusion process. The presence of both phosphatidylethanolamine and cholesterol appears to be essential for measurable fusion effects to occur with low levels of phospholipid hydrolysis. Optimal fusion rates are observed with about 10–20 enzyme molecules per large unilamellar vesicle. This system of catalytically induced liposome fusion may be of relevance for the interpretation of physiological membrane fusion processes.

Membrane fusion is a key step in many events of cell physiology and pathology. Its mechanism has been studied both in cell and in model membranes, but a detailed description is still to be achieved. Since it was first proposed by Lucy (1970), the idea that amphipathic molecules may act as intermediates in membrane fusion has received considerable experimental support. These amphiphiles are believed to perturb the lipid bilayer, perhaps through the formation of intramembranous "inverted micellar intermediates" (Verkleij, 1984; Ellens et al., 1986); membrane–membrane contact through these relatively unstable regions would in turn lead to bilayer fusion (Siegel, 1986a,b; Rand & Parsegian, 1986).

As in many other problems of membrane biology, the use of liposomes has been extremely valuable in the study of membrane fusion. Taupin and McConnell (1972) were the first to describe fusion of sonicated phospholipid vesicles; data that could be related to surfactant-induced liposome fusion have been published by our group (Alonso et al., 1982). Studies on liposome fusion have been greatly helped by the introduction of fluorescence techniques, allowing the independent assay of mixing of vesicle contents and mixing of bilayer lipids [see, e.g., Wilschut and Papahadjopoulos (1979) and Ellens et al. (1985)].

In recent years, diacylglycerols have attracted the attention of scientists, both because of their putative role as metabolic regulators (Berridge, 1987) and because of their capacity to destabilize lipid bilayers (Dawson et al., 1984; Epand, 1985; Das & Rand, 1986). The latter authors have specifically suggested that the ability of diacylglycerols to form nonbilayer structures may constitute the first destabilizing step leading to membrane fusion. The present work intends to go one step further in exploring the mechanisms of bilayer fusion; phospholipase C, an enzyme giving rise to diacylglycerols in the lipid bilayer, has been used to promote fusion of large and small unilamellar liposomes.

Materials and Methods

Phospholipase C (EC 3.1.4.1) from *Bacillus cereus* was purchased from Boehringer Mannheim and used without further purification. SDS–PAGE revealed that our enzyme preparation was 91% pure; this figure was used for subsequent calculations of enzyme:lipid mole ratios. Egg phosphatidylcholine (PC)[1] was purified from fresh egg yolks according to Singleton et al. (1965); phosphatidyethanolamine (PE) was obtained through Avanti (Birmingham, AL), and cholesterol (CHOL) was from Sigma. 8-Aminonaphthalene-1,3,6-trisulfonic acid (ANTS), *p*-xylylenebis(pyridinium bromide) (DPX), and octadecylrhodamine B (R_{18}) were obtained from Molecular Probes (Eugene, OR).

Large unilamellar vesicles (LUV) of different compositions were prepared by extrusion and sized by using 0.1-μm pore-size Nuclepore membranes as described by Mayer et al. (1986). Trapped volume measurements, according to the above authors, indicate that our liposomes contain about 1.5 lamellae/vesicle. Small unilamellar vesicles were obtained by sonication of aqueous lipid dispersions (Alonso et al., 1982).

Vesicle contents mixing and leakage were measured by using the ANTS/DPX system according to Ellens et al. (1985). In these assays, liposomes contained either (a) 50 mM ANTS, 100 mM NaCl, 10 mM Hepes, and 10 mM $CaCl_2$, or (b) 180 mM DPX, 10 mM Hepes, and 10 mM $CaCl_2$, or (c) 25 mM ANTS, 90 mM DPX, 50 mM NaCl, 10 mM Hepes, and 10 mM $CaCl_2$. Liposomes were separated from unencapsulated material by column chromatography on Sephadex G-75 using 10 mM Hepes, 200 mM NaCl, and 10 mM $CaCl_2$ (pH 7.0) as the elution buffer. The same buffer was used for all the leakage and fusion experiments as well as for the enzyme activity and light-scattering assays. All of those vesicles when assayed showed equal susceptibility to phospholipase action. The 100% fluorescence level (or 0% fusion) was set by using a 1:1 mixture of ANTS and DPX liposomes at 0.3 mM final lipid concentration. The fluorescence level corresponding to 100% mixing of contents was determined from 0.3 mM liposomes containing coencapsulated ANTS and DPX; the value so obtained corresponds to either 100% fusion or 0% leakage. The 100% fluorescence level for leakage was obtained by detergent lysis of the liposomes containing both ANTS and DPX. Corrections for differences in the amount of entrapped

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[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; CHOL, cholesterol; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium bromide); R_{18}, octadecylrhodamine B; LUV, large unilamellar vesicle(s); SUV, small unilmellar vesicle(s).

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solutes in the various vesicle preparations were routinely carried out after measuring the ratio of ANTS fluorescence before and after the addition of excess detergent (5 mM Triton X-100). The fluorescence change of a preparation containing 0.15 mM ANTS liposomes plus 0.15 mM "empty" liposomes (i.e., buffer-loaded) was routinely subtracted from the ANTS/DPX fluorescence signal, in order to account for scattering and other possible artifacts. Fusion rates have been calculated as in Ellens et al. (1986). Excitation was at 355 nm and emission at 530 nm; a Schott 06505 cutoff filter (515 nm) was used to avoid scattered light. Occasionally, release of vesicle contents was measured with glucose-loaded liposomes, as described elsewhere (Ruiz et al., 1988).

Vesicle/lipid mixing was measured by dilution in the bilayer of the self-quenching probe R_{18} as described by Hoekstra et al. (1984). In this case, the 0% fluorescence level (or 0% fusion) was determined from a 1:4 mixture of 8 mol % R_{18} containing liposomes and R_{18}-free liposomes. The fluorescence of the same amount of liposomes with the diluted probe uniformly distributed, i.e., 1.6 mol % R_{18}-containing liposomes, was taken as the 100% fluorescence level, or 100% fusion. Light scattering of the samples was monitored by fixing the excitation and the emission at 276 mm. Fluorescence and light-scattering measurements were performed in a RF-540 Shimadzu spectrofluorometer.

Enzyme activity was assayed by determination of phosphorus contents in the aqueous phase of an extraction mixture (choloform/methanol 2:1) after addition of aliquots from the reaction mixture at different times. All the assays were carried out at 37 °C. Occasionally, simultaneous measurements of phosphate release and diacylglycerols present in enzyme-treated liposomes were carried out, always with good correlation. In these cases, the enzyme activity was stopped at the appropriate times by increasing the pH to ca. 10; enzyme-treated vesicles were then collected by centrifugation, and diacylglycerols were quantitated according to Ortiz et al. (1988). Phosphorus contents of the samples were determined by the method of Bartlett (1959). Protein concentration was assayed according to Lowry et al. (1951).

Results

When LUV composed of PC/PE/CHOL (50:25:25 mole ratio) are treated with phospholipase C, vesicled fusion occurs while very small amounts of phospholipid are hydrolized, and without significant release of vesicle contents (Figure 1). Fusion is detected as mixing of vesicle contents and mixing of bilayer lipids, respectively in panels A and B of Figure 1. The fusion signal (from mixing of vesicle contents) was not corrected for leakage (Ellens et al., 1985), since the latter was undetectable in the time scale of our observations. Significant leakage could only be observed after periods on the order of minutes (data not shown). Vesicle fusion is accompanied by a concomitant increase in scattered light (Figure 1B).

The above results were obtained in the presence of 1.6 IU mL^{-1} phospholipase C and 0.3 mM total lipid, corresponding roughly to 10 enzyme molecules per vesicle [assuming average lipid areas as indicated by Papahadjopoulos and Kimelberg (1974)]. The enzyme:lipid ratio was found to be important for the observation of fusion, judging from measurements of leakage-free mixing of aqueous contents. As seen in Figure 1A, the fusion curve is sigmoidal and shows a lag period; both the maximum slope (i.e., maximum fusion rate) and the lag period are modified by the enzyme:lipid ratio (Figure 2). Fusion is observed within a given range of enzyme:lipid ratios, i.e., from about 0.1 to 250 enzyme molecules per vesicle (Figure 2A). Under our conditions, fusion is optimally observed with about 10–20 enzyme molecules per vesicle. The lag period for the mixing of vesicle contents is monotonically decreased with increasing enzyme concentrations (Figure 2B). When the enzyme is preincubated in the presence of 50 mM *o*-phenanthroline, a specific inhibitor of phospholipase C (Little & Otnaess, 1975), neither phospholipid hydrolysis nor liposome fusion is observed.

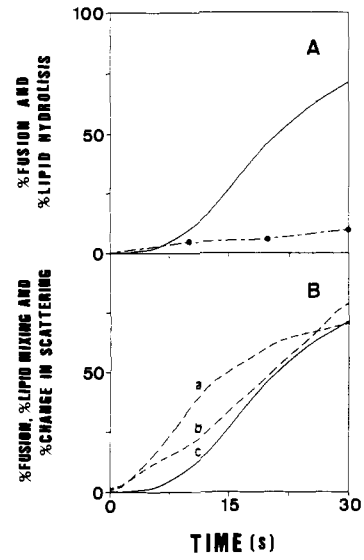

FIGURE 1: Fusion of liposomes (PC/PE/CHOL, 50:25:25 mole ratio) in the presence of phospholipase C. Total lipid concentration was 0,3 mM; enzyme activity was 1.6 IU/mL. (A) Continuous line, fusion as mixing of vesicle contents. Dashed lines, percent phospholipid hydrolyzed by the enzyme; the dots correspond to experimental values (average of three measurements, SEM/smaller than the dot size). (B) (a) Fusion as mixing of bilayer lipids; (b) percent change in scattered light; (c) fusion as mixing of vesicle contents (redrawn here to facilitate comparison).

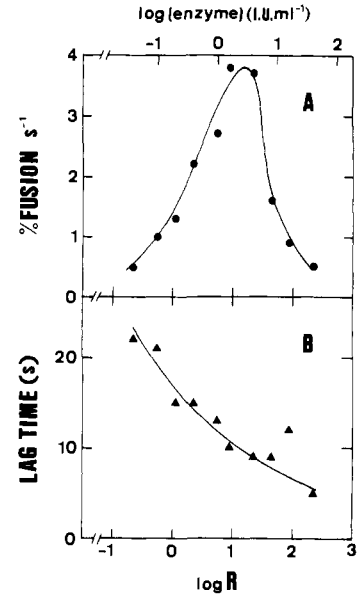

FIGURE 2: Influence of enzyme:lipid ratio on phospholipase C induced liposome fusion (contents mixing). (A) Maximum fusion rates. (B) Lag time of fusion. $R \equiv$ approximate number of enzyme molecules per vesicle (see text).

The question of the influence of bilayer composition on the fusogenic effects of phospholipase C was addressed by repeating the above experiments with liposomes containing varying proportions of PC, PE, and CHOL. Our results are summarized in Table I, while a few representative examples are depicted in Figure 3. The optimal enzyme concentration

Table I: Influence of Bilayer Composition on Phospholipase C Induced Liposome Fusion

lipid compn (% PC:PE:CHOL mol ratio)	type of vesicle	% lipid hydrolyzed when max fusion rate is reached	time for reaching max fusion rate (s)	% fusion at 10% hydrolyzed lipid P	time for 10% lipid hydrolysis (s)	enzyme concn (IU/mL)
25:50:25	LUV	5	13	39	25	0.1
25:25:50	LUV	3	3	25	11	16
33:33:33	LUV	19	6	15	3	4
50:50:0	LUV	41	132	5	84	1.6
50:25:25	LUV	6	20	71	30	1.6
75:25:0	LUV	16	119	3	80	1.6
70:0:25	LUV	17	270	3	156	1.6
50:25:25	SUV	20	10	21	5[a]	1.6
75:25:0	SUV	22	35	13	15	1.6

[a] 10% lipid P hydrolysis obtained from extrapolation of experimental values shown in Figure 4.

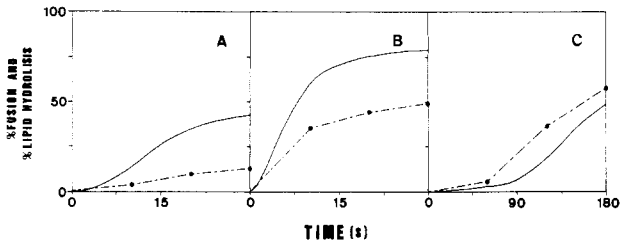

FIGURE 3: Influence of bilayer composition on phospholipase C activity and phospholipase C induced fusion. Continuous lines, contents mixing. Dashed lines, percent hydrolyzed phospholipid, as in Figure 1. Mole ratios: (A) PC/PE/CHOL, 25:50:25; (B) PC/PE/CHOL, 33:33:33, (C) PC/PE, 50:50. Note the different time scales.

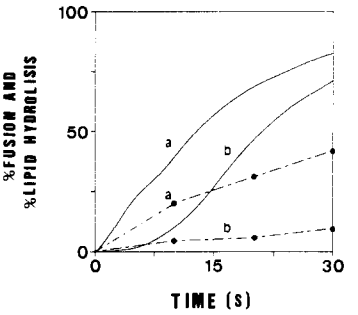

FIGURE 4: Comparison of phospholipase C effects on large and small unilamellar vesicles. (a) SUV, PC/PE/CHOL, 50:25:25 mole ratio. (b) LUV, as in Figure 1. Continuous lines, contents mixing. Dashed lines, percent hydrolyzed phospholipid, as in Figure 1.

was determined separately in each case, and the corresponding value is also included in Table I. Preliminary experiments showed that, under our conditions, PC and PE are hydrolyzed at similar rates by phospholipase C. For purposes of comparison, the results in Table I have been normalized to the percent lipid hydrolyzed at the time point where the fusion rate attains its maximum value for each hydrolysis/fusion time course. The percent fusion (mixing of vesicle contents) obtained when 10% of the phospholipid has been hydrolyzed by the enzyme is also included. It is obvious that the bilayer composition is rather critical and that both PE and CHOL are essential, in addition to PC, for significant fusion to occur with low levels of phospholipid hydrolysis.

Fusion of sonicated vesicles (SUV) in the presence of phospholipase C was also considered (Table I, bottom, and Figure 4). As expected, these vesicles are a better substrate than LUV for phospholipase C; i.e., the initial rates of lipid hydrolysis are much higher. Consequently, fusion also occurs at an earlier stage, mainly because of a decreased lag period; however, the extent of fusion and the maximum fusion rates are similar than with LUV.

Discussion

Our results show that phospholipase C, when added to a liposome suspension, can induce (a) mixing of bilayer lipids and, simultaneously or immediately afterward, (b) leakage-free mixing of vesicle contents (Figure 1). These two observations, taken together, are indicative of liposome–liposome fusion (Ellens et al., 1985). The extent of fusion (>50%) implies that each final fusion product is composed of more than two of the original liposomes (Bentz et al., 1988). None of the above phenomena are observed when the enzyme activity is inhibited by *o*-phenanthroline; thus, vesicle fusion is being promoted specifically by the catalytic activity of phospholipase C. Since this enzyme cleaves phospholipids releasing diacylglycerols, it can be reasonable assumed that the latter lipids are responsible for the observed phenomena. This is in agreement with the idea of Lucy (1970) that amphiphile-dependent bilayer destabilization is a step in the process of membrane fusion, and also with the more recent studies on the effects of diacylglycerols on bilayer architecture (Epand, 1985; Das & Rand, 1984, 1986; Ortiz et al., 1988).

The precise role of diacylglycerols in the fusion process is difficult to ascertain. It should be noted that both PE and CHOL are required, in addition to PC, in order to observe significant amounts of fusion at low levels of phospholipid hydrolysis (Table I). This means that diacylglycerols alone are not capable of inducing fusion of bilayers whatever their composition. On the other hand, PE and CHOL tend to destabilize the bilayer structure under certain conditions (Cullis & de Kruijff, 1978; Tilcock et al., 1982, 1984); diacylglycerols may potentiate the destabilizing properties of those two lipids. Alternatively, diacylglycerols would just facilitate either vesicle aggregation and/or what has been termed "close approach of the surfaces" (Bentz et al., 1988); PE and CHOL would then induce membrane destabilization. The existence of a stage of apposition of the outer monolayers in our system, with some degree of lipid exchange, is shown by the fact that lipid mixing occurs before contents mixing (Figure 1B); this phenomenon has been equally observed in other systems (Wilschut et al., 1985; Bentz et al., 1988).

In the past 2 decades, there have been numerous instances of cell and vesicle fusion induced by a variety of chemicals [see Sowers (1987) for a review]. The present report is, however, to the authors' knowledge, the first to show liposome fusion induced by a *catalytic* agent, namely, phospholipase C. [Morero et al. (1985) described vesicle fusion induced by glyceraldehyde-3-phosphate dehydrogenase, but no hint of a relationship between enzyme activity and fusogenic ability of that protein was given.] The biological relevance of our observations remains to be established; it is, however, interesting to note that high phospholipase C activities have been found in mammalian cells from brain (in synaptosomes), sperm, or secretory glands, where frequent fusion processes are expected to occur (Michell et al., 1981; Dawson et al., 1983; Waite,

1986; Ribbes et al., 1987). It has also been shown that phospholipase C induces, via diacylglycerol, the formation of stomatocytes or echinocytes in human red blood cells (Allan et al., 1978).

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