Poly(ethylene glycol)-lipid conjugates inhibit phospholipase C-induced lipid hydrolysis, liposome aggregation and fusion through independent mechanisms

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Abstract Poly(ethylene glycol)-phosphatidylethanolamine (PEG-PE) conjugates have been introduced in liposomal compositions. The resulting large unilamellar vesicles were subjected to the action of phospholipase C. Enzyme-promoted vesicle aggregation and fusion were assayed in liposomes containing various proportions of PEG-PE. At PEG-PE concentrations above 1 mol% the rate of phospholipid hydrolysis decreases, perhaps because the PEG moiety hinders the enzyme from reaching the membrane surface. At concentrations above 0.1 mol% vesicle aggregation occurs at a slower rate, presumably because of the repulsive barrier properties or surface-grafted PEG. Lipid mixing decreases in parallel with vesicle aggregation. Finally, liposomal fusion rates measured as mixing of vesicle aqueous contents are decreased at or even below 0.1 mol%. The latter inhibition is due, apart from the reduced rates of lipid hydrolysis, vesicle aggregation and lipid mixing, to a PEG-PE-based stabilization of the lipid bilayer structure. Thus the observed low rates of contents mixing arise from three combined and independent inhibitory effects of PEG-PE.

1. Introduction

Poly(ethylene glycol) (PEG)-derivatized phosphatidylethanolamines (PEG-PE) have been used in liposomal formulations for clinical use in the last decade, with the aim of reducing liposomal uptake by the mononuclear phagocytic system, thus increasing the vesicle lifetimes in circulation [1–5]. From the early studies, the membrane stabilizing properties of PEG-PE were thought to parallel those of the ganglioside GM1 [6], both being molecules with a phospholipid moiety and a large polar headgroup. Mori et al. [7] described that both PEG-PE and GM1 prolonged the circulation time of liposomes, although only the former lipid showed a significant steric barrier activity, as judged from liposome agglutination studies.

As a part of a wider project on the structural effects of phospholipase C on lipid bilayers, we have recently examined the effects of a variety of gangliosides, including GM1, on the phospholipase C-promoted fusion of liposomes [8]. Two different inhibitory effects of gangliosides on the fusion process were described, namely inhibition of the enzyme phosphohydrolase activity and inhibition of the lamellar to non-lamellar transition of the lipid structures. In view of the similarities, both in structure and in liposome stabilizing properties, between gangliosides and PEG-PE, we tested the influence of the latter compound on the phospholipase C-induced fusion process mentioned above. Although the overall effect of PEG-PE on liposomal fusion is inhibitory, just as that of gangliosides, some additional mechanisms appear to operate in PEG-PE inhibition. In particular, the steric barrier effect of surface-grafted PEG inhibits vesicle aggregation even with intact phospholipase C activities. As a result, the phenomena of phospholipid hydrolysis and liposome aggregation, that had been conceptually distinguished and causally related in our previous papers [9–11] are now physically separated.

2. Materials and methods

PEG-PE (MW of PEG ≈2000) was purchased from Avanti (Birmingham, AL). This particular molecular weight of PEG was selected according to the data of Mori et al. [7]. Phospholipase C (EC 3.1.4.1) from *Bacillus cereus* was supplied by Boehringer Mannheim. All lipids were from Lipid Products (South Nutfield, UK). Fluorescent probes were purchased from Molecular Probes (Eugene, OR).

The methods have been described in detail in our previous publications. Briefly, large unilamellar vesicles (LUV) were prepared by the extrusion method of Mayer et al. [12]. The lipid composition of these liposomes was PC/PE/Ch (2:1:1 mol ratio). When required, the appropriate amounts of PEG-PE were added. These amounts are indicated as *additional* mole percentages. The aqueous lipid suspensions were extruded through Nuclepore filters, 0.1-μm pore diameter.

Phospholipase C activity was determined from measurements of water-soluble phosphorous, and vesicle aggregation was estimated by the increase in scattered light [9,10]. Lipid mixing was measured by dilution in the bilayer of the self-quenching probe octadecylrhodamine B (R18), as described by Hoekstra et al. [13]; (see for details [8,9]). Mixing of aqueous vesicle contents was estimated using the ANTS/DPX fluorescent probe system described by Ellens et al. [14] (details as in [8,9]).

Steady-state fluorescence anisotropy of TMA-DPH was recorded in a MPF-66 Perkin Elmer fluorometer as described by Nieva et al. [15]. Lamellar to non-lamellar transitions were recorded as shown by Basáñez et al. [16]. ^{31}P-NMR spectra were recorded in a KM360 Varian spectrometer operating at 300 MHz for protons; the spectra were plotted with a line broadening of 80 Hz [8,16].

3. Results

3.1. PEG-PE and liposomal fusion

The inhibitory effect of 1 mol% PEG-PE on the phospholipase C-promoted liposomal fusion is shown in Fig. 1. The effect on vesicle aggregation and lipid mixing consists of decreasing the maximum rates while keeping constant saturation values (saturation not shown for lipid mixing in Fig. 1A). The

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Abbreviations: ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylene-*bis*(pyridinium bromide); LUV, large unilamellar vesicle; PEG, poly(ethylene glycol); PEG-PE, distearoylphosphatidylethanolamine-poly(ethylene glycol 2000); R18, octadecylrhodamine B; TMA-DPH,1-(trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene

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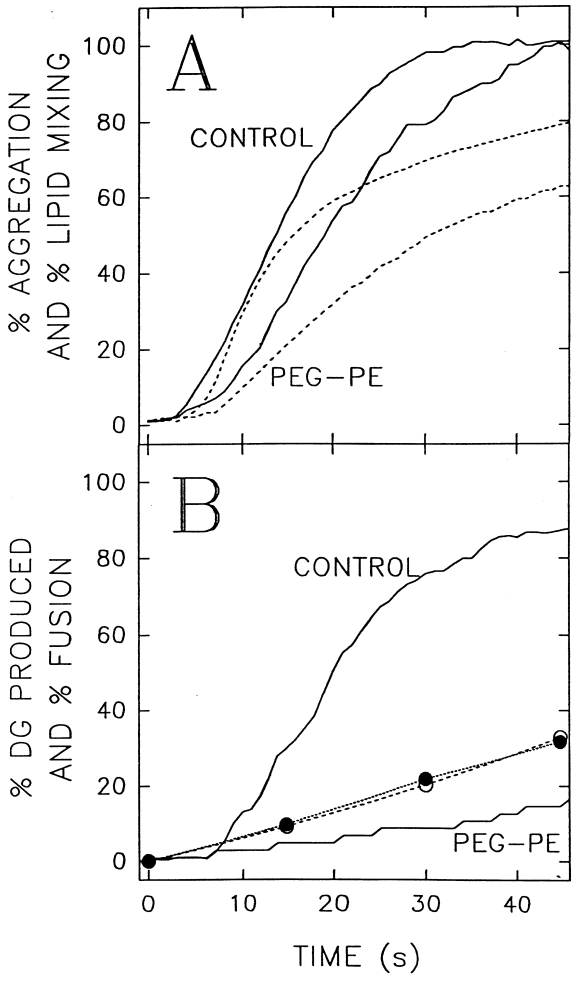

Fig. 1. PEG-PE inibition of phospholipase C-promoted vesicle fusion. Control: large unilamellar vesicles consisting of PC/PE/Ch (2:1:1 mol ratio). PEG-PE: plus 1 mol% PEG-PE conjugate. A: Continuous lines, vesicle aggregation; broken lines, lipid mixing. B: Continuous lines, mixing of vesicle aqueous contents (fusion); (○) % diacylglycerol production, control; (●) % diacylglycerol production in the presence of 1 mol% PEG-PE.

inhibitory effect of 1% PEG-PE on liposomal fusion (mixing of aqueous contents) is particularly remarkable (Fig. 1B). Note that, unlike in our previous studies with fusion inhibitors, PEG-PE is inhibiting fusion without altering the rate of phospholipid hydrolysis.

The effects of PEG-PE at concentrations ranging from 0.1 to 9% are shown in Fig. 2 for lipid hydrolysis, vesicle aggregation, lipid mixing and mixing of vesicle aqueous contents. The minimum concentration of PEG-PE that has a detectable effect varies according to the effects observed. The threshold appears to be above 1 mol% for phospholipid hydrolysis (Fig. 2A), at 0.5 mol% for vesicle aggregation (Fig. 2B), between 0.1 and 0.5 mol% for lipid mixing (Fig. 2C), and at or even below 0.1 mol% for contents mixing (Fig. 2D). Thus the more complex the event, the lower the threshold concentration of PEG-PE; in increasing order of sensitivity, enzyme activity < aggregation ≈ lipid mixing < contents mixing. This suggests that a number of independent inhibition mechanisms are accumulating their effects along the process. The observations in Fig. 2 are confirmed and clarified by the data in Table 1, that summarizes the maximum rates for each process in the presence of increasing concentrations of PEG-PE. The data in Table 1 show more accurately that vesicle aggregation may be already partially inhibited at 0.5% PEG-PE. Also noticeable is the parallel inhibition of aggregation and lipid mixing, suggesting that PEG-PE does not prevent lipid mixing once the liposomes are in contact.

3.2. PEG-PE and lipid phase behaviour

We have recently shown that measurements of TMA-DPH fluorescence polarisation could be used to detect lamellar to nonlamellar phase transitions in lipid systems [16]. In view of the putative involvement of nonlamellar intermediates in membrane fusion ([11], and references therein), and of the role of gangliosides in stabilizing lamellar phases [8], we tested the effect of PEG-PE on the lamellar to nonlamellar transition(s) of PC/PE/Ch/diacylglyceride through changes in TMA-DPH fluorescence polarisation. Parallel studies were carried out with ^{31}P-NMR.

The data in Fig. 3A,B correspond respectively to the mixtures PC/PE/Ch/diacylglyceride 50:25:25:5 and 50:25:25:10 (mol ratios). According to changes in TMA-DPH fluorescence anisotropy, these mixtures have a phase transition at 57°C and 48°C, respectively [16]. Similar measurements have been carried out with the above lipid mixtures, to which increasing amounts of PEG-PE (0.5–9 mol%) have been added. It is clear that the PEG-PE conjugate stabilizes the lamellar phase, shifting to higher temperatures the phase transition to the point that, in some cases, the mixture remains lamellar in the whole temperature range of our study, i.e. up to 80°C (Fig. 3A).

These results were fully confirmed by ^{31}P-NMR spectroscopy. As an example, Fig. 4 shows spectra corresponding to a PC/PE/Ch/diacylglyceride (50:25:25:5) mixture, in the absence (control) or presence of 1% PEG-PE. The control mixture exhibits a lamellar to nonlamellar transition, that is

Table 1
Percent inhibitory effect of PEG-PE on the rates of phospholipase C activity, vesicle aggregation, lipid mixing, and vesicle fusion (mixing of aqueous contents)

% PEG-PE	Hydrolisis rate	Aggregation rate	Lipid mixing rate	Fusion rate
0	100	100	100	100
0.1	99	103	ND	80
0.5	105	95	82	25
1	103	83	63	5
3	80	23	16	1
6	28	3.5	2.5	0
9	9	0	ND	0

The original mixture (0% PEG-PE) contains PC/PE/Ch (2:1:1). The data are taken from experiments as shown in Fig. 2; the figures are average values of three experiments.
ND, not determined.

perfectly detectable at 60°C by the presence of an isotropic signal as the main feature in the spectral line. At 70°C, any remnants of a bilayer signal have disappeared, and the spectral lineshape shows a combination of isotropic and hexagonal components. In the presence of 1 mol% PEG-PE, the lamellar phase is very effectively stabilized, since it is shown to prevail even at 70°C. According to our previous studies [8,11,16], the observed stabilization of the lamellar phase by PEG-PE explains the difference between the inhibition caused by this reagent on vesicle aggregation (+lipid mixing) and the one caused on mixing of vesicle aqueous contents.

4. Discussion

The stability provided by PEG-PE to cell and model membranes has been described by a number of authors [5,7,17–20]. In particular, inhibition of membrane fusion has been mentioned as one of the effects of surface-grafted PEG [21,22]. Käsbauer et al. [23] have described an interesting system in which PEG-PE inhibits PEG-induced liposomal fusion. However, the results in this paper present a more detailed description of the inhibitory effects of PEG-PE on the various steps leading to bilayer fusion.

4.1. The steps of membrane fusion and levels of fusion inhibition

It has become apparent in the recent years, as a result of multiple experimental studies on cell and model membranes, that the process of membrane fusion is in fact the result of at least three events, namely membrane apposition, mixing of lipids in the outer bilayers and (through the establishment of a fusion structural intermediate) full lipid mixing with mixing of aqueous contents [24]. Among other laboratories, we were able to show, with free poly(ethylene glycol) and sonicated vesicles of egg phosphatidylcholine, that different PEG concentrations were required to reach the various stages mentioned above [25], thus contributing to support the idea that those stages, periods or steps had each its separate physical entity, being more than ideal concepts. Much of the work behind these ideas has been reviewed in Bentz [26]. In the model system for membrane fusion that has been explored by this group in the past years, namely phospholipase C-induced liposome fusion, the hydrolytic action of the enzyme is a sort of 'zeroth stage' that brings about the remaining ones [9,10,27].

In our recent study on fusion inhibition by gangliosides [8] we were able to distinguish two levels of inhibition, respectively the 'phosphohydrolase activity' and the 'lamellar to nonlamellar transition' levels, at which gangliosides acted independently and additively. This was already a step forward with respect to our previous observations on fusion inhibition (e.g. by lysophospholipid [10]) in which only inhibition of the final event, i.e. contents mixing, was considered. In the present work, the use of PEG-PE has allowed us to distinguish a further level of inhibition, namely membrane apposition (or vesicle aggregation), as separate from phospholipid hydroly-

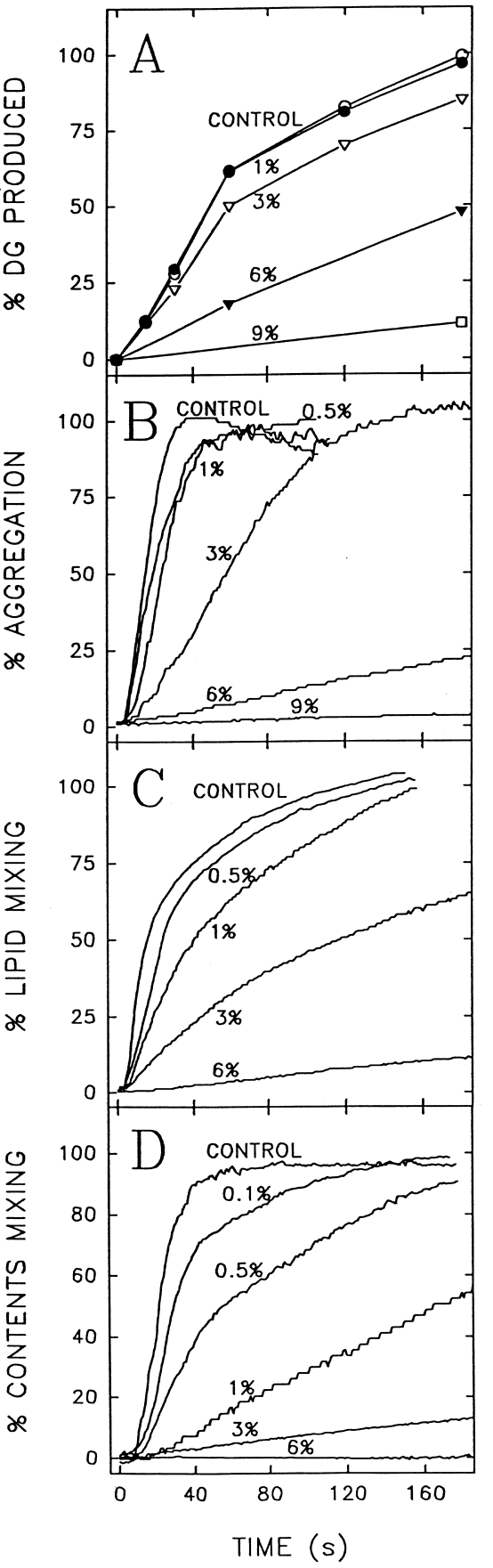

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Fig. 2. Correlation among PEG-PE inhibition of (A) phosphohydrolase activity, (B) vesicle aggregation, (C) lipid mixing, and (D) contents mixing, induced by phospholipase C. Control: as in Fig. 1. The figures by the curves correspond to the PEG-PE concentrations in the bilayers (as mol%).

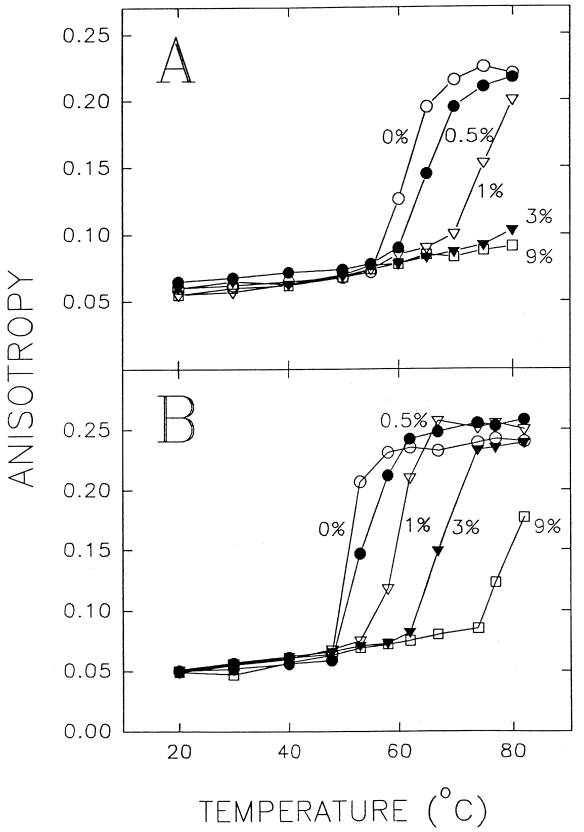

Fig. 3. Thermotropic phase transitions of phospholipid/cholesterol aqueous dispersions as detected by changes in anisotropy of TMA-DPH. The figures by the curves correspond to the concentration of PEG-PE in the bilayer (expressed as mol%) in each case. The basic lipid mixtures (0%) were (A) PC/PE/Ch/diacylglycerol (50:25:25:5, mol ratio) and (B) PC/PE/Ch/diacylglycerol (50:25:25:10, mol ratio).

sis, that had not been observed as a separately regulable entity in this series of studies up to now.

The results summarized in Table 1 show that PEG-PE is inhibiting every step involved in liposome fusion. However, the inhibitory effect is not the same on every stage examined. The enzyme phosphohydrolase activity is clearly inhibited at PEG-PE concentrations above 1% (mol ratio). This inhibition can be reasonably attributed to either, or both, of two factors. First, PEG-PE may be inhibiting phospholipase C in much the same way as gangliosides, either by modifying the catalytic activity of the adsorbed enzyme or by altering the availability of substrates in an appropriate conformation [8,28]. The inhibitory effect of gangliosides on phospholipases A_2 and C increases with the complexity of the polar head group [29–31], in this respect PEG-PE offers a particularly large head group. But PEG-PE may be operating as well through a second mechanism, namely by hindering enzyme access to the vesicle surface. Torchilin et al. [32] have presented theoretical and experimental arguments in support of the idea that even low proportions of PEG-PE (in the 0.2–1 mol% range) may prevent opsonizing protein molecules from contacting the liposomes. For the same reason PEG-PE would prevent access of phospholipase C to its substrate, causing an apparent enzyme inhibition.

In another recent study [33] the origin of the lag time that is observed when phospholipase C is added to LUV consisting of pure egg phosphatidylcholine was examined. It was concluded that, irrespective of the experimental conditions, the end of the lag time was concomitant with the start of vesicle aggregation, so that the 'burst' of enzyme activity was virtually coincident with the increase in turbidity due to liposome aggregation. In previous papers [10,11] we had proposed that formation of diacylglycerol patches on the liposomal surface, as a result of phospholipase C activity, lead almost necessarily to liposome aggregation. In all of our previous experiments, changes in enzyme activity were invariably linked to parallel changes in aggregation rate. PEG-PE allows us the opportunity to dissect these two phenomena. While it will be interesting to examine the effect of this conjugate on the enzyme lag period, the data in Table 1 show clear divergences between the effects of PEG-PE on lipid hydrolysis and vesicle aggregation rates. At 1 mol%, PEG-PE does not inhibit enzyme activity, while the aggregation rate is reduced by about 20%. At 3% PEG-PE, enzyme activity is only inhibited by 20%, and the aggregation rate is decreased by 80%. These data show that, in addition to inhibiting phospholipase C, PEG-PE is impairing membrane apposition through an independent mechanism, so that the observed low rates of aggregation are the result of two added inhibitory events. It has been known for several years that PEG-PE provides a strong repulsive barrier, of steric origin, that prevents the close approach of bilayers, according to X-ray diffraction studies [17], and also prevents agglutination of immunoliposomes [7]. The same mechanism is probably preventing LUV aggregation in our case, even in the presence of diacylglyceride. Holland et al. [21] have described the inhibition by PEG-PE of Ca^{2+}-induced fusion of liposomes containing phosphatidylserine. Inhibition was attributed to the steric barrier effect of PEG-PE. Note that these authors did not perform direct measurements of vesicle aggregation, but only of lipid mixing (through a resonance energy transfer procedure).

When the data in Table 1 corresponding to vesicle aggregation and lipid mixing are compared, a good parallelism is observed. This would suggest that PEG-PE had no direct effect on the mixing of lipids, and that the inhibition of the latter event was a direct consequence of decreased vesicle aggregation. These data confirm for our system what had been implied by Holland et al. [21] in their case, i.e. that inhibition of lipid mixing was only a result of the steric barrier effect. Note however that a direct inhibition of lipid mixing by PEG-PE cannot be entirely ruled out in the light of the present experiments.

In the last step of fusion communication is established between the cavities of the apposed vesicles, and their contents are allowed to mix. Comparison of the columns corresponding to lipid mixing and contents mixing in Table 1 again shows an additional inhibitory mechanism that prevents contents mixing even with reasonable rates of lipid mixing. The situation is reminiscent of the one found for ganglioside inhibition [8] and, as shown in Figs. 3 and 4, the same explanation applies here: PEG-PE, just as gangliosides, stabilizes the lamellar phase, thus preventing the formation of the highly-curved intermediate that, according to the currently accepted theories [34,35], is required in the last step of fusion. Holland et al. [36] have shown a similar bilayer-inducing behaviour of PEG-PE for a different lipid system (dioleoylphosphatidylethanolamine/cholesterol).

In summary, PEG-PE has three independent effects that

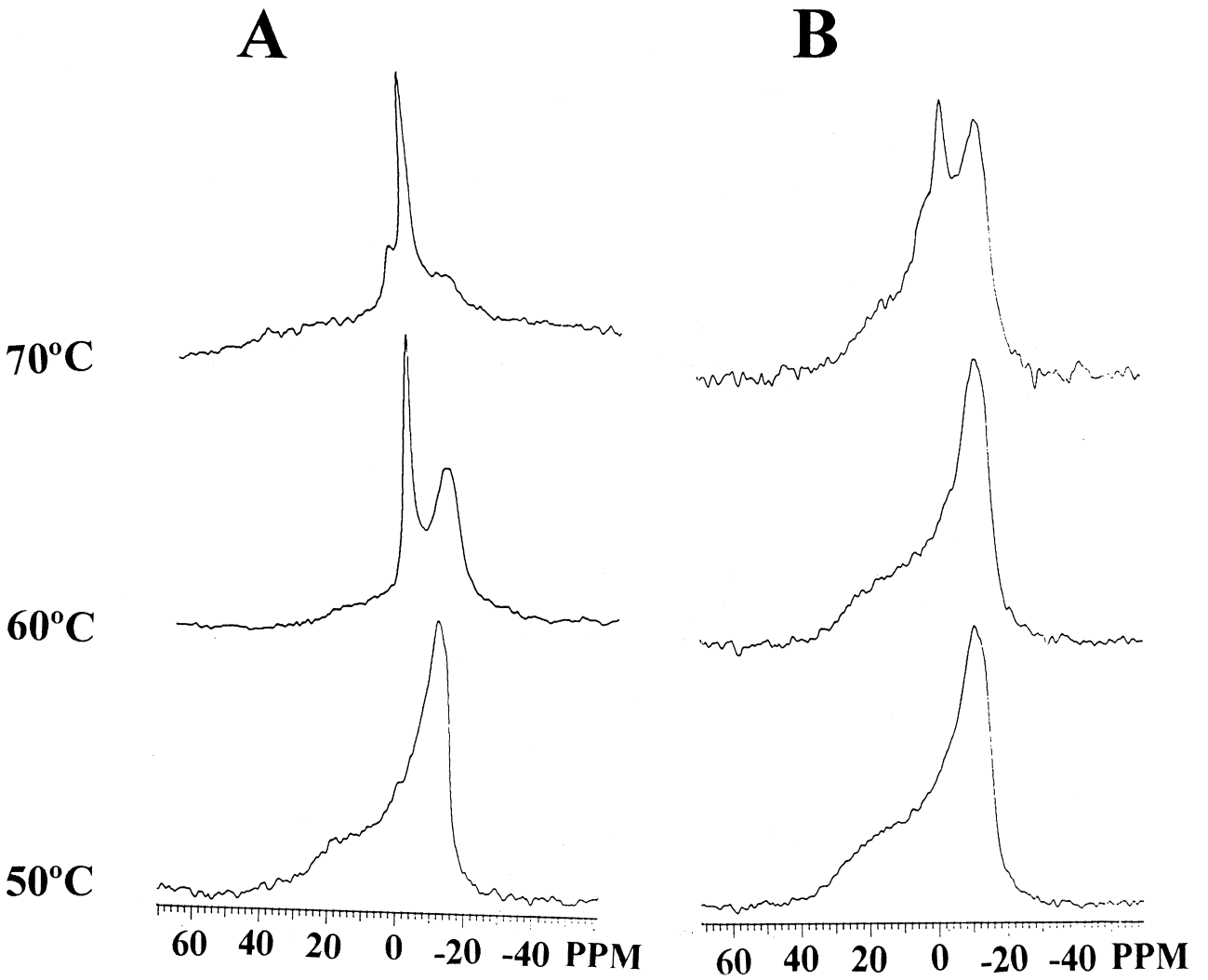

Fig. 4. ^{31}P-NMR spectra of phospholipid/cholesterol aqueous dispersions, recorded at different temperatures. A: PC/PE/Ch/diacylglycerol (50:25:25:5, mol ratio). B: As in (A), but with an additional 1 mol% PE-PE. Spectral shifts in ppm relative to orthophosphoric acid. Lipid concentration $\approx$0.2 M. Temperatures (in °C) are given by each curve.

may act together inhibiting phospholipase C-induced liposome fusion, namely inhibition of enzyme activity, prevention of membrane apposition and stabilization of the lamellar phase. The last two effects should operate in any membrane fusion system.

From the point of view of liposome stability in blood, the observations in this paper add a new factor explaining the stabilizing properties of PEG-PE, namely its phospholipase-inhibiting ability. The similar effects of PEG-PE and gangliosides on phospholipase C (this paper, and also Basáñez et al. [8]) suggest that a variety of lipases may be inhibited similarly by PEG-PE as they are by gangliosides [28], the conjugate thus contributing significantly to an increased vesicle lifetime in biological fluids.

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