Effect of Single Chain Lipids on Phospholipase C-Promoted Vesicle Fusion. A Test for the Stalk Hypothesis of Membrane Fusion†

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ABSTRACT: The effect of low proportions (up to 5 mol %) of single-chain lipids on phospholipase C-promoted fusion of large unilamellar vesicles has been investigated with the aim of testing the so-called stalk model of membrane fusion. This model is known in two main versions, the one originally published by Kozlov and Markin [Kozlov, M. M. and Markin, V. S. (1983) *Biofizika 28*, 255−261] and what is known as the "modified stalk model" [Siegel, D. P. (1993) *Biophys. J. 65*, 2124−2140], that differ in a number of predictions. In the view of the latter author, hydrocarbons or other nonpolar lipids should help fusion by decreasing the interstitial energy of the stalk connecting the two apposed bilayers. We show that small amounts of hexadecane or squalene increase significantly the fusion rates in our system. Changes in monolayer curvature are the object of different predictions by the original and modified stalk theories. According to the original form, fusion would be promoted by lipids inducing a negative curvature in the closest (cis) monolayers of the fusing membranes and inhibited by the same lipids in the trans monolayers; the opposite would happen with lipids inducing a positive curvature. The modified stalk model predicts that fusion is helped by increasing the negative curvature of both monolayers. In our system, symmetrically distributed arachidonic acid, which increases the negative curvature, enhances lipid and content mixing, and the opposite is found with symmetrically distributed lysophosphatidylcholine or palmitoylcarnitine, which facilitate a positive monolayer curvature. In addition, fluorescence polarization and ^{31}P NMR studies of the lamellar-to-isotropic (Q^{224} cubic) thermotropic transition of a lipid mixture corresponding to our liposomal composition reveal that all lipids that facilitate fusion decrease the transition temperature, while fusion inhibitors increase the transition temperature. Moreover, fusion (content mixing) rates show a maximum at the lamellar-to-isotropic transition temperature. These observations support the involvement of inverted lipid structures, as occurring in the inverted cubic phases, in membrane fusion. All these data are in full agreement with the stalk model of membrane fusion, particularly in its modified version.

Membrane fusion is a complex process involved in a variety of physiological and pathological phenomena. Its molecular mechanism is not completely understood yet. One rather obvious step in the fusion of any two lipid bilayers is the formation, albeit transient and local, of a nonbilayer structure. Among the theoretical models that have been proposed to explain the formation of the nonlamellar intermediate, the so-called "stalk hypothesis" has received considerable attention in recent years (*1*, *2*). The stalk is proposed to be a semitoroidal lipidic structure having a negative curvature (in this work the convention is followed that the curvature of a monolayer in the inverted hexagonal H_{II}[1] phase is negative) that would allow the merger of the closest (cis) leaflets of apposed membranes. Indirect evidence supporting the stalk intermediate has been accumulating in the past years (reviewed in ref *3*), although direct proof of its existence is still lacking, perhaps because of its transient nature (*4*).

The poststalk molecular events in membrane fusion are even less clear, particularly the steps leading to the opening of an aqueous connection, the fusion pore, between the two contacting vesicles or cells. In the original version of the stalk model (*1*, *5*) it was proposed that the stalk would expand radially so that the trans monolayers would dimple inward and come in contact through their hydrophobic regions, giving rise to what has been called a trans membrane contact (TMC). TMC would expand, forming a single bilayer diaphragm, until the accumulated tension would break this structure, giving rise to the fusion pore. A further version of the stalk hypothesis (*2*) underlines (a) the energy contribution of the hydrophobic interstices arising from the coexistence of positively- and negatively curved monolayers in the

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[1] Abbreviations: ANTS, 8-aminonaphthalene 1,3,6-trisulfonate; Ch, cholesterol; DPX, *p*-xylenebis(pyridinium bromide); H_{II}, inverted hexagonal phase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Q_{II}, inverted cubic phase; R18, octadecylrhodamine B; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene; TMC, trans monolayer contacts.

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stalk and (b) the curvature energy of the trans monolayers, which would oppose dimpling. According to the latter author, TMCs would not grow laterally but they would instead reduce their free energy, relieving the unfavorable curvature and interstitial energy by forming a pore. Both versions of the stalk hypothesis emphasize the importance of changes in membrane curvature in the fusion process, but they also differ in a number of predictions. In particular, the "modified stalk theory" (*2*) predicts that alkanes or other nonpolar lipids will contribute to filling in the interstices and increase the fusion rates. Also, according to the modified theory, lipids that facilitate positive curvatures (e.g., lysolecithin) should decrease the rate of TMC formation and fusion, while, according to the original formulation of the stalk−pore theory, as summarized by Chernomordik (*3*), lysolecithin and similar lipids would inhibit stalk formation but promote pore opening.

Previous studies from this group have shown that the catalytic action of phospholipase C promotes fusion of large unilamellar vesicles. Optimum fusion has been obtained with the liposomal composition PC:PE:Ch (2:1:1, molar ratio) (*6*−*8*). Moreover, phospholipase C-induced vesicle fusion has been related to the formation of inverted cubic phase elements, which involve negatively curved monolayers (*4*, *9*, *10*). In addition, the fusion process has been shown to be inhibited by lipids promoting positive monolayer curvature, e.g., lysolecithin (*7*), glycosphingolipids (*11*), or poly(ethylene glycol)-derivatized PE (*12*). These data have been interpreted as supporting the stalk hypothesis in its more general sense.

The present work is intended as a detailed test of the stalk−pore model in its two main variants (*1*, *2*) as applied to phospholipase C-promoted liposome fusion, a model system in which fusion is induced by the catalytic action of an enzyme. The test consists of studying the effects of low (usually up to 5 mol %) proportions of single-chain lipids, either nonpolar or amphiphilic, negative- or positive-curvature inducers, on phospholipase C-induced fusion and on the phase behavior of aqueous PC/PE/Ch/diacylglycerol dispersions. The results are in agreement with the predictions of the modified stalk model.

MATERIALS AND METHODS

Phospholipase C (EC 3.1.4.1) from *Bacillus cereus* was supplied by Boehringer-Mannheim. Egg phosphatidylcholine (PC), egg lysophosphatidylcholine (lyso PC), and egg phosphatidylethanolamine (PE) were from Lipid Products (South Nutfield, England); cholesterol (Ch), fatty acids, hexadecane, squalene, and palmitoylcarnitine were from Sigma (St. Louis, MO). Octadecylrhodamine B (R18), 8-aminonaphthalene 1,3,6-trisulfonate (ANTS), and *p*-xylenebis(pyridinium bromide) (DPX) were purchased from Molecular Probes (Eugene, OR).

The experimental procedures have been given in detail in previous papers (*6*, *9*, *11*, *13*). Briefly, large unilamellar vesicles composed of PC:PE:Ch (2:1:1 mole ratio) of ≈100 nm average diameter were prepared by the extrusion method. The desired vesicle composition was obtained by mixing the various components in organic solvent, before liposome preparation. When required, the amounts of single-chain lipids added are indicated as additional mole percentages; thus a liposome composition containing 2% squalene is PC/PE/Ch/squalene (50:25:25:2 mole ratio). Phospholipase C was assayed by determining water-soluble phosphorus in the reaction mixture at regular intervals. Vesicle aggregation was estimated by the increase in scattered light at 520 nm. Lipid mixing was measured by dilution in the bilayer of the self-quenching probe R18. Mixing of aqueous vesicle contents, as well as vesicle leakage, was estimated using the ANTS/DPX fluorescent probe system. ^{31}P NMR spectra were recorded in a VXR 300 Varian spectrometer, operating at 300 MHz for protons (121. 4 MHz for ^{31}P); samples contained ≈0.2 M lipid, and spectra were plotted with a line broadening of 80 Hz. Phase transitions were also detected through changes of TMA-DPH fluorescence polarization (*9*).

RESULTS AND DISCUSSION

"Void-Filling" Nonpolar Lipids: Hydrocarbons. The modified stalk theory (*2*) predicts that small amounts of very hydrophobic lipids, which will tend to be located within the nonpolar lipid matrix when incorporated into membranes, will increase the fusion rates by reducing the interstitial energy of the stalks and trans monolayer contacts. Several authors have shown that long-chain hydrocarbons promote the transition from lamellar to nonlamellar (reversed hexagonal or "isotropic", perhaps cubic) phases (*14*−*19*). This is significant in view of the implication of nonlamellar structures in membrane fusion (*10*). In fact, Walter et al. (*20*) were able to show that hexadecane increases divalent cation-induced lipid mixing rates between phosphatidylserine large unilamellar vesicles.

We have examined the effect of hexadecane or squalene, at 0.5, 2, and 4 mol %, on the aggregation, lipid mixing, and content mixing induced by phospholipase C in LUV composed of PC:PE:Ch (2:1:1). The results are summarized in Figure 1 and Table 1. Very similar effects are seen for both hydrocarbons: enzyme activity and aggregation rates are increased, except for 0.5% hexadecane, but the rates of lipid mixing and, in particular, content mixing are markedly enhanced in all cases. The effects on lipid mixing rates are quantitatively similar to those observed by Walter et al. (*20*). Squalene is more potent than hexadecane as an enhancer of vesicle aggregation and fusion. This is probably related to the observation by Siegel et al. (*17*) that squalene is more effective than hexadecane in stabilizing nonlamellar phases (see below). The larger positive effect of hydrocarbons on vesicle content mixing than on lipid mixing reminds us that the inhibitory effect of gangliosides (*11*) or poly(ethylene glycol)-derivatized PE (*12*) was also more clearly seen on content mixing rates. This is interpreted in support of the involvement of inverted lipid phases in the formation of the fusion pore. Moreover, the comparatively smaller effect on lipid mixing will be discussed in detail below.

"Negative-Curvature" Lipids: Fatty Acids. The shape theory of lipid structure of Israelachvili et al. (*21*) predicts that fatty acids will promote negative curvature and destabilize the bilayer, particularly when they are unsaturated. Experimental studies of hexagonal phase formation and lipid mixing (*22*) have shown that the effect of fatty acids may be more complex than predicted; however, at least for the C_{22} series at pH 7.4, it was demonstrated that unsaturation facilitates inverted phase formation. Arachidonic and other

Table 1: Effect of Single Chain Lipids on the Maximum Rates of Phospholipase C-Promoted Vesicle Aggregation, Lipid and Contents Mixing[a]

lipid comp	mol % additive	hydrolysis rate	aggregation rate	lipid mixing rate	content mixing rate
control[b]		100	100	100	100
+hexadecane	0.5	95 ± 4.5	99 ± 3.7	107 ± 5.5	158 ± 7.0
	2	108 ± 9.2	120 ± 7.8	139 ± 6.8	215 ± 8.9
	4	117 ± 7.0	133 ± 8.8	147 ± 62	241 ± 10.1
+squalene	0.5	105 ± 6.1	107 ± 5.8	131 ± 5.9	179 ± 8.0
	2	125 ± 5.2	132 ± 8.2	159 ± 7.7	271 ± 12.3
	4	130 ± 9.0	150 ± 10.4	191 ± 8.6	298 ± 14.2
+arachidic acid	1	98 ± 5.0	103 ± 6.2	107 ± 7.7	105 ± 5−5
	5	101 ± 9.0	106 ± 3.8	102 ± 4.0	122 ± 4.1
	10	97 ± 6.6	100 ± 8.2	109 ± 6.5	129 ± 7.6
+arachidonic acid	1	107 ± 4.3	100 ± 4.2	111 ± 6.0	158 ± 3.7
	5	119 ± 3.2	132 ± 5.7	129 ± 8.5	232 ± 11.1
	10	128 ± 7.5	135 ± 6.1	159 ± 4.3	278 ± 12.0
+LysoPC	1	101 ± 3.3	95 ± 4.4	87 ± 2.9	44 ± 5−0
	5	88 ± 9.1	79 ± 6.0	80 ± 7.7	22 ± 2.7
	10	70 ± 7.2	68 ± 3.9	51 ± 8.1	10 ± 1.9
+palmitoylcarnitine	1	n.d.	97 ± 6.0	85 ± 6.1	47 ± 6−1
	5	n.d.	77 ± 4.8	69 ± 3.3	18 ± 2.9
	10	n.d.	60 ± 3.0	58 ± 4.4	14 ± 3.0

[a] Data are presented as percent changes with respect to the control lipid mixture and correspond to average values ± SEM ($n = 3$). Maximum rates are computed from curves such as those shown in Figures 1−3. [b] Control vesicles contain PC/PE/Ch (mole ratio 2:1:1). Total lipid concentration is 0.3 mM. Absolute rates for the control lipid mixture: hydrolysis, 1.9 nmol of phospholipid s^{-1}; aggregation, 4.4 fluorescence units s^{-1}; lipid mixing, 2.6 fluorescence units s^{-1}; content mixing, 1.9 fluorescence units s^{-1}.

cis-unsaturated fatty acids have been described as enhancing fusion in biological systems [reviewed in (*3*)). In our case, one saturated and one polyunsaturated fatty acid, respectively arachidic ($C_{20:0}$) and arachidonic ($C_{20:4}$), have been tested on the phospholipase C fusion system. The corresponding results are shown in Figure 2 and Table 1. Arachidic acid has hardly any effect, while its polyunsaturated homologue arachidonic is a clear enhancer of liposomal fusion, its effects being qualitatively similar to those of the hydrocarbons, although the fatty acid is less potent. Again arachidonic acid action is more noticeable on vesicle content mixing than on lipid mixing; this issue will be experimentally addressed below.

"Positive-Curvature" Lipids. Lipids with a tendency to form micelles [cone-shaped, in the terminology of Israelachvili et al. (*21*)] stabilize the lamellar phase under conditions otherwise favoring the lamellar-inverted hexagonal transition. Among the many examples that have been described in the literature, lysoPC and palmitoylcarnitine have been studied in the context of our model fusion system. LysoPC has been found to stabilize the lamellar phase in lamellar-hexagonal transitions (*9*, *14*, *23*, *24*) and to inhibit cell and model membrane fusion (*25*−*30*). LysoPC inhibition of phospholipase C-promoted liposome fusion was already reported by us (*7*) but is now studied here in a more detailed way, and also included for comparison. Palmitoylcarnitine is less well-known; it is a membrane-active metabolite (*31*, *32*) and also a stabilizer of the lamellar phase (*33*, *34*).

The effects of these two cone-shaped lipids on our system can be seen in Figure 2 and Table 1. Figure 2A shows a peculiar aspect of the behavior of lysoPC, namely the induction of a long lag-time in phospholipase C activity. The same happens with palmitoylcarnitine (data not shown). The origin of lag times in phospholipase C activity has been studied in detail by us recently (*35*) and the effect of lysoPC is currently the object of specific research. Briefly, a lag period occurs when pure PC is used as a substrate because the enzyme cleaves the lipid at a very low rate until a given fraction of the final product diacylglycerol is formed, at which point the physical properties of the bilayer change, perhaps due to the formation of structural defects, and the burst of enzyme activity ensues. Virtually no lag time is observed with PC:PE:Ch bilayers (Ruiz-Argüello et al., manuscript in preparation), but in the presence of lysoPC, or of palmitoylcarnitine, these cone-shaped molecules counter the effect of the inverted cone-shaped diacylglycerol, and as a result, an extra amount of diacylglycerol has to be formed in the low-rate regime, thus originating a measurable lag time even in those bilayers.

Apart from increasing the lag period, lysoPC and palmitoylcarnitine have an inhibitory effect on the aggregation rate (Table 1). This is also explained by the mutually compensating shapes of these lipids and diacylglycerol, since the latter is considered to be directly responsible for vesicle aggregation in our system (*7*, *10*, *35*). Under the conditions of the experiment in Figure 2 and Table 1, lysoPC and palmitoylcarnitine have a considerable effect on content mixing, while the inhibition of lipid mixing appears to be merely the result of the decreased aggregation. This point is considered in detail in the following section.

Lipid mixing is assayed in our system with the R18 probe, according to ref 36. In this assay, 80% of the vesicles are initially probe-free, while the remaining 20% contain as much as 8 mol % of symmetrically distributed R18, a molecule consisting of a single hydrocarbon chain with a bulky headgroup, i.e., a cone-shaped lipid. Thus R18 is likely to provide lipid mixing rates somewhat lower than real. While we cannot rule out this possibility, the effect of R18 should be essentially similar in all the various vesicle compositions tested; thus it should not interfere with our conclusions.

Monolayer Curvature and the Predictions of the Stalk Models. According to the stalk hypothesis in any of its versions, lipid mixing between apposed vesicles occurs as a direct result of stalk formation. Thus experimental measurements of lipid mixing should be a very sensitive test for any changes in the rate or extent of stalk generation and/or stabilization. However, the above results (Figures 1 and 2, and Table 1) with interstice-filling or curvature-changing

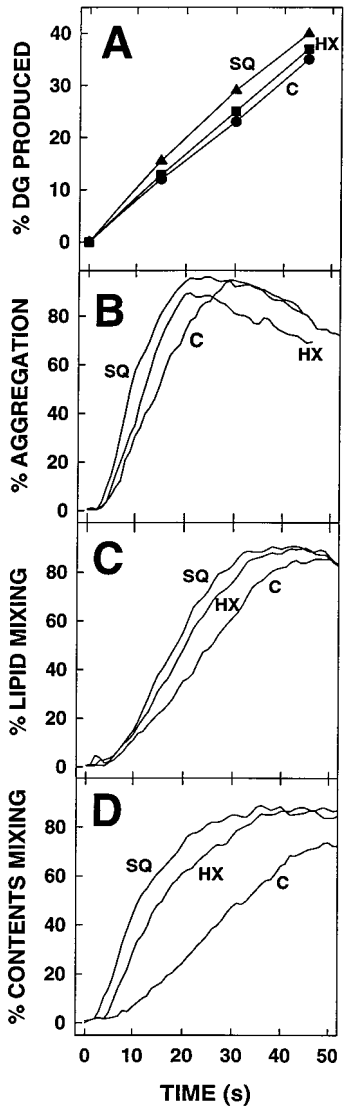

FIGURE 1: Effect of hydrocarbons on phospholipase C-promoted vesicle fusion: (A) production of diacylglycerol; (B) liposome aggregation; (C) mixing of liposomal lipids; (D) mixing of liposomal contents; (curves C) control vesicles; (curves HX) +2 mol % hexadecane; (curves SQ) +2 mol % squalene.

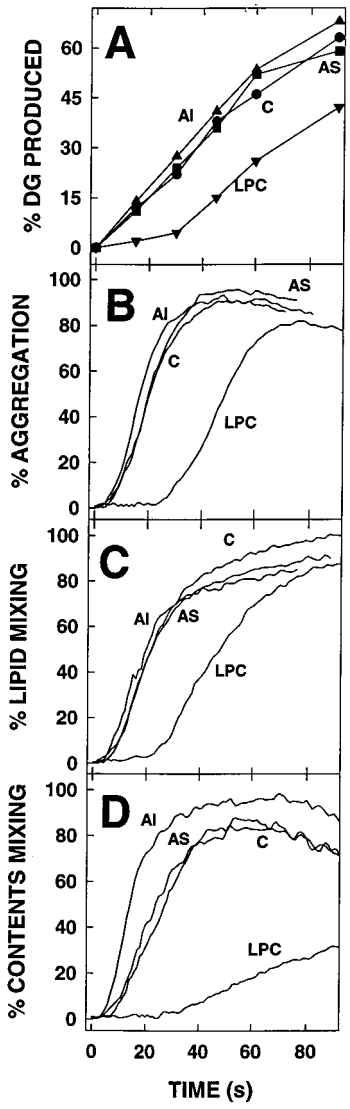

FIGURE 2: Effect of free fatty acids and lysophosphatidylcholine on phospholipase C-promoted vesicle fusion: (A) production of diacylglycerol; (B) liposome aggregation; (C) mixing of liposomal lipids; (D) mixing of liposomal contents; (curves C) control vesicles; (curves AS) +5 mol % arachidic acid; (curves AI) +5 mol % arachidonic acid; (curves LPC) +5 mol % lysophosphatidylcholine.

lipids show much more limited effects, if any, on lipid mixing than on content mixing. One possible explanation for this lack of effect would be that, as our system was optimized for maximum and fastest fusion (*6*), stalk formation would be taking place at maximum velocity, in a zero-order kinetic regime with respect to substrate; thus the effect of low amounts of inhibitors or activators would go largely undetected. This hypothesis was experimentally tested by decreasing the phospholipase C activity, and consequently stalk formation, in two different ways, namely decreasing the temperature while the enzyme concentration was maintained and lowering the lipase concentration at the usual temperature of 37 °C. The results are shown in Figure 3. Note the extended time scale in the abscissa. Under this slower regime, squalene and arachidonic acid are clearly seen to enhance lipid mixing, while lysoPC and palmitoylcarnitine markedly inhibit the process. The effects of 5 mol % arachidonic acid, lysoPC, and palmitoylcarnitine fully support the notion of a negatively curved stalk intermediate, while the increase in lipid mixing produced by squalene speaks in favor of the relevance of interstitial energy in stalk formation and stabilization.

The results in Figures 1−3 may also help to understand a further point in the mechanism of membrane fusion. As was mentioned in the introduction, the stalk−pore model in its original formulation predicted the formation of a diaphragm by the trans monolayers, a result of the radial expansion of the stalk. Rupture of the diaphragm would originate a pore, stabilized by positive-curvature lipids (see Figure 2 in ref 3). Thus, according to this view, positive-curvature lipids, e.g., lysoPC, would inhibit stalk formation but facilitate pore formation, or more specifically, lysoPC in the cis monolayers would inhibit stalk formation, and thus fusion, but the same lipid in the trans monolayers would have the opposite effect, stimulating fusion. These predictions appear to be confirmed by experiments in which liposomes are fused to planar bilayers (*37*). In our case, low amounts of lysoPC or palmitoylcarnitine are symmetrically distributed in the bilayer. According to the original stalk hypothesis, pore formation, and thus content mixing, should be enhanced by

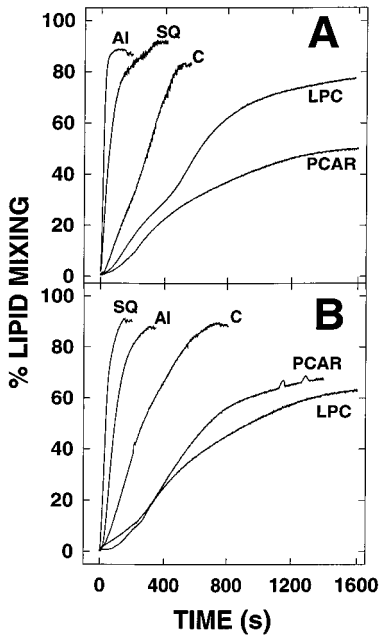

FIGURE 3: Effect of slowing down enzyme activity on the regulatory properties of various single-chain lipids in phospholipase C-induced fusion: (A) 1.6 U/mL phospholipase C, 25 °C; (B) 0.06 U/mL phospholipase C, 37 °C; (curves C) control vesicles; (curves AI) +5 mol % arachidonic acid; (curves SQ) +2 mol % squalene; (curves LPC) +5 mol % lysophosphatidylcholine; (curves PCAR) +5 mol % palmitoylcarnitine. In all cases fusion (as lipid mixing) is plotted as a function of time.

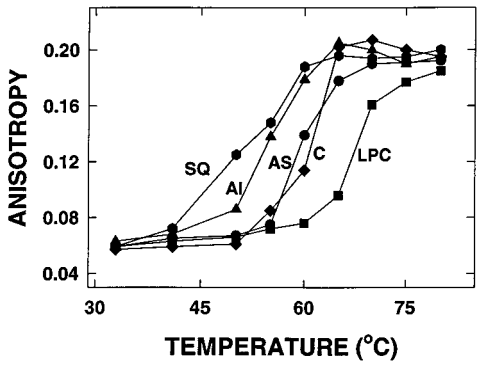

FIGURE 4: Detection of lamellar-to-nonlamellar thermotropic transitions in aqueous lipid dispersions through changes in TMA-DPH fluorescence polarization. TMA-DPH anisotropy is plotted as a function of temperature. Experimental details can be found in Basáñez et al. (*4*). Lipid mixtures: (C) control, PC/PE/Ch/diacylglycerol (47:23:25:5 mole ratio); (AS) +5 mol % arachidic acid; (AI) +5 mol % arachidonic acid; (SQ) +2 mol % squalene; (LPC) +5 mol % lysophosphatidylcholine.

lysoPC and inhibited by arachidonic acid, while the opposite is observed (Figures 1 and 2). In fact, our observations are in better agreement with the modified stalk theory (*2*) according to which pore opening results from the rupture of the dimpled monolayers, which releases both the curvature and interstitial energies (see Figure 2 of ref *2*) without requiring an intermediate pore structure with a curvature opposite that of the stalk.

The asymmetric requirements for optimal fusion by the original stalk−pore model, in particular the presence of positive-curvature lipids in the trans monolayers, are difficult to meet experimentally. The evidence produced up to now is not as clear as desired. In the experiments involving liposomes and plane bilayers (*37*) the estimated concentrations of lysoPC in the trans monolayers are 1 order of magnitude above those used in this paper, and some of the membranes are made of asolectin, that is likely to contain significant (>1%) proportions of lysolipids that will become symmetrically distributed. Wu et al. (*38*) achieved asymmetric distributions of lysoPC in liposomes that were later fused in the presence of poly(ethylene glycol), but the authors themselves interpreted their observations as resulting from altered lipid packing in the vesicle outer leaflets rather than from the asymmetric distribution of lysoPC. In our own experience, attempts to enrich selectively in lysoPC the trans monolayers by treating with bovine serum albumin vesicles containing symmetrically distributed lysoPC (*37*) failed to provide any meaningful information because albumin interfered with phospholipase C, inhibiting its action. In contrast, studies in which lysoPC is simply added externally, so that it incorporates chiefly in the outer (cis) monolayers, or is premixed with other lipids in model membrane preparations, so that an approximately symmetrical distribution is achieved, show unequivocally inhibition of the fusion process, be it detected as lipid mixing or as content mixing (*7*, *25*−*29*, *37*, and results in this paper). All this accumulating evidence speaks in favor of the stalk hypothesis although it does not support the proposed existence of a pore intermediate consisting of positively curved lipids.

It should be noted that, in phospholipase C-promoted membrane fusion, the fusogenic agent is the enzyme product diacylglycerol, a typical example of a negative-curvature-inducing lipid (*39*). It has been estimated that our vesicles aggregate and fuse when 5−20% of the phospholipid has been hydrolyzed and converted into diacylglycerol (*7*). Still, vesicles prepared with compositions of the type PC/PE/Ch/diacylglycerol (43:22:25:10) are perfectly stable (*7*), although they fuse readily after addition of the enzyme. The role of the enzyme has been suggested to be the asymmetric (and localized) generation of diacylglycerol. The energetic advantage of an asymmetric curvature for fusion to occur has been put forward by the stalk theory in its various formulations (see Figure 7 of ref *2*).

Fusion and the Role of Nonbilayer Structures. The transient formation of nonbilayer structural intermediates is an unavoidable requirement of membrane fusion. It is also an essential tenet of the stalk hypothesis, since the stalk itself is a nonbilayer structure, in which the monolayers have a negative curvature, such as seen in inverted lipid phases, H_{II} hexagonal, or Q_{II} cubic. "Nonlamellar" has been equated in practice to "reversed hexagonal" in the context of membrane fusion (*2*, *17*), although isotropic ^{31}P NMR signals, which may be compatible with, among others, inverted cubic phases, have also been associated with fusion intermediates (*18*, *28*, *40*, *41*). Siegel and Epand (*42*) have recently suggested that TMC intermediates play a role in lamellar-to-nonlamellar phase transitions and that they can either rupture to form fusion pores that modulate transitions to Q_{II} inverted cubic phases or assemble into bundles of H_{II} inverted hexagonal phase tubes. Nieva et al. (*10*) showed a direct correlation between bilayer compositions and temperatures giving optimum fusion and those leading to the formation of an "isotropic" component, which was identified with a bicontinuous inverted cubic phase Q^{224} by X-ray diffraction. Both the stalk and the pore, as predicted by the

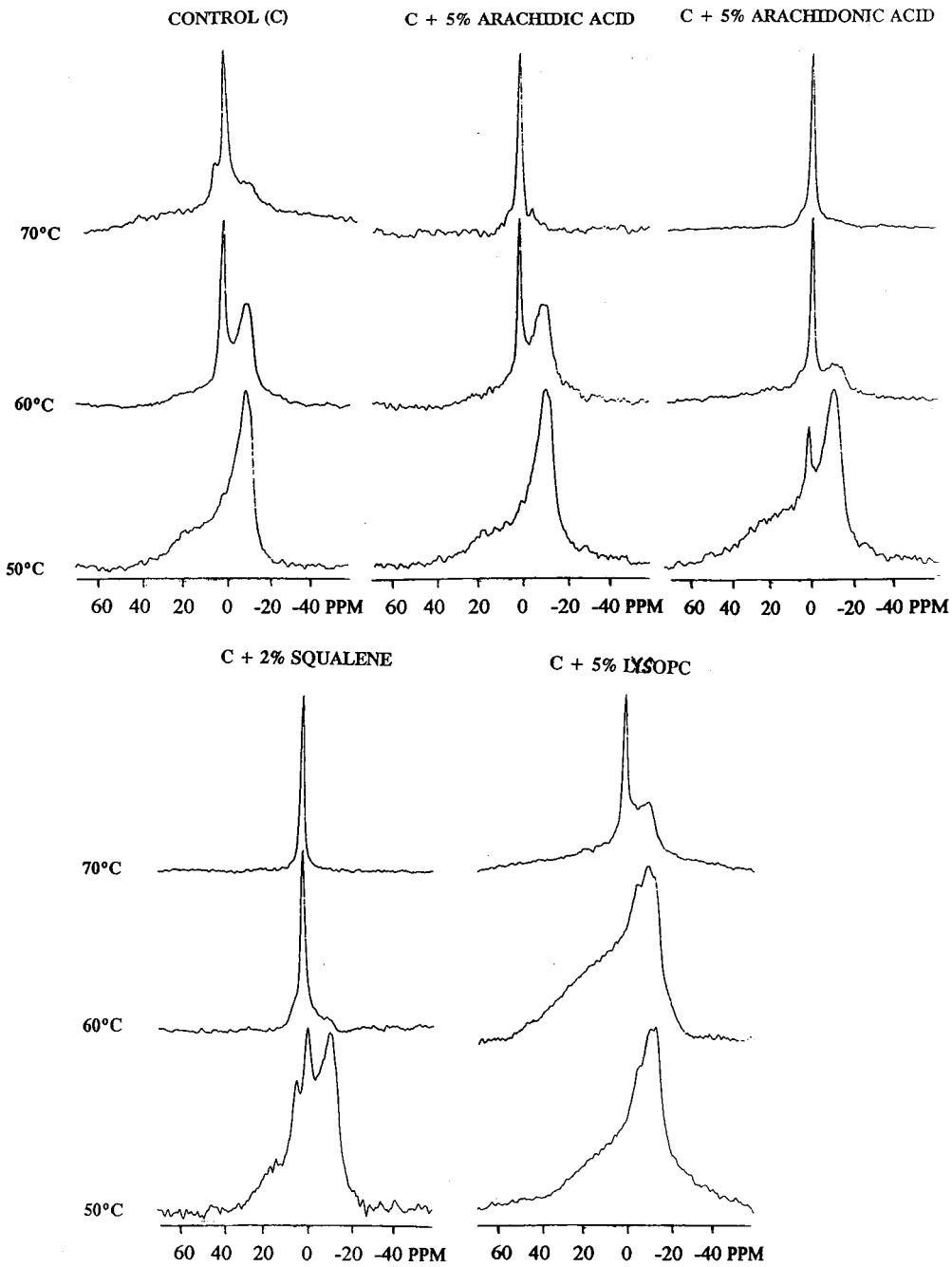

FIGURE 5: Detection of lamellar-to-nonlamellar thermotropic transitions in aqueous lipid dispersions through changes in ^{31}P NMR spectroscopy. Spectra were recorded at the temperatures indicated in each case. Lipid mixtures were based on PC/PE/Ch/diacylglycerol (47:23:25:5 mole ratio), designated in the figure as control (C), to which various additions were made as indicated on each series of spectra.

modified stalk theory, have geometries that can be related to that of the Q^{224} phase. In our previous studies of fusion inhibition by positive-curvature lipids, gangliosides and poly(ethylene glycol)-modified PE (*11, 12*), a good correlation has been shown between the inhibitory effects of those lipids and the increased temperatures in the corresponding lamellar-to-nonlamellar transitions. This point has also been explored using a fluorescence polarization technique by Basáñez et al. (*9*).

The effects of a variety of single-chain lipids on the lamellar-to-nonlamellar (isotropic, Q^{224}) transition of a PC/PE/Ch/diacylglycerol (50:25:25:5, mole ratio) mixture have been studied by fluorescence polarization (Figure 4) and ^{31}P NMR (Figure 5). A very good correlation is observed between the modification of phase transition temperature and fusion activity. Squalene and arachidonic acid, which were found to enhance lipid and content mixing, are seen to facilitate the lamellar−isotropic transition, and the opposite occurs with the positive-curvature lipid lysoPC. Arachidic acid was neutral both with respect to fusion and with respect to phase transition. These results are in obvious agreement with the stalk model.

Previously published data on phospholipase C-induced liposomal fusion can be reinterpreted in the light of the predictions of the modified stalk theory. Siegel (*2*) suggests that when the lipid in the bilayers is very close to the T_h, lamellar-to-hexagonal transition temperature, stalks may form H_{II} phase precursors, and any TMCs that form should have a tendency to radially expand, decreasing the driving force for fusion pore formation. However, the expanded TMC

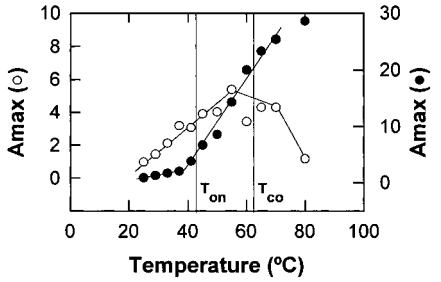

FIGURE 6: Relationship between rates of vesicle aggregation, rates of vesicle fusion (content mixing), and lamellar-to-nonlamellar transitions. The maximum rates of phospholipase C-induced vesicle aggregation (●) and fusion (○) are plotted as a function of temperature. Aggregation and lipid mixing change in parallel in this system. The vesicle composition was PC:PE:Ch (2:1:1 mole ratio). The vertical lines correspond to the onset (T_{on}) and completion (T_{co}) temperatures of the L_α fluid lamellar to the Q^{224} bicontinuous cubic phase transition of a PC/PE/Ch/diacylglycerol (47:23:25:5) mixture. Data are redrawn from Nieva et al. (*7*, *10*).

would make a large, comparatively stable lipid connection between apposed bilayers, which would promote extensive lipid mixing. It has been observed that the content mixing rate often increases with temperature and goes through a maximum at $T \approx T_h$ (*17*, *39*, *43*, *44*), decreasing thereafter, while the lipid mixing rate increases monotonically. Combining our data on phospholipase-induced fusion as a function of temperature (*7*) with those on the phase behavior of our lipid mixtures (*10*), we can show in Figure 6 the correlation that is found in our case for fusion rates and transition temperatures.

The figure includes data of vesicle aggregation rates as a function of temperature (under those conditions aggregation and lipid mixing always go in parallel), data of fusion (content mixing) rates as a function of temperature, and two vertical lines, marked T_{on} and T_{co}, respectively, corresponding to the onset and completion temperatures of the lamellar−Q^{224} cubic transition of a PC/PE/Ch/diacylglycerol (47:23:25:5) mixture. This mixture was selected because 5 mol % diacylglycerol is the minimum amount of this lipid that allows fusion to occur (*7*). The mixture does not undergo a direct lamellar−cubic transition, but instead, lamellar, hexagonal, and cubic phases appear to coexist between T_{on} and T_{co} (*10*). Fusion and phase behavior studies are not strictly comparable, since they consist of kinetic and equilibrium measurements, respectively. Still it can be seen in Figure 6 that vesicle aggregation increases monotonically with temperature, while content mixing has a maximum in the temperature region corresponding to the lamellar-to-nonlamellar (in our case cubic) transition, in agreement with the above-mentioned predictions and observations. Thus the structural "fusion-intermediate" whose existence was predicted from our kinetic studies (*7*) corresponds probably to the stalk−TMC−pore.

CONCLUSIONS

Low proportions of single-chain lipids modify in a dose-dependent way the rates of lipid mixing and content mixing in the fusion of large unilamellar vesicles induced by phospholipase C. Hydrocarbons and the polyunsaturated arachidonic acid enhance fusion, lysoPC and palmitoylcarnitine inhibit both lipid and content mixing, and the saturated arachidic acid has no effect. Fusion enhancers also lower the lamellar−cubic transition temperature of the lipid mixture in the liposomes, while fusion inhibitors increase the transition temperature. All these data are interpreted in support of the stalk hypothesis of membrane fusion, first proposed by Kozlov and Markin (*5*), although the experimental observations fit better the modified form of this hypothesis, as described by Siegel (*2*). Our studies also support the validity of phospholipase C-promoted vesicle fusion as a model for biological membrane fusion.

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REFERENCES

1. Chernomordik, L. V., Melikyan, G. B., and Chimadzhev, Y. A. (1987) *Biochim. Biophys. Acta 906*, 309−352.
2. Siegel, D. P. (1993) *Biophys. J. 65*, 2124−2140.
3. Chernomordik, L. V. (1996) *Chem. Phys. Lipids 81*, 203−213.
4. Basáñez, G., Ruiz-Argüello, M. B., Alonso, A., Goñi, F. M., Larlsson, G., and Edwards, K. (1997) *Biophys. J. 72*, 2630−2637.
5. Kozlov, M. M. and Markin, V. S. (1983) *Biofizika 28*, 255−261.
6. Nieva, J. L., Goñi, F. M., and Alonso, A. (1989) *Biochemistry 28*, 7364−7367.
7. Nieva, J. L., Goñi, F. M., and Alonso, A. (1993) *Biochemistry 32*, 1054−1058.
8. Goñi, F. M., Nieva, J. L., Basáñez, G., Fidelio, G. D., and Alonso, A. (1994) *Biochem. Soc. Trans. 22*, 839−844.
9. Basáñez, G., Nieva, J. L., Rivas, E., Alonso, A., and Goñi, F. M. (1996) *Biophys. J. 70*, 2299−2306.
10. Nieva, J. L., Alonso, A., Basáñez, G., Goñi, F. M., Gulik, A., Vargas, R., and Luzzati, V. (1995) *FEBS Lett. 368*, 143−147.
11. Basáñez, G., Fidelio, G. D., Goñi, F. M., Maggio, B., and Alonso, A. (1996) *Biochemistry 35*, 7506−7513.
12. Basáñez, G., Goñi, F. M., and Alonso, A. (1997) *FEBS Lett. 411*, 281−286.
13. Ruiz-Argüello, M. B., Basáñez, G., Goñi, F. M., and Alonso, A. (1996) *J. Biol. Chem. 271*, 26616−26621.
14. Epand, R. M. (1985) *Biochemistry 24*, 7092−7095.
15. Tate, M. W. and Gruner, S. M. (1987) *Biochemistry 26*, 231−236.
16. Sjölund, M., Rilfors, L., and Lindblom, G. (1989) *Biochemistry 28*, 1323−1329.
17. Siegel, D. P., Banschbach, J., and Yeagle, P. L. (1989) *Biochemistry 28*, 5010−1019.
18. van Gorkom, L. C. M., Nie, S. Q., and Epand, R. M. (1992) *Biochemistry 31*, 671−677.
19. Lohner, K., Degovics, G., Laggner, P., Gnamusch, E., and Paltauf, F. (1993) *Biochim. Biophys. Acta 1152*, 69−77.
20. Walter, A., Yeagle, P. L., and Siegel, D. P. (1994) *Biophys. J. 66*, 366−376.
21. Israelachvili, J. N., Marcelja, S., and Horn, R. G. (1980) *Q. Rev. Biophys. 13*, 121−200.
22. Epand, R. M., Epand, R. F., Ahmed, N., and Chen, R. (1991) *Chem. Phys. Lipids 57*, 75−80.
23. Madden, T. D. and Cullis, P. R. (1982) *Biochim. Biophys. Acta 684*, 149−153.
24. Salgado, J., Villalaín, J., and Gómez-Fernández, J. C. (1993) *Biochim. Biophys. Acta 1145*, 284−292.
25. Chernomordik, L. V., Vogel, S. S., Leikina, E. A., Sokoloff, A., Onaram, H. O., and Zimmerberg, J. (1993) *FEBS Lett. 318*, 71−76.
26. Chernomordik, L. V., Leikina, E., Frolov, V., Bronk, P., and Zimmerberg, J. (1997) *J. Cell. Biol. 136*, 81−93.
27. Vogel, S. S., Leikina, E. A., and Chernomordik, L. V. (1993) *J. Biol. Chem. 268*, 25764−25768.

28. Yeagle, P. L., Smith, F. T., Young, J. E., and Flanagan, T. D. (1994) *Biochemistry 33*, 1820−1827.
29. Martin, I. and Ruysschaert, J. M. (1995) *Biochim. Biophys. Acta 11240*, 95−100.
30. Shangguan, T., Alford, D., and Bentz, J. (1996) *Biochemistry 35*, 4956−4965.
31. Requero, M. A., Goñi, F. M., and Alonso, A. (1995) *Biochemistry 34*, 10400−10405.
32. Goñi, F. M., Requero, M. A., and Alonso, A. (1996) *FEBS Lett. 390*, 1−5.
33. Epand, R. M., Epand, R. F., and Lancaster, C. R. D. (1989) *Biochim. Biophys. Acta 945*, 161−166.
34. Veiga, M. P., Requero, M. A., Goñi, F. M., and Alonso, A. (1996) *Mol. Membr. Biol. 13*, 165−172.
35. Basáñez, G., Nieva, J. L., Goñi, F. M., and Alonso, A. (1996) *Biochemistry 35*, 15183−15187.
36. Hoekstra, D., de Boer, T., Klappe, K., and Wilschut, J. (1984) *Biochemistry 23*, 5675−5681.
37. Chernomordik, L. V., Chanturiya, A., Green, J., and Zimmerberg, J. (1995) *Biophys. J. 69*, 922−929.
38. Wu, H., Zheng, LX., and Lentz, B. R. (1996) *Biochemistry 35*, 12602−12611.
39. Leikin, S., Kozlov, M. M., Fuller, N. L., and Rand, R. P. (1996) *Biophys. J. 71*, 2623−2632.
40. Ellens, H., Siegel, D. P., Alford, D., Yeagle, P. L., Boni, L., Lis, L. J., Quinn, P. J., and Bentz, J. (1989) *Biochemistry 28*, 3692−3703.
41. Luzzati, V. (1997) *Curr. Opin. Struct. Biol. 7*, 661−668.
42. Siegel, D. P., and Epand, R. M. (1997) *Biophys. J. 73*, 3089−3111.
43. Ellens, H., Bentz, J., and Szoka, F. C. (1986) *Biochemistry 25*, 285−294.
44. Ellens, H., Bentz, J., and Szoka, F. C. (1986) *Biochemistry 25*, 4141−4147.

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