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Effects of platelet activating factor and related lipids on phase transition of dipalmitoylphosphatidylcholine

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Recent evidence localizing the inflammatory mediator, platelet activating factor, (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) to the membranes of stimulated neutrophils raises the possibility that PAF may, in addition to its activities as a mediator, alter the physical properties of membranes. Accordingly, the effects of PAF and related alkyl ether and acyl analogs on phase transition thermodynamics of dipalmitoylphosphatidylcholine (DPPC) were studied using fluorescence polarization of the fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH). PAF, its ester analog (1-palmitoyl-2-acetylphosphatidylcholine) and both the corresponding alkyl and acyl lysophospholipid analogs (each at a concentration of 10 mol%) significantly decreased the phase transition temperature and broadened the phase transition of DPPC ($P < 0.05$). The relative potency of the lipids in causing this effect was ester-PAF ≥ PAF ≥ lyso-PAF > lyso-PC suggesting that the fluidization of the synthetic membranes was attributable to both the 2-position acetyl group and the 1-position alkyl linkage. Furthermore, using various related compounds, increases in chain length and degree of unsaturation in the 2-position were shown to enhance the depression in transition temperature and broadening of the phase transition. Phase transition thermodynamics were also assessed using differential scanning calorimetry. Similar depression in the phase transition temperature was measured for PAF and both the alkyl and acyl lysophospholipids. Broadening of the phase transition for DPPC by the various analogs was assessed by calculation of transition peak width and cooperative unit. Data from fluorescence polarization and differential scanning calorimetry provide similar though not identical results and support the hypothesis that the unique features of PAF may alter membrane physical properties and could ultimately explain some of its biologic actions.

Abbreviations:

PAF,	platelet activating factor, 1-*O*-hexadecyl-2-acetylphosphatidylcholine;
ester-PAF,	1-palmitoyl-2-acetylphosphatidylcholine;
lyso-PAF,	1-*O*-hexadecyllysophosphatidylcholine;
lyso-PC,	1-palmitoyllysophosphatidylcholine;
methyl-PAF,	1-*O*-hexadecyl-2-*O*-methylphosphatidylcholine;
PAF-C18,	1-*O*-octadecyl-2-acetylphosphatidylcholine;
lyso-PC-C18,	1-stearoyllysophosphatidylcholine;
PAF-C18:1,	1-*O*-(*cis*-9-octadecenyl-2-acetylphosphatidylcholine;
PAPC,	1-palmitoyl-2-arachidonoylphosphatidylcholine;
HAPC,	1-*O*-hexadecyl-2-arachidonoylphosphatidylcholine;
DPPC,	dipalmitoylphosphatidylcholine;
DPH,	1,6-diphenyl-1,3,5-hexatriene;
DSC,	differential scanning calorimetry;
T_m,	transition temperature;
W_t,	transition peak width at half height.

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Platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is an unusual phospholipid because of both the ether linkage in the 1-position and the acetyl group in the 2-position of the glycerol backbone. This compound produces biological actions at extraordinarily small extracellular concentrations. For example, 10^{-11} to 10^{-10} M activates rabbit platelets [1] and 10^{-9} to 10^{-8} M stimulates adhesion and degranulation of human neutrophils [2]. More recently, activation of neutrophils with other stimuli has been found to produce considerable quantities of PAF intracellularly, most of which is retained within the cell [3,4]. This intracellular PAF appears to partition into cell membranes and achieves significant concentrations in certain subcellular membranes. For example, phagolysosomal membranes prepared from neutrophils stimulated with opsonized zymosan contain PAF at a concentration greater than 0.5 mol% [5]. These findings raise the question of whether PAF might alter the physical properties of cellular membranes. In the only study that has addressed this issue, Fink and Gross [6] found that 1.5 mol% PAF disordered the inner core of myocardial sarcolemmal membranes.

Synthesis of PAF occurs concomitant with cellular activation, secretion, and extensive fusion and remodeling of intracellular membranes. Recent studies demonstrate that activation of leukocytes [7] and platelets [8] by certain stimuli, involve changes in membrane fluidity. Furthermore, membrane fusion may be dependent on lipid physical properties such as acyl chain ordering and lateral phase separations [9,10]. Thus, an understanding of the effects of PAF on membrane physical properties may be important in understanding the role of this lipid in cellular activation.

In the present study, we compared the effects of PAF, ester-PAF, lyso-PAF, lyso-PC and other related lipids on the physical properties of phospholipid vesicles. These lipids were chosen because they allow evaluation of the importance of the 2-acetyl group and the 1-ether linkage. The effects of these lipids on the phase transition of dipalmitoylphosphatidylcholine were studied using fluorescence polarization and differential scanning calorimetry.

Materials and Methods

Materials. 1-Palmitoyl-2-arachidonoyl- and dipalmitoyl-L-phosphatidylcholine (PAPC, and DPPC, respectively) were obtained from Avanti Polar Lipids (Birmingham, AL). 1-*O*-Alkyl-2-acetyl-L-phosphatidylcholine (PAF), 1-*O*-hexadecyl-L-lysophosphatidylcholine (lyso-PAF), 1-palmitoyl-L-lysophosphatidylcholine (lyso-PC), and 1-*O*-hexadecyl-2-*O*-methyl-L-phosphatidylcholine (methyl-PAF) were obtained from Sigma Chemical Co. (St. Louis, MO). Analysis of the PAF by fast atom bombardment mass spectrometry indicated that it consisted of only the hexadecyl species. 1-*O*-Octadecyl-2-acetyl-L-phosphatidylcholine (PAF-C18) and 1-*O*-(*cis*-9-octadecenyl)-2-acetyl-L-phosphatidylcholine (PAF-C18 : 1) were obtained from Bachem, Inc. (Bubendorf, Switzerland). The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes, Inc. (Junction City, OR). 1-*O*-Hexadecyl-2-arachidonoyl-L-phosphatidylcholine (HAPC) and 1-palmitoyl-2-acetyl-L-phosphatidylcholine (ester-PAF), were synthesized by acylation of the corresponding lysophospholipid with the appropriate acid chloride. Following purification with TLC, fast atom bombardment mass spectrometry confirmed the identities of each compound and the absence of the lysophospholipid precursors. Approximate concentrations of HAPC and ester-PAF were determined by comparison of ion currents generated from simultaneous application of known amounts of DPPC. Accurate calibration of both compounds was then determined by gas chromatographic analysis of the fatty acid content of aliquots of the HAPC and ester-PAF solutions.

Preparation of lipid vesicles. Lipids (90 mmol total, dissolved in chloroform) were mixed with 0.1 nmol of DPH dissolved in tetrahydrofuran. All solutions were stored under argon at $-20\,^{\circ}$C. The mixtures of lipids and probe were dried to a thin film by a stream of nitrogen and stored overnight under vacuum. One milliliter of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 20 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesul-

fonic acid (pH 7.4)) was added to samples containing DPH. The tubes were purged with argon and incubated in the dark with occasional vortexing at a temperature above the phase transition temperature (about 50 °C). The samples were then placed in a bath-type sonicator for 1 minute. After vigorous vortexing, they were transferred to a 10×4 mm quartz cuvette for fluorescence measurements. This method has been shown to produce multilamellar vesicles [11].

Fluorescence measurements. Fluorescence polarization of DPH was measured with an HH-1 T-format polarization spectrofluorimeter (H and L Instruments, Burlingame, CA). Fixed excitation and emission polarization filters were used to measure fluorescence intensity parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarization plane of the exciting light. Polarization of fluorescence $(I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$ and intensity of fluorescence $(I_{\parallel} + 2I_{\perp})$ were calculated by an on-line microprocessor. The excitation wavelength was 362 nm and a 03FCG001 filter (Melles Griot, Irvine, CA) was used in the excitation beam and KV389 filters were used for the emitted light. The use of an excitation filter reduced light scattering to negligible levels. Cuvette temperature was maintained by a circulating water bath and monitored continuously by a thermister inserted into the cuvette to a level just above the light beam. For temperature scans, the sample was heated to the highest temperature to be measured and cooled at a rate of about 0.5 C°/min. Temperature, polarization and total fluorescence data (collected only during cooling unless otherwise noted) were stored on disk by an Apple II computer interfaced with the fluorimeter. The derivative of each individual cooling scan was used to determine phase transition temperature (T_m) and to calculate peak width at half height (W_t) as an approximation of the size of the cooperative unit [12].

Differential scanning calorimetry (DSC). A Perkin-Elmer DSC-2c instrument was used. Lipid mixtures (0.2–0.3 mg) were dissolved in chloroform, placed in 75 μl aluminum calorimeter pans (washed with chloroform) and dried to a thin film under vacuum. PBS (50 μl) was added and the pans sealed. Multiple heating and cooling scans were performed at scan rates of 2.5 C°/min. The third heating and cooling scans were used for calculations. A chart recorder plotted the excess heat capacity as a function of temperature. The midpoint temperature of the phase transition was estimated visually, the area under the curve was determined by cutting and weighing the paper. After completing the scans, the amount of lipid in the sample was determined by extracting the pan with chloroform and determining the amount of phosphorus [13]. These data were used to calculate the enthalpy change of the transition and the size of the cooperative unit [12].

Results

Fluorescence polarization studies

The lipid order and phase transition behavior of DPPC vesicles containing PAF and related lipids was studied by fluorescence polarization of DPH. Vesicles composed of DPPC alone displayed the characteristic abrupt and marked change in polarization at about 41 °C, the phase transition temperature (Fig. 1). The most notable effect of PAF was lowering of the transition temperature and broadening of the phase transition. A PAF concentration of 10 mol% significantly lowered the transition temperature slightly more than one degree (Table I), while a concentration of 1 mol% produced a small but detectable de-

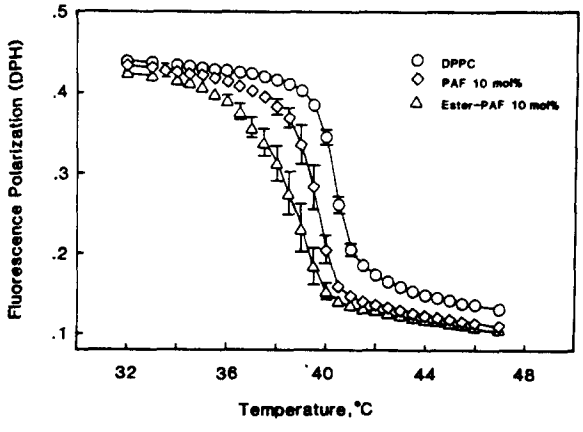

Fig. 1. Temperature (cooling) scan of DPPC (○) demonstrating the abrupt increase in fluorescence polarization at the T_m of 41 °C. Addition of PAF (◇) or ester-PAF (△) (10 mol%) lowers the T_m, broadens the phase transition and increases fluidity above the phase transition. Data represent mean ± S.E. values ($n = 5$ different preparations). Calculated values from these and other vesicle preparations are given in Table I.

TABLE I

EFFECT OF PAF AND RELATED LIPIDS (10 MOL%) ON TRANSITION TEMPERATURE (T_m) AND TRANSITION WIDTH (W_t) OF DPPC VESICLES

Lipid	N	Decrease in T_m [a]	Increase in W_t [a]
Lyso-PC	3	0.48 ± 0.02 [b,c]	0.27 ± 0.12 [b]
Methyl-PAF	4	0.63 ± 0.11 [b,c]	0.09 ± 0.15
Lyso-PAF	3	1.15 ± 0.18 [b]	0.10 ± 0.20
PAF	12	1.19 ± 0.14 [c]	0.40 ± 0.09 [b]
Ester-PAF	5	1.56 ± 0.20 [b]	0.39 ± 0.13 [b]
PAF C18:1	2	1.97	0.78
PAPC	3	2.45 ± 0.08 [b,c]	0.49 ± 0.17 [b]
HAPC	2	2.20–? (too broad)	1.25–? (too broad)

[a] Values are C° expressed as mean change ± S.E. relative to DPPC. For DPPC, $T_m = 40.84 \pm 0.12$° C; $W_t = 0.59 \pm 0.07$° C ($N = 14$).

[b] Significant effect of the lipid (i.e., significantly different from DPPC alone), $P < 0.05$ by t-test for unpaired samples.

[c] Significantly different from PAF, $P < 0.05$, by t-test for unpaired samples.

crease (not shown). PAF also had fluidizing effects on DPPC in the liquid-crystalline state as demonstrated by decreased polarization at temperatures above the phase transition (Fig. 1). These effects were even more pronounced for ester-PAF (the ester analog of PAF) at a concentration of 10 mol% (Fig. 1). Effects of lyso-PAF were similar to those of PAF. In contrast, lyso-PC (at a concentration of 10 mol%) produced only a slight decrease in the transition temperature (Table I) significantly less than that seen with PAF and did not alter polarization above or below the transition temperature. Hence, the importance of the *sn*-2 acetyl group and the 1-position ether linkage in altering phase transition characteristics were demonstrated. Lengthening of the alkyl chain to 18 carbons (PAF-C18) produced similar effects to those of PAF (data not shown), while the presence of unsaturation in PAF-C18:1 had more pronounced effects on phase transition characteristics (Table I). Comparisons of the alkyl phospholipids revealed similar effects of changes in chain length and degree of saturation in the *sn*-2 position. Whereas shortening the *sn*-2 group as in lyso-PAF and methyl-PAF had no significant effect on broadening the phase transition as measured by peak width at half height, chain lengthening and the presence of unsaturation produced a marked decrease in transition temperature and broadening of the phase transition as shown for HAPC (Table I).

Differential scanning calorimetry

Effects of PAF, lyso-PAF and lyso-PC on the phase transition of DPPC were also examined by differential scanning calorimetry (DSC). The effects of ester-PAF were not evaluated because of limited availability. All of these compounds broadened the calorimetry transitions and shifted them to lower temperatures. Representative scans are shown in Fig. 2, and calculated values are given in Table II. Upon heating, PAF broadened the transition and produced a characteristic

TABLE II

EFFECTS OF LIPIDS ON THE PHASE TRANSITION OF DPPC AS DETERMINED BY DSC USING BOTH HEATING AND COOLING SCANS

The concentration of PAF, lyso-PAF and lyso-PC were 10 mol%. Values are mean ± S.E., $N = 4$–6 different preparations.

Lipid	T_m [a]		Enthalpy [b]		W_t [c]		Cooperative unit [d]	
	heating	cooling	heating	cooling	heating	cooling	heating	cooling
DPPC	41.4 ± 0.1	39.3 ± 0.1	7.7 ± 0.3	8.0 ± 0.3	0.41 ± 0.04	0.45 ± 0.03	196 ± 19	179 ± 15
PAF	40.6 ± 0.1 [e]	38.1 ± 0.2 [e]	8.4 ± 0.3	8.6 ± 0.3	0.90 ± 0.03 [e]	0.52 ± 0.02	90 ± 3 [e]	155 ± 7
Lyso-PAF	40.3 ± 0.2 [e]	38.1 ± 0.2 [e]	7.7 ± 0.4	7.7 ± 0.5	0.62 ± 0.05 [e]	0.58 ± 0.05 [e]	130 ± 10 [e]	139 ± 9 [e]
Lyso-PC	40.5 ± 0.1 [e]	38.4 ± 0.1 [e]	8.4 ± 0.4	8.5 ± 0.5	0.72 ± 0.05 [e]	0.79 ± 0.05 [e]	112 ± 8 [e]	103 ± 9 [e]

[a] Transition midpoint (° C).

[b] Transition enthalpy (kcal/mol).

[c] Transition width at half height (° C).

[d] Number of lipid molecules in the cooperative unit.

[e] Significantly different from DPPC, $P < 0.05$, t-test for unpaired samples.

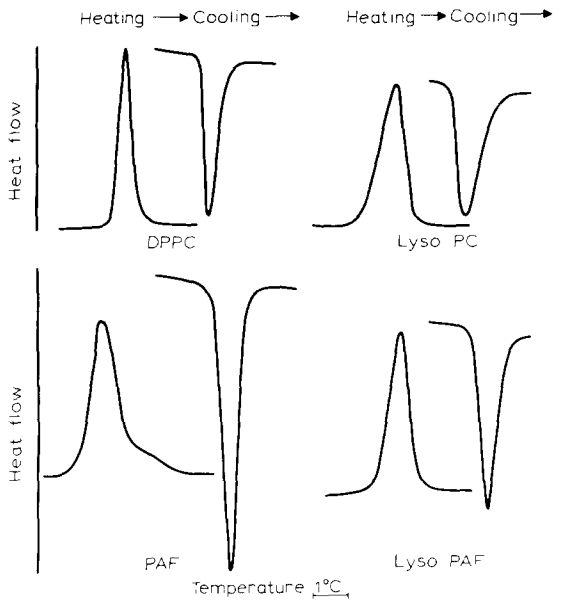

Fig. 2. Representative heating and cooling differential scanning calorimetry tracings for DPPC, lyso-PC, PAF and lyso-PAF. Calculated values from these and other preparations are given in Table II.

shoulder on the peak. These effects were not seen with cooling scans (Fig. 2 and Table II). This difference between heating and cooling could be observed repeatedly with the same sample and was reproducible between samples. In contrast, lyso-PAF and lyso-PC produced similar changes with heating and cooling scans. None of the compounds altered the enthalpy of the calorimetric transitions, but all decreased the size of the cooperative unit indicating that they decrease the cooperative interaction between phospholipid molecules [12]. PAF was the most effective in changing the cooperative unit during heating scans but all three compounds were about equieffective in decreasing the transition temperature (Table II). The control values (transition temperature, enthalpy and size of cooperative unit) obtained for DPPC in this study are in excellent agreement with those reported by Mountcastle et al. [12].

The data reported above for fluorescence polarization were obtained from cooling scans. However, examination of repeated heating and cooling scans revealed that PAF produced a similar broadening of the transition measured by fluorescence polarization regardless of whether it was determined by heating or cooling (data not shown). Thus, the fluorescence and calorimetric studies show similar, but not identical, effects of PAF and related lipids on the phase properties of DPPC.

Discussion

PAF, ester-PAF, lyso-PAF and lyso-PC all decreased the phase transition temperature and broadened the transition of DPPC. Analysis of effects of these lipids on the phase transition in the context of ideal solution theory indicates that they partition preferentially into fluid phases [14]. Measurement of the phase transition by fluorescence polarization demonstrated that the potency varied as ester-PAF $\geq$ PAF $\geq$ lyso-PAF > lyso-PC, indicating that both the acetyl group and the ether linkage increase the lipid disordering action of the lipids. In addition, increase in the 2-position chain length or degree of unsaturation in either chain made progressively greater alterations in phase transition characteristics when compared to PAF. These data are consistent with previous work by Lee and Fitzgerald [15] who showed a lower transition temperature for a pure monoether (α-hexadecyl-β-oleoyl-GPC) in aqueous dispersion than its diester (α-palmitoyl-β-oleoyl-GPC) analog and with the results of Fink and Gross [6] demonstrating that PAF increased the fluidity of myocardial sarcolemmal membranes, while lyso-PC did not change membrane fluidity (as measured by electron spin resonance of doxylstearate probes). Furthermore, in the work of Demediuk et al. [16], the addition of a 1-position ether lipid (plasmalogen) to vesicles of diacylphosphatidylcholine and phosphatidylethanolamine resulted in an initial increase and then decrease in membrane fluidity (as measured by electron spin resonance spectroscopy) with increasing concentrations of plasmalogen. In these latter experiments, relatively large concentrations of plasmalogen (20 mol% or more) were employed and findings could not be attributed more specifically to either the ether-linkage or the double bond between carbon-1 and -2 of the alkyl chain.

In contrast to the above work, our DSC studies showed PAF, lyso-PAF and lyso-PC to be equally potent in decreasing the phase transition temperature. The effectiveness of lyso-PC in reducing the

transitions measured by both fluorescence polarization and DSC was somewhat surprising in view of DSC studies showing that 10–20 mol% lyso-PC either does not affect [17] or even increases [18] the transition temperature of DPPC. The shape of the transitions were differentially affected by the lipids and PAF was the most potent in broadening the phase transition for heating scans, but the least potent for cooling scans. The markedly different effects of PAF during heating and cooling may be due to formation of PAF-rich domains in the liquid crystalline phase but not in the gel phase. It is unclear why some of the fluorescence and calorimetric results differ, but could be related to technical differences, e.g., the heating and cooling rates were 0.5 C°/min for fluorescence but the lower sensitivity of the calorimeter required rates of 2.5 C°/min. The faster temperature changes could magnify metastable states. Also, multilamellar vesicles were used for fluorescence, whereas lipid films were needed for calorimetry.

Notably, most investigation aimed at characterizing physical changes in membranes attributed to ether lipids have contrasted diethers to the corresponding diester analogs. In contrast to effects of a pure monoether [15] and our data with mixtures of phospholipids containing small amounts of 1-position monoethers, diether phospholipids appear to increase the gel to liquid-crystalline transition temperature as shown by DSC [19,20]. This increase in transition temperature has been attributed to attractive forces resulting in denser molecular packing among diether phospholipids than diester analogs [21]. However, these changes in molecular packing do not appear to alter the cooperative unit [20] perhaps because of increased water penetration in the interfacial region of diether phospholipids [22]. Recent reports using DSC and ^{31}P-NMR have suggested that the intermolecular attractive forces associated with the ether linkage may also promote the lamellar to hexagonal phase transition resulting in a rank ordering of this transition temperature for diether < 1-ether-2-acyl ≪ diacyl phosphatidylethanolamines [23]. In another report small amounts of alkanes (< 1 mol%), while having little influence on the gel to liquid-crystalline transition, significantly decreased the lamellar to hexagonal transition temperature [24]. Thus, it is possible that ether lipids such as PAF may promote such phase transition. To date, such analysis has not been applied to evaluate 1-position monoethers.

Recent investigation using Raman and NMR spectroscopies [25] has shown that PAF-C18 and related compounds (both the ester-linked analog and lyso-PC-C18) in pure aqueous dispersion undergo a lamellar to micellar transition at around 21°C. Greater rotational freedom along the glycerol backbone was noted at temperatures below the phase transition for PAF-C18 and attributed to the lack of an anchoring carbonyl group in the 1-position. However, these differences between the analogs were not noted at 25°C in the micellar phase. Rotational freedom of PAF in DPPC bilayers undergoing phase transition has not been determined, but a high degree of rotational freedom is consistent with the shifts in phase transitions observed in the present study.

The present demonstration of effects of PAF on membrane phase transition properties raises the question of the possible physiological importance of these changes. One critical issue is whether the membrane concentrations used in these studies are achieved in vivo. Phagolysosomal membrane preparations from stimulated neutrophils contain concentrations of PAF in the range of 0.5–1 mol% [5], and we demonstrated small effects of 1 mol% PAF in the present studies of phase transitions. In addition, it is likely that improved membrane separation techniques will reveal membranes that contain higher concentrations of PAF than those measured thus far. As PAF is concentrated in some membranes and very low in others (Ref. 5, and Riches, unpublished), the measured concentrations are highly dependent on membrane purity. In addition, the phase transition studies indicate that PAF selectively partitions into fluid phases suggesting that the concentration in these domains will be larger than the bulk membrane concentration.

In view of the possibility that stimulated cells may accumulate sufficient PAF to alter the physical properties of cellular membranes, it is of interest to consider the functional consequences of membrane changes. PAF is produced in neutrophils (activated by various stimuli) during endo- and exocytosis and may be involved in membrane fusion. Its ability to fluidize membranes is con-

sistent with promotion of membrane fusion by fluid phases [26,27]. Furthermore, the formation of hexagonal phase lipids may be important for fusion to occur and other ether lipids have been shown to promote the lamellar to hexagonal transition [23], suggesting that PAF may also enhance fusion by promoting this transition. Finally, changes in membrane fluidity can alter the activity of membrane-bound enzymes and transmembrane ion channels [28,29]. In this context, it is important to consider consequences of metabolism of ether lipids. Recent work with intact cells (hepatocytes) suggests that the net loss of arachidonic acid results in decreased membrane fluidity [30]. The precursor of PAF in neutrophils is 1-hexadecyl-2-arachidonyl-PC (HAPC) which is cleaved by phospholipase A_2 to release arachidonate and lyso-PAF which is then converted to PAF [31]. Therefore, the conversion of HAPC to lyso-PAF with loss of arachidonate could produce a marked decrease in membrane fluidity that would be partially offset by conversion and accumulation of PAF. Thus, formation of PAF may serve to maintain a fluid membrane despite the release of arachidonic acid. This model, however, assumes that these lipids do not move to other membranes or membrane domains during their metabolism; the validity of this assumption remains to be tested. The effects of PAF and related lipids on the physical properties of model membranes suggest several possible mechanisms for regulation of cellular function by these lipids, but studies with biological membranes will be required to define the physiological significance of the membrane actions of PAF.

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