Biochimica et Biophysica Acta, 943 (1988) 211–219
Elsevier

BBA 74095

Effects of platelet activating factor on calcium–lipid interactions and lateral phase separations in phospholipid vesicles

Donna L. Bratton [a], R. Adron Harris [b,c], Keith L. Clay [c] and Peter M. Henson [a]

[a] *National Jewish Center for Immunology and Respiratory Medicine,* [b] *Denver V.A. Medical Center, and* [c] *Department of Pharmacology, University of Colorado Health Science Center, Denver, CO (U.S.A.)*

(Received 16 February 1988)

Key words: Platelet activating factor; Lateral phase separation; Calcium-lipid binding; Phospholipid vesicle; (Dansylated probe)

Recent studies localizing the inflammatory mediator, platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), to the membranes of stimulated neutrophils, raise the possibility that PAF may, in addition to its activities as a mediator, alter the physical properties of membranes. This, and the increasing evidence that calcium–lipid interactions may have central importance in membrane organizational structure and in functions of cell homeostasis and stimulus-response coupling, prompted us to study the effects of PAF on calcium–lipid interactions in lipid vesicles. Using fluorescence polarization of dansylated probes located in the glycerol portion of the membrane bilayer, PAF (at a concentration as low as 1 mol%) was shown to reduce membrane rigidification significantly during calcium-induced lateral phase separations. This effect of PAF was structurally dependent on both the 1-position alkyl linkage and the 2-position acetyl group as shown by studies of related lipid analogs. Furthermore, using a self-quenching probe, it was shown that inhibition of lateral phase separation did not account for this reduction in the calcium-induced membrane rigidification attributed to PAF. Data suggest that PAF at low concentrations may alter phospholipid head packing and, thereby, change membrane surface features during calcium–lipid interactions, effects which may ultimately explain some of its biological actions.

Abbreviations: PAF, platelet activating factor, 1-*O*-hexadecyl-2-acetyl-phosphatidylcholine; ester-PAF, 1-palmitoyl-2-acetyl-phosphatidylcholine; lyso-PAF, 1-*O*-hexadecyllysophosphatidylcholine; lyso-PC, 1-palmitoyllysophosphatidylcholine; HAPC, 1-*O*-hexadecyl-2-arachidonoylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; PS, phosphatidylserine; DPPC, dipalmitoylphosphatidylcholine; egg PC, egg phosphatidylcholine; dansyl-PS, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)phosphatidylserine; dansyl-DPPE, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)dipalmitoyl-L-phosphatidylethanolamine; TMA-DPH, 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene; DPH, 1,6-diphenyl-1,3,5-hexatriene; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; R18, octadecyl Rhodamine B, chloride salt; MLV, multilamellar vesicles; SUV, small unilamellar vesicles.

Correspondence: D.L. Bratton, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206, U.S.A.

Introduction

In stimulated human neutrophils, the synthesis of platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a unique ether phospholipid, occurs concomitant with cellular activation, secretion, and extensive fusion and remodeling of intracellular membranes. Recent subcellular fractionation studies of activated neutrophils demonstrate that considerable quantities of PAF produced intracellularly remain localized within certain cell membranes in significant concentration [1,2]. For example, phagolysosomal membranes prepared from neutrophils stimulated with opsonized zymosan contain PAF at a concentration greater than 0.5 mol% (Ref. 3; Riches, personal communication). This rises the possibility of whether PAF might alter the physical properties of membranes and thereby exert additional effects in the cell. Membrane fusion events in endocytosis and exocytosis may be dependent on lipid physical properties, such as acyl chain ordering and membrane fluidity [4–6]. Indeed, activation of leukocytes [7–9] and platelets [10] is thought to involve changes in membrane fluidity. Furthermore, in the only study that has addressed effects of PAF in biologic membranes, Fink and Gross [11] found that 1.5 mol% PAF disordered the inner core of myocardial sarcolemmal membranes. We have demonstrated similar findings in model membranes, and have defined the importance of the 1-ether linkage and 2-position acetyl group in these effects [12].

Membrane events in cellular activation and the central role of calcium as a second messenger in cell stimulus-response coupling are areas of intensive investigation. Recent studies suggest that the binding of calcium to membranes may contribute to organizational structure and ultimately to function [13–15], and may well play a role in membrane fusion [16–18]. Calcium-induced lateral phase separations have been demonstrated in natural [19], as well as model membranes, and may be important in membrane permeability [20,21], and facilitation of membrane fusion events [21,22]. In the present study we compare the effects of PAF and related lipids, lyso-PAF, lyso-PC and ester-PAF on calcium–lipid interactions and calcium-induced lateral phase separation in phospholipid vesicles. These related lipids were chosen to evaluate the importance of the 2-position acetyl group and the 1-position ether linkage. Calcium–lipid interactions were studied using steady-state fluorescence polarization with a variety of probes (dansyl-DPPE, dansyl-PS, TMA-DPH, and DPH) enabling us to evaluate several regions of the lipid bilayer. Calcium-induced lateral phase separations and vesicle fusion were studied using the self-quenching probes NBD-PE and R18.

Methods

Materials. Bovine brain phosphatidylserine (PS), 1-*O*-alkyl-2-acetyl-L-phosphatidylcholine (PAF), 1-*O*-hexadecyl-L-lysophosphatidylcholine (lyso-PAF), 1-palmitoyl-L-lysophosphatidylcholine (lyso-PC), and dipalmitoylphosphatidylethanolamine (DPPE) 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. Egg phosphatidylcholine (egg PC), dipalmitoyl-L-phosphatidylcholine (DPPC) and *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (NBD-PE) were obtained from Avanti Polar Lipids (Birmingham, AL). 1-*O*-Hexadecyl-2-arachidonoyl-L-phosphatidylcholine (HAPC) was obtained from Biomol (Philadelphia, PA). *N*-(5-Dimethylaminonaphthalene-1-sulfonyl)dipalmitoyl-L-phosphatidylethanolamine (dansyl-DPPE), the phosphatidylserine analog (dansyl-PS), 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH), 1,6-diphenyl-1,3,5-hexatriene (DPH) and octadecyl Rhodamine B, chloride salt (R18) were purchased from Molecular Probes, Inc. (Junction City, OR). 1-Palmitoyl-2-acetyl-L-phosphatidylcholine (ester-PAF) was synthesized from the corresponding lysophospholipid with the appropriate acid chloride. Following purification with TLC, fast atom bombardment mass spectrometry confirmed the identity of the compound and the absence of the lysophospholipid precursor. The approximate concentration of ester-PAF was determined by comparison of ion currents generated from simultaneous application of known amounts of DPPC. Accurate calibration was then determined by gas chromatographic analysis of the

fatty acid content of aliquots of the ester-PAF solution.

Preparation of lipid vesicles. DPPE and PS in a 2:1 molar ratio were used in all experiments utilizing the dansyl probes, TMA-DPH or DPH. Lipids (90 nmol total, dissolved in chloroform or chloroform/methanol) were mixed with 0.2 nmol of DPH dissolved in tetrahydrofuran, TMA-DPH dissolved in tetrahydrofuran/water, or 1.0 nmol of dansyl-DPPE or dansyl-PS dissolved in chloroform. All solutions were stored under argon at −20°C. The mixtures of lipids and probe were dried to a thin film by a stream of nitrogen. Samples were suspended in 1.2 ml Hepes-buffered EGTA saline (HES) (145 mM NaCl, 3 mM KCl, 0.1 mM EGTA, 20 mM Hepes, pH 7.4). The tubes were purged with argon and incubated in the dark with occasional vortexing at a temperature above the phase transition temperature. The samples were then placed in a bath-type sonicator for one 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 (MLV) [23]. The appropriate concentration of $CaCl_2$ was then added sequentially and manually stirred before each fluorescence polarization reading.

Similarly, in experiments with NBD-PE, probe (5 mol%) was mixed with PS and varying amounts of PAF, lyso-PAF, lyso-PC, ester-PAF, or egg PC (90 nmol total lipid) dissolved in chloroform. The mixtures of lipid were dried to a thin film by a stream of nitrogen. Samples were suspended in HES with $CaCl_2$ added in the indicated amounts. Samples were vortexed for one minute, incubated for five minutes at 45°C and sonicated in a bath-type sonicator for one minute. Vesicle suspensions were then transferred to a 10×4 mm quartz cuvette for fluorescence measurements. Stock solutions containing the lipids and NBD-PE were stored at −20°C with argon.

Small unilamellar vesicles (SUV) of PS were prepared by the method of Hoekstra et al. [24]. For unlabeled SUV, PS or PS with 10 mol% DPPC, lyso-PC or PAF (96 nmol total lipid) was dried to a thin film by a stream of nitrogen. Labeled SUV were made similarly with 23 nmol PS and 1 nmol R18 dissolved in chloroform/methanol (1:1, v/v). Each sample was brought up in 2.4 ml HES buffer, vortexed vigorously for 1 min, placed under argon and sonicated with a probe sonicator using a 50% pulse cycle for 5 min. Samples were then centrifuged in a microfuge at 13000×*g* for 2 min. Aliquots of labeled and unlabeled SUV were mixed for a final ratio of 1:4 (labeled/unlabeled) in a 10×4 mm quartz cuvette for fluorescence determinations.

Fluorescence measurements. Fluorescence polarization of dansyl-DPPE, dansyl-PS, TMA-DPH and DPH were 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 for dansyl-DPPE and dansyl-PS was 350 nm, for TMA-DPH, 362 nm, and for DPH, 363 nm. 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 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. The sample temperature was 37°C for all experiments.

In separate experiments lateral phase separation was monitored by measuring the fluorescence intensity of NBD-PE [22] in a Farrand MKI spectrofluorimeter. The excitation and emission wavelengths were 467 and 530 nm, respectively. To prevent photobleaching, the light path was blocked except when readings were taken. Temperature was maintained at 37°C while fluorescence intensity readings were taken. Initial studies had shown a steady decrease in fluorescence over the first several minutes during calcium-induced lateral phase separation in which the self-quenching probe is isolated into microdomains separated from PS. Fluorescence intensity for each sample was read after stabilization of the signal (at 15 min). Control samples containing DPPE in place

of NBD-PE were prepared and analyzed in the same manner to determine light scattering. These values were then subtracted from total fluorescence of the corresponding NBD-PE samples.

Calcium-induced fusion of SUV was monitored using the self-quenching probe R18 by the method of Hoekstra et al. [24]. Baseline fluorescence intensity for the mixture of probe-labeled and unlabeled SUV (see above) was measured in a Farrand spectrofluorimeter with the excitation and emission wavelengths set at 560 nm and 590 nm, respectively, and using 5 nm slits. After obtaining a baseline fluorescence signal, an aliquot of $CaCl_2$ was added to achieve a final calcium concentration of either 2 or 4 mM, and the sample vigorously stirred. An increase in fluorescence intensity is seen as calcium-induced vesicle fusion occurs and the probe is diluted in the enlarging bilayer surface area. Fluorescence intensity was recorded 5 sec after addition of calcium, and every 30 s thereafter for a total of 15 min. At the end of each experiment, Triton X-100 (1% v/v final concentration) was added to the sample to achieve a maximal signal (infinite probe dilution) [24]. Data for all time points were expressed as the percent of the maximal (total) signal. Early fusion experiments demonstrated that incorporation of PAF (or the other related lipids) into either the labeled or unlabeled SUV population produced identical results. Data shown are for experiments in which PAF (or related lipid) was incorporated in the unlabeled SUV population prior to fusion.

Statistics. Concentration-response curves were compared by analysis of variance for repeated measurements. Individual points were compared by a *t*-test for unpaired samples. A P value < 0.05 was considered significant.

Results

Effects of PAF and related lipids on lateral phase separation

Dansyl-DPPE and dansyl-PS studies. The binding of calcium to vesicles containing acidic lipids produces lateral phase separations due to formation of clusters of calcium-lipid complexes [6,20]. We examined the effects of PAF and related lipids on calcium–lipid interaction in multilamellar vesicles of DPPE and brain PS containing the fluorescent probes dansyl-DPPE and dansyl-PS. These probes are located in the glycerol region of the lipid bilayer [25] and are quite sensitive to effects of calcium on lipids (Refs. 26, 27, and Harris R.A., unpublished data). Use of both dansyl probes in separate experiments allowed study of calcium-lipid interactions in both DPPE and PS microdomains during lateral phase separation. Addition of calcium to DPPE/PS vesicles markedly increased fluorescence polarization of both dansyl-DPPE (Fig. 1 solid circles) and dansyl-PS (Table I). This marked rigidification is likely due to calcium-induced separation of the rigid DPPE from the calcium-PS phase [4]. Addition of PAF to the multilamellar vesicles markedly reduced this rigidification in a concentration-dependent manner (Fig. 2). Addition of as little as 1 mol% PAF resulted in significant fluidization of the glycerol region of the bilayer as sensed by the dansyl-DPPE probe (Fig. 2, left panel), while 3 mol% resulted in a similar effect with dansyl-PS (Fig. 2, right panel).

The structural specificity of this fluidizing effect during calcium-induced lateral phase separation was determined using related lipid analogs. As can be seen in Fig. 1, the relative potency in reducing the rigidifying effects of calcium was

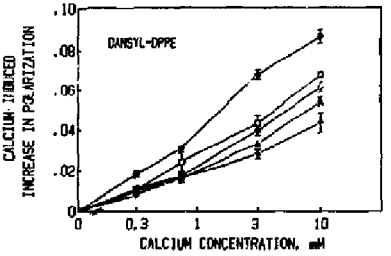

Fig. 1. The rigidifying effect of calcium (as measured by increased polarization of dansyl-DPPE) on vesicles of DPPE/PS (2:1) (●) and those containing 10 mol% PAF (◆), ester-PAF (△), lyso-PAF (○) or lyso-PC (□). Points represent mean ± S.E.; N = four preparations. Although significant reduction in membrane rigidification was seen with all compounds, the interaction of calcium and the acetyl compound containing vesicles (those with PAF and ester-PAF) was significantly greater than with those containing the corresponding lyso compounds (lyso-PAF and lyso-PC) by ANOVA (see text).

TABLE I

EFFECT OF PAF ON MEMBRANE RIGIDIFICATION DURING LATERAL PHASE SEPARATION [a]

Comparison of two methods of calcium addition [b]

		Calcium concentration (mM)				
		0	0.3	0.7	3	10
DPPE/PS 2:1						
+PAF 0 mol%	post	0.179±0.004	0.197±0.004	0.209±0.001	0.246±0.004	0.266±0.005
	prior	0.180±0.006	0.210±0.005	0.238±0.002	0.264±0.006	0.283±0.005
+PAF 3 mol%	post	0.177±0.004	0.188±0.003	0.197±0.003	0.224±0.003	0.247±0.005
	prior	0.178±0.004	0.209±0.003	0.218±0.017	0.251±0.005	0.266±0.006
+PAF 10 mol%	post	0.167±0.003	0.183±0.004	0.188±0.003	0.206±0.005	0.221±0.002
	prior	0.166±0.002	0.191±0.005	0.203±0.003	0.217±0.006	0.250±0.010

[a] Polarization data for dansyl-PS.

[b] Paired data±S.E. for each PAF concentration are shown. First of each pair labeled 'post' is obtained by addition of calcium after formation of MLV. Second of pair labeled 'prior' is obtained by addition of calcium before formation of MLV by sonication. N is four or five.

PAF ≥ ester-PAF ≥ lyso-PAF ≥ lyso-PC. Analysis of variance of the effect of each of the acetylated lipids revealed significant differences from the effect of the corresponding lysophospholipids (PAF vs. lyso-PAF $F(1,6) = 5.27$. $P = 0.05$; ester-PAF vs. lyso-PC $F(1,6) = 18.61$, $P < 0.005$). Thus, while both the 2-position acetyl group and the 1-position ether linkage contribute to this effect, the acetyl group appears to exert the greater influence. The effect of ester-PAF, however, may in part be explained by a significant reduction in baseline rigidity seen with ester-PAF but not with the other compounds (Table II). The effect of increased chain length in the 2-position was examined utilizing HAPC, a precursor of PAF in neutrophils [28]. Calcium-induced membrane rigidification in vesicles containing HAPC was found to be no different than that seen in vesicles containing equimolar PAF (data not shown). Thus it appears that surface effects of calcium binding as sensed by the dansyl probes are dependent on the 1-ether linkage and the 2-position ester linkage with the α-methylene segment. (See Discussion).

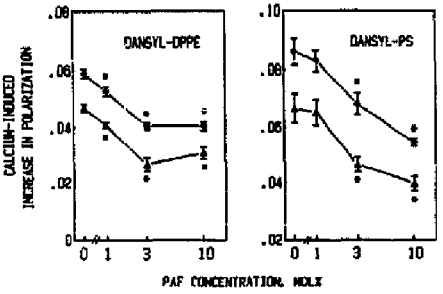

Fig. 2. PAF reduces membrane rigidification of DPPE/PS (2:1) vesicles (as measured by a smaller change in polarization from baseline) that accompanies calcium addition: 3 mM (▲) and 10 mM (◆). Left panel data are for vesicles probed with dansyl-DPPE; right panel for vesicles probed with dansyl-PS during calcium-induced lateral phase separation. Points represent mean±S.E.; N = four or five preparations. (*) Notes values that are significantly different from baseline changes in polarization in the absence of PAF ($P < 0.05$ by t-test for unpaired samples).

To assure that the reduction of rigidification was not due to altered penetration of calcium within the MLV structures in the presence of PAF, similar experiments using the dansyl probes were performed with calcium added at each concentration prior to lipid sonication. These experiments provide qualitatively identical results (Table I).

TMA-DPH and DPH studies. To determine the depth of rigidification within the lipid bilayer during binding of calcium and lateral phase separation and the subsequent influence of PAF, the probes TMA-DPH and DPH were used. The fluorescent hydrophobic tail of the probe TMA-DPA localizes near the glycerol region anchored by its

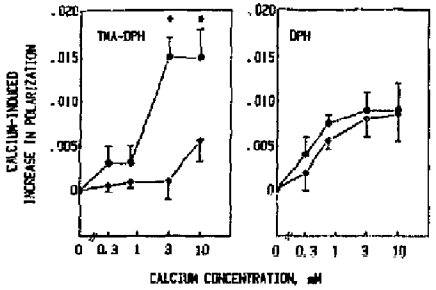

Fig. 3. (Left panel) In the glycerol region (probed with TMA-DPH), the presence of PAF (10 mol%) (◆) reduces membrane rigidification in vesicles of DPPE/PS (2:1) (●) during calcium addition. (Right panel) Within the hydrophobic core region (probed with DPH), PAF (10 mol%) has no effect on membrane rigidification during calcium-induced lateral phase separation. Points represent mean ± S.E.; $N = 6$ preparations. (*) Notes values that are significantly different in the absence of PAF ($P < 0.05$ by t-test for unpaired samples).

polar head group and senses a significantly more rigid environment with the addition of calcium (Fig. 3, left panel). The magnitude of this effect of calcium was not as marked as that seen with the dansyl probes but was significantly reduced in the presence of PAF. Alternatively, PAF had little influence on the hydrophobic core of the bilayer probed with DPH in identical calcium addition experiments (Fig. 3, right panel). Of note, baseline increases in fluidity attributable to PAF (prior to addition of calcium) were demonstrated for both probes (Table II) and were similar to those seen with vesicles of dipalmitoylphosphatidylcholine in the presence of PAF [12].

NBD-PE studies. Because of the profound surface effects of PAF during calcium-lipid binding, we sought evidence for alteration or inhibition of calcium-induced lateral phase separations using the self-quenching probe NBD-PE. Phase separation increases the local concentration of this probe resulting in self-quenching and a decrease in fluorescence intensity [22]. This decrease in fluorescence is calcium-dependent and clearly evident at 3 mM (Fig. 4, hatched bars). To the extent that a phospholipid inhibits calcium-induced lateral phase separation, self-quenching of the probe is reduced (hence the signal is preserved). Addition of PAF (3 or 10 mol%) to lipid vesicles

TABLE II

EFFECT OF PAF AND RELATED COMPOUNDS ON BASELINE FLUORESCENCE POLARIZATION OF THE VARIOUS PROBES [a]

Lipid [b]	Dansyl-DPPE	TMA-DPH	DPH
DPPE/PS (2:1)	0.152 ± 0.001	0.348 ± 0.003	0.371 ± 0.010
+ PAF	0.149 ± 0.004	0.338 ± 0.002 [c]	0.346 ± 0.004 [c]
+ ester-PAF	0.133 ± 0.003 [c]	–	–
+ lyso-PAF	0.151 ± 0.002	–	–
+ lyso-PC	0.144 ± 0.003	–	–

[a] Baseline values (prior to the addition of calcium) expressed as mean ± S.E. $N = 4$–6 preparations each.

[b] The concentration of PAF, ester-PAF, lyso-PAF, and lyso-PC is 10 mol%.

[c] Significantly different from DPPE/PS, $P < 0.01$ by t-test for unpaired samples.

of PS appears to reduce lateral separation in a concentration-dependent manner but, notably, is no more effective on an equimolar basis in inhibiting lateral phase separation than other related lipids. Each molecular species (at 10 mol% concentration in data shown) appears to be equally effective as a 'spacer' molecule in inhibiting self-quenching of the probe (Fig. 4). These studies would suggest that inhibition of lateral separation

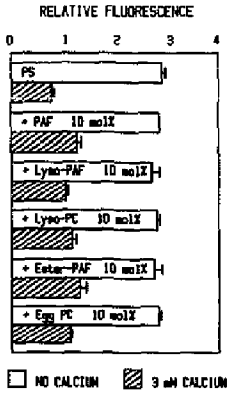

Fig. 4. Lateral phase separation monitored by fluorescence of NBD-PE. A loss of fluorescence occurs with the addition of 3 mM calcium (hatched bars) to vesicles of PS with or without the related phosphatidylcholine compounds (present at 10 mol% concentration). Quenching is lessened similarly by addition of any of the phosphatidylcholine compounds.

does not adequately explain the surface effects of PAF during calcium binding sensed by the dansyl probes and TMA-DPH. More likely, PAF influences polar head packing, maintaining surface fluidity during calcium-induced lateral phase separation.

Effects of PAF and related lipids on calcium-induced fusion in PS vesicles

R18 studies. Work by several groups suggests that the inhibition of fusion by phosphatidylcholines incorporated in anionic vesicles is due to the increased polar head hydration of the bulky choline groups [29,30]. While the affinity for binding of calcium to phosphatidylcholine itself is quite low [31], it is the increase in hydration about the phosphatidylcholine head group that is thought to prevent the dehydrated trans-PS-calcium binding between apposing vesicles required for fusion [32]. Recent studies suggesting that increased water penetration occurs in the interfacial area of diether phospholipids [33] prompted us to ask whether potential differences in hydration may explain our findings for the different analogs in the dansyl studies and could influence vesicle fusion. In these studies the effects on vesicle fusion of PAF, lyso-PC (lyso-PC being the least potent of the phosphatidylcholine compounds in reducing the calcium-induced rigidification sensed by the dansyl probes) and DPPC were compared. Calcium-induced fusion of SUV of PS was monitored by the self-quenching probe, R18, by the method of Hoekstra et al. [24]. As shown in Fig. 5, the addition of calcium (2 mM) to a mixture of labeled and unlabeled PS vesicles results in a rapid increase in fluorescence as the vesicles fuse and the probe is redistributed over an increasing bilayer surface area. The addition of PAF at 3 mol% or greater (10 mol% is shown) to the vesicles results in a decrease in fusion (both rate and extent), as would be expected with the addition of any phosphatidylcholine to the vesicles. Notably, the addition of lyso-PC or DPPC in equimolar amounts does not slow and limit fusion to the same extent as PAF. Fusion induced with 4 mM calcium demonstrated the same rank ordering (data not shown). Though these differences are subtle, they would suggest that greater hydration in the interfacial region of the monoether lipid

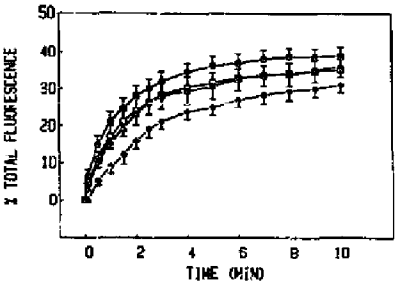

Fig. 5. SUV fusion monitored by fluorescence of R18. Addition of 2 mM calcium at time zero to SUV of PS (●) results in fusion (measured as an increase in % total fluorescence, see text). The addition to SUV of 10 mol% PAF (◆) results in greater inhibition of fusion than seen with either 10 mol% DPPC (◇) or lyso-PC (□). Points represent mean ± S.E.; N = four preparations.

PAF may explain both the greater limitation of fusion and reduction of the rigidification imposed by surface-bound calcium.

Discussion

Calcium-lipid interactions may play an important role not only in remodeling of intracellular membranes during processes of endocytosis and exocytosis [17,18], but also in the function of membrane-bound proteins [34] and organizational structure of cell membranes [13–15]. The presence of lateral phase separations have been documented in biologic membranes and may result in important structure-function relationships [19,35]. In this study we have examined the effects of PAF, a unique ether lipid known to accumulate in the membranes of activated neutrophils, on calcium-lipid binding and lateral phase separations. PAF was found to markedly reduce the membrane rigidification accompanying calcium-induced lateral phase separations in vesicles made of DPPE/PS. This effect involved the glycerol region of the bilayer as demonstrated by decreased fluorescence polarization of dansyl-DPPE, dansyl-PS and TMA-DPH, all probes localizing to this area (Fig. 1–3). The use of lipid analogs allowed the evaluation of the 1-position ether linkage and the 2-position acetyl group, the unique features of PAF, in contributing to this effect. The

rank ordering of PAF ≥ ester-PAF ≥ lyso-PAF ≥ lyso-PC demonstrates the greater effect of the acetyl compounds over that demonstrated by the lyso analogs and the potency of the ether linkage over that of the ester for each analog pair. Recent data utilizing NMR and Raman spectroscopy suggest that in the micellar phase greater rotational mobility exists among the ether-linked phospholipids when compared to the ester-linked analogs in pure aqueous dispersion [36]. Although rotational freedom of PAF has not been examined during calcium-induced lateral phase separation in mixed bilayers, greater mobility in the glycerol region is consistent with the reduction of calcium-induced rigidification observed in the present study. The finding that phospholipid vesicles containing equimolar concentrations of either HAPC or PAF behaved identically during calcium addition suggests that increasing chain length in the 2-position has no effect on surface binding of calcium. The data are consistent with the observation that for phospholipids in bilayers the 2-position ester linkage and α-methylene group extend from the glycerol backbone parallel to the bilayer surface [36]. Thus, the interfacial region configuration appears to be little affected by the remainder of the 2-position acyl chain of HAPC which bends to parallel to the *sn*-1 chain in the hydrophobic region of the bilayer. HAPC, a precursor of PAF in neutrophils is cleaved by phospholipase A_2 to release arachidonate and lyso-PAF which is then acetylated to form PAF [28]. The conversion of HAPC to lyso-PAF in a membrane bilayer may result in marked rigidification due to calcium-lipid binding that is then offset by acetylation of lyso-PAF to PAF. Hence, synthesis and retention of PAF may serve a homeostatic role in the neutrophil.

Lateral phase separation was also studied using NBD-PE. Each of the lipid analogs studied exhibited the same degree of inhibition of probe self-quenching (Fig. 4). Inhibition was dependent on the concentration of added lipid and may have resulted from simple dilution of the probe as it is concentrated within microdomains (segregated from PS) in the bilayer. Notably, at 10 mol% of each analog tested, the phosphatidylcholine to probe molar concentration was 2 : 1. These findings suggest that PAF may alter polar head packing and surface fluidity during calcium-lipid binding rather than inhibit lateral-phase separation.

The low affinity of phosphatidylcholines for binding calcium has been demonstrated in recent studies [31] and, thus, we would expect PAF and the other related lipid analogs examined in this study to inhibit fusion of anionic vesicles as has been demonstrated for other phosphatidylcholines [29,30]. Though subtle, the greater inhibition of fusion seen with PAF compared to lyso-PC or DPPC (Fig. 5) may be explained by increased polar head hydration as has been demonstrated in diether lipids [31]. This finding would suggest that increased polar head hydration may be one mechanism by which PAF exerts its marked effect on the vesicle surface during calcium binding and lateral-phase separation.

The synthesis and accumulation of PAF occurs during activation of human neutrophils during which endocytosis, exocytosis of secretory granules and extensive remodeling of intracellular membranes occur. The finding that PAF limited calcium-induced fusion of anionic vesicles would at first appear contrary to the notion that PAF may play a role in these membrane events. We do not feel, however, that inhibition of fusion by PAF in this simple model utilizing anionic vesicles necessarily precludes a facilitory role in fusion of physiologic membranes, where other factors such as calcium-binding proteins and membrane fluidity are likely of critical importance [4,5,17,18].

Additionally, PAF has been shown to fluidize the membrane hydrophobic core and to lower the phase transition in vesicles of DPPC as demonstrated by fluorescence polarization of DPH [12]. In our previous study, the use of lipid analogs demonstrated the importance of both the acetyl group and the ether linkage in contributing to this effect. Taken together, these findings raise the possibility that PAF may exert some of its bioactions by alteration of membrane physical properties and thereby affect membrane-bound proteins or transmembrane ion channels as has been demonstrated for other lipid perturbants in cell membranes [34,37]. We have examined the effects of PAF on MLV composed of DPPE and brain PS. While the concentrations of DPPE and PS and acyl group composition (in the case of DPPE) are not found in natural membranes, these phos-

pholipid types are enriched in cell membrane inner leaflets where PAF may exert its effects. Furthermore, in addition to alteration of polar head packing and membrane fluidization, it is possible that PAF may promote formation of nonbilayer structures [36]. Indeed, 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 of phosphatidylethanolamines [38]. Future studies of biological membranes will be required for further definition of the physiologic significance of these membrane effects of PAF.

Acknowledgments

Supported in part by funds from the Veterans Administration and U.S. Public Health Service, grant AA 06399, AM 34914 and NIH grant HL 34303. We thank Sue McQuilkin, Anne McClard and Larry Zacarro for excellent technical assistance, and Helga Cole, Julie Gunnerson and Judy Franconi for typing the manuscript.

References

1 Ludwig, J.C., McManus, L.M., Clark, P.O., Hanahan, D.J. and Pinckard, R.N. (1984) Arch. Biochem. Biophys. 232, 102–110.
2 Lynch, J.M. and Henson, P.M. (1986) J. Immunol. 137, 2653–2661.
3 Riches, D.W.H., Young, S.K., Seecombe, J.F., Lynch, J.M. and Henson, P.M. (1985) Fed. Proc. 44, 737.
4 Silvius, J.R. and Gagne, J. (1984) Biochemistry 23, 3232–3240.
5 Wilschut, J., Düzgünes, N., Hoekstra, D. and Papahadjopoulos, D. (1985) Biochemistry 24, 8–14.
6 Düzgünes, N., Paiement, J., Freeman, K.B., Lopez, N.G., Wilschut, J. and Papahadjopoulos, D. (1984) Biochemistry 23, 3486–3494.
7 Valentino, M., Governa, M., Fiorini, R. and Curatola, G. (1986) Biochem. Biophys. Res. Commun. 141, 1151–1156.
8 Bultmann, P.D., Allmendinger, P., Raus, R.U., Melzner, I., Haferkamp, O., Eggers, H. and Gruler, H. (1984) Am. J. Pathol. 116, 46–55.
9 Berlin, R.D. and Fera, J.P. (1977) Proc. Natl. Acad. Sci. USA 74, 1072–1076.
10 Steiner, M. and Luscher, E.F. (1984) Biochemistry 23, 247–252.
11 Fink, K.L. and Gross, R.W. (1984) Circ. Res. 55, 585–594.
12 Bratton, D.L., Harris, R.A., Clay, K.L. and Henson, P.M. (1988) Biochim. Biophys. Acta 941, 76–82.
13 Bendu, F., Marche, P., Viret, J., Daveloose, D., Leterrier, F., Lévy-Toledano, S. and Caen, J.P. (1985) Nouv. Rev. Fr. Hematol. 27, 293–297.
14 Sauerheber, R.D., Zimmermann, T.S., Esgate, J.A., Vanderlaan, W.P. and Gordon, L.M. (1980) J. Membr. Biol. 52, 201–219.
15 Langer, G.A. and Nudd, L.M. (1983) Circ. Res. 53, 482–490.
16 Hoekstra, D., Wilschut, J. and Scherphof, G. (1985) Eur. J. Biochem. 146, 131–140.
17 Hong, K., Düzgünes, N. and Papahadjopoulos, D. (1982) Biophys. J. 37, 297–305.
18 Knight, D.E. and Baker, P.F. (1982) J. Membr. Biol. 68, 107–140.
19 Ashley, R.H. and Brammer, M.J. (1984) Biochim. Biophys. Acta 769, 363–369.
20 Smaal, E.B., Mandersloot, J.G., Demel, R.A., De Kruijff, B. and De Gier, J. (1987) Biochim. Biophys. Acta 897, 180–190.
21 Papahadjopoulos, D., Vail, W.J., Newton, C., Nir, S., Jacobson, K., Poste, G. and Lazo, R. (1977) Biochim. Biophys. Acta 465, 579–598.
22 Hoekstra, D. (1982) Biochemistry 21, 2833–2840.
23 Tillack, T.W., Wong, M., Allietta, M. and Thompson, T.E. (1982) Biochim. Biophys. Acta 691, 261–273.
24 Hoekstra, D., De Boer, T., Klappe, K. and Wilschut, J. (1984) Biochemistry 23, 5675–5681.
25 Waggoner, A.S. and Stryer, L. (1970) Proc. Natl. Acad. Sci. USA 67, 579–589.
26 Harris, W.E. (1977) Chem. Phys. Lipids 19, 243–254.
27 Harris, W.E. and Stahl, W.L. (1976) Mol. Pharmacol. 12, 115–126.
28 Chilton, F.H., Ellis, J.M., Olson, S.C. and Wykle, R.L. (1984) J. Biol. Chem. 259, 12014–12019.
29 Sundler, R., Düzgünes, N. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 649, 751–758.
30 Düzgünes, N., Wilschut, J., Fraley, R. and Papahadjopoulos, D. (1981) Biochim. Biophys. Acta 642, 182–195.
31 Altenbach, C. and Seelig, J. (1984) Biochemistry 23, 3913–3920.
32 Ekerdt, R. and Papahadjopoulos, D. (1982) Proc. Natl. Acad. Sci. USA 79, 2273–2277.
33 Massey, J.B., She, H.S. and Pownall, H.J. (1985) Biochemistry 24, 6973–6978.
34 Sandermann, H. (1978) Biochim. Biophys. Acta 515, 209–237.
35 Viret, J. and Leterrier, F. (1976) Biochim. Biophys. Acta 436, 811–824.
36 Huang, C. and Mason, J.T. (1986) Biochim. Biophys. Acta 864, 423–470.
37 Harris, R.A. and Bruno, P. (1985) J. Neurochem. 44, 1274–1281.
38 Boggs, J.M., Stamp, D., Hughes, D.W. and Deber, C.M. (1981) Biochemistry 20, 5728–5735.