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A model for the extracellular release of PAF: the influence of plasma membrane phospholipid asymmetry

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Recent studies suggesting that cellular activation leads to enhanced transbilayer movement of phospholipids and loss of plasma membrane phospholipid asymmetry lead us to hypothesize that such events may govern the release of PAF, a potent, but variably released, lipid mediator synthesized by numerous inflammatory cells. To model these membrane events, we studied the transbilayer movement of PAF across the human erythrocyte and erythrocyte ghost plasma membrane, membranes with documented phospholipid asymmetry which can be deliberately manipulated. Utilizing albumin to extract outer leaflet PAF, transbilayer movement of PAF was shown to be significantly enhanced in erythrocytes and ghosts altered to lose membrane asymmetry when compared to movement in those with native membrane asymmetry. Verification of membrane changes was demonstrated using merocyanine 540 (MC540), a dye which preferentially stains loosely packed or hydrophobic membranes, and acceleration of the modified Russell's viper venom clotting assay by externalized anionic phospholipids. Utilizing the erythrocyte ghost loaded with PAF in either the outer or the inner leaflet, enhanced transbilayer movement to the opposite leaflet was seen to accompany loss of membrane asymmetry. Studies utilizing ghosts loaded with albumin intracellularly demonstrated that 'acceptor' molecules binding PAF further influence the disposition of PAF across the plasma membrane. Taken together, these findings suggest that the net release of PAF from activated inflammatory cells will depend on localization of PAF to the plasma membrane, transbilayer movement, which is facilitated by alteration of membrane phospholipid asymmetry, and removal from the membrane by extracellular and intracellular 'acceptor' molecules.

Introduction

Little is known of the mechanism(s) of secretion of platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), a unique ether-linked phospholipid and potent inflammatory mediator. Although PAF is clearly released from a variety of synthesizing cells [1] and such liberated material may have important stimulatory mediator functions, the molecule is also retained to a significant extent within the cell of origin [1,3–5]. Previous work by Ludwig et al. has shown that PAF is solubilized by albumin [6] and that its release is dependent on the presence of albumin in the extracellular media [5]. Additionally, release of PAF appears to be affected by cell type [7], species [3], agonist [3,6,7], cell density [8–11], 'dynamic removal' of medium [11], pH [9], calcium concentration [4], state of adherence [7,12] and 'phase of response' [13]. Thus, it appears that release of PAF is under as yet undefined cellular regulatory processes.

While the precise route(s) of release of PAF from synthesizing cells has not been determined, it seems likely that transbilayer movement (flip-flop) of PAF across the plasma membrane may be an integral step in passage from an intracellular site of synthesis [14], to its release extracellularly. This assumption leads us to ask whether the state of the plasma membrane of a PAF-synthesizing cell may in fact govern release of PAF. Emerging evidence from several laboratories suggests that while the plasma membrane of circulating cells may exhibit asymmetry of the various phospholipid classes across the resting plasma membrane [15,16],

Abbreviations: PAF, platelet activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; MC540, merocyanine 540; RBC, red blood cell (erythrocyte); PC, choline-linked phosphoglycerides; PE, ethanolamine-linked phosphoglycerides; PS, serine-linked phosphoglycerides; PBS, phosphate-buffered saline; RVV, Russell's viper venom.

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profound physical changes that lead to enhanced flip-flop and loss of endogenous phospholipid asymmetry occur during the process of cellular activation [17-9]. Furthermore, such changes in the plasma membrane of activated platelets [20], and manipulated red blood cells (RBCs) [21,22] can be correlated with staining by the fluorescent dye MC540, a dye which preferentially stains loosely packed membranes or membranes of enhanced hydrophobicity [23]. Similarly, several other inflammatory cells (lymphocytes, neutrophils, monocytes) stain with MC540 following activation, suggesting that such changes of the plasma membrane occur more widely in inflammatory cells [24].

We hypothesize that under some circumstances cellular activation results in enhanced transbilayer movement (flip-flop) of plasma membrane phospholipids, and that this event, in an inflammatory cell synthesizing PAF, can result in the transbilayer movement of PAF to the plasma membrane outer leaflet where it can be released extracellularly. Thus, we suggest that the ultimate disposition of PAF (intracellular/extracellular) and its net release will depend on the dynamics of (1) PAF localization to the plasma membrane inner leaflet, (2) transbilayer movement (flip-flop) and (3) removal to intracellular and extracellular 'acceptors' (see Discussion). While serum albumin appears to affect release of PAF [5] and likely serves as a candidate for such an extracellular 'acceptor', other proteins, intracellular [25] and extracellular [26], appear to bind PAF and may be involved in removal of PAF from the plasma membrane. As a consequence of this hypothesis, we would predict that under such circumstances of enhanced transbilayer movement (flip-flop) resulting in loss of endogenous membrane asymmetry, that enhancement of transbilayer movement of exogenously added PAF from plasma membrane outer leaflet to inner leaflet would be demonstrated (i.e., increased uptake of PAF should occur).

To model these events as they relate to plasma membrane asymmetry, transbilayer movement of PAF into and out of the human erythrocyte (RBC) and RBC ghost was studied. This cell was chosen because it lacks internal membranes, is generally not endocytic and has a well defined plasma membrane asymmetry which can be manipulated. We present data to support the hypothesis that loss of plasma membrane asymmetry is associated with enhanced transbilayer movement either into, or out of the cell, of PAF added exogenously to either side of the plasma membrane. Our data suggest that net release of PAF from an inflammatory cell may be in part governed by the loss of plasma membrane asymmetry that likely accompanies cellular activation.

Materials and Methods

Materials. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) was obtained from Calbiochem Corporation (San Diego, CA) and radiolabelled, 1-*O*-[*hexadecyl*-1′,2′(n)-^{3}H]PAF, specific activity 56.7 Ci/mmol, from New England Nuclear (Boston, MA). 1-[1-^{14}C]Palmitoyl-2-hydroxyl-*sn*-glycero-3-phosphocholine, specific activity 59 mCi/mmol, was obtained from Amersham (Arlington Heights, IL) for synthesis of radiolabelled 1-[1-^{14}C]palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine, the ester analogue of PAF by the method described previously [27]. Egg phosphatidylcholine was obtained from Avanti Polar Lipids (Pelham, AL). Sodium tetrathionate, merocyanine 540 (MC540) and gramicidin from *Bacillus brevis* (a mixture of gramicidin A, B, C and D) were obtained from Fluka Chemical (Ronkonkoma, NY). Diamide (diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)) 1-*O*-hexadecyl-2-hydroxyl-*sn*-glycero-3-phosphocholine, Russell's viper venom and rabbit brain cephalin were obtained from Sigma (St. Louis, MO). Delipidated (fatty acid and phospholipid-free) human serum albumin was obtained from Miles Diagnostics (Kankakee, IL), lipopolysaccharide-free human serum albumin obtained from Biocell Laboratories (Carson, CA), bovine serum albumin from Fisher Scientific (Pittsburgh, PA) and [^{14}C]methylated bovine serum albumin, specific activity 50 μCi/mg, from Amersham (Arlington Heights, IL). Silica Gel G thin-layer chromatography plates were obtained from Analtech (Newark, DE) and Trypan blue stain from Gibco Laboratories (Grand Island, NY).

Buffers. Phosphate-buffered saline (PBS) was made by the method of Williamson et al. [22] with 137 mM NaCl, 2.7 mM KCl, 10.6 mM Na_2HPO_4 8.5 mM KH_2PO_4 (pH 7.4) with additions of 1 mM $MgCl_2$, 100 μM EGTA and 1 mM $CaCl_2$ where designated. Cell incubations with diamide were done by the method of Bergmann et al. [28] in buffer containing 44 mM sucrose, 90 mM KCl, 45 mM NaCl, 12.5 mM Na_2HPO_4/NaH_2PO_4, (pH 8.0) and incubations with tetrathionate in the same buffer with the substitution of 10 mM Na_2HPO_4/NaH_2PO_4 by the method of Haest and Deuticke [29]. Cell incubations with gramicidin were carried out using a buffer modified from Classen et al. [30] containing 150 mM NaCl, 5 mM Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 100 mM sucrose.

Erythrocyte/ghost preparation. Whole blood was obtained by venipuncture from healthy donors and anticoagulated with sodium citrate. Erythrocytes (RBCs) were separated from other blood cells and serum by first centrifuging the cells at $250 \times g$ for 20 min and aspirating and discarding the supernatant and buffy coat. Two additional washes were performed by centrifuging the cells at $1000 \times g$ for 10 min and aspirating and discarding the supernatant and top cell layer each time. RBC ghosts both asymmetric and 'symmetric' were prepared by the method of Williamson et al. [22]. Cells were hypotonically lysed at 4°C at a concentra-

tion of 1:5 cells to 1/50 dilution of PBS with magnesium chloride (1 mM) and EGTA (100 μM) added for asymmetric ghosts, and in the presence of calcium chloride (1 mM), for 'symmetric' ghosts. After incubating for 60 min, isotonicity was restored with 5-fold concentrated PBS (with 1 mM magnesium/100 μM EGTA) and ghosts were resealed by warming to 37°C for 1 h. To induce loss of membrane asymmetry in ghosts after resealing, asymmetric ghosts were prepared as above, resealed at 37°C for 1 h and then calcium chloride (1 mM) was added to the suspending medium.

To load ghosts internally with albumin, ghosts were prepared as above but prior to resealing, were suspended at 4°C in media containing 2% delipidated HSA for 60 min. Extracellular albumin was then removed from the surrounding media by repeated washing in albumin-free PBS. In pilot studies utilizing radiolabelled BSA, 0.1 μCi added to 2% HSA presented to open ghosts resulted in approx. 0.1 mg of HSA sealed internally in 10^8 ghosts upon warming to 37°C for 1 h.

To load PAF or the related ester analogue, 1-palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine, in the inner leaflet of unsealed ghosts, asymmetric unsealed ghosts were incubated at 4°C with lipid at 10^{-6} M in 1/50 dilution of PBS containing 0.025% delipidated HSA for 60 min, after which ghosts were resealed in 5-fold concentrated PBS at 37°C for 1 h and washed in fresh PBS with 1% HSA to remove adherent and outer leaflet lipid.

Erythrocyte integrity for all assays was measured by the spectrophotometric assessment of hemoglobin release. Hemolysis in all assays was less than 1% except at the highest gramicidin concentration (10 μM for 60 min) where hemolysis averaged 2.3%. Sealed ghosts were judged for integrity by their exclusion of Trypan blue with the number of stained cells always less than 0.1% in all preparations.

Incubation of RBCs/ghosts with PAF or ester analogue to measure transbilayer movement from the outer to inner leaflet. Briefly, the method used was that employed by Schneider et al. [31] with minor modifications. Radiolabelled (1–5%) and nonlabelled PAF or radiolabelled 1-palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine (the ester analogue) were dried down in a glass tube under a stream of nitrogen and then resuspended to a final concentration of 10^{-6} M in PBS containing 0.025% delipidated HSA by vigorous vortexing of the tube over 30 min. RBCs or ghosts were prepared as above and incubated at 37°C at $4 \cdot 10^8$ cells or ghosts/ml in a shaking water bath. At specified times, duplicate samples of 10^8 ghosts/cells were removed to 5 ml polypropylene tubes and either pelleted at 4°C to determine total associated lipid (inner and outer leaflets) or washed in HSA to determine the concentration of lipid having undergone transbilayer movement to the inner leaflet (see below). The above method differs from that of Schneider et al. [31] in that lipid is 'presented' to ghosts or RBCs on albumin to avoid fluidizing effects on the plasma membrane of ethanol addition [32] and in an effort to simulate a likely physiological event. Regardless of this minor modification, our data for the transbilayer movement of PAF in RBCs and asymmetric ghosts closely reproduce previously published values for transbilayer movement of PAF in native RBCs (see Results) [31].

Albumin extraction of outer leaflet PAF. The use of albumin to remove outer leaflet lyso-PCs has been extensively utilized in the literature to measure the rates of transbilayer movement across the red cell membrane [28,30,33]. As previously published by Schneider et al. [31], the affinity of HSA for PAF has also been similarly employed to extract outer leaflet PAF. Outer leaflet PAF was extracted from red cells by washing three times in 2% albumin (4 ml, 4°C), while ghosts were twice washed in 1% albumin (4 ml, 4°C) as washing in more concentrated albumin resulted in disruption of fragile ghosts. The cell/ghost sample was then pelleted and the remaining, nonextractable radiolabelled lipid quantified. As in the studies of Schneider et al. [31], we found that a small amount of immediately associated PAF could not be washed from cells (< 0.3 pmol/10^8 RBCs or < 0.5 pmol/10^8 ghosts, and accepted this as a small, consistent overestimate of inner leaflet material. It should be noted that the ester analogue of PAF was slightly less readily removed by HSA than PAF at the zero time point and thus the inner leaflet concentrations were consistently overestimated by 0.8–2.0 pmol/10^8 RBCs or 3.0–5.0 pmol/10^8 ghosts. Thus, for any given time point it may be argued that albumin washing does not entirely remove outer leaflet PAF/ester analogue. Accepting this caveat, however, the transbilayer movement of radiolabelled PAF to a 'nonextractable pool' considered to be largely localized to the inner leaflet, can be studied [31].

Measuring the transbilayer movement of PAF from inner to outer leaflet. For these studies asymmetric ghosts were prepared as described above, loaded with PAF, sealed and washed free of outer leaflet PAF in 1% HSA. This procedure was found to remove 54.7 ± 2.6% (mean ± S.E.) of the total loaded PAF. Ghost samples so prepared were then sampled (at time zero) to determine the amount of PAF located in the inner leaflet and this was designated as 100%. The ghosts were then placed in media (PBS) alone or in media containing magnesium (1 mM)/EGTA (100 μM) or calcium (1 mM) at 37°C, and samples removed at specified times for albumin washing to determine movement of PAF to the outer leaflet (into the albumin extractable pool).

Liquid scintillation. RBC or ghost pellets were lysed in 250 μl of doubly distilled H_2O, transferred to scintillation vials containing 100 μl scintillation cocktail and bleached to prevent quenching by hemoglobin by the

method of Mohandas et al. [33] with 250 μl 30% H_2O_2 overnight. Scintillation cocktail was added and samples counted on a Beckman 3801 LS counter.

Measurement of phospholipid acylation. Samples were extracted using the method of Rose and Oklander [34], spotted on thin-layer plates along with known standards for diacyl-PC and lyso-PAF, and developed in a solvent system of chloroform/methanol/acetic acid/water (50:25:8:4) by the method of Chilton [35]. Lipids were visualized after exposure to iodine vapor and areas cut, scraped, eluted and counted by liquid scintillation or plates were scanned by a Berthold Automatic TLC-Linear Analyzer with automated quantification of area under the curve.

MC540 staining and flow cytometry. Stock solution of MC540 was prepared the day before use at a concentration of 1 mg/ml in 50% ethanol and stored in foil covered tubes at 4°C. Cells and ghosts were prepared as above and washed twice in PBS. Cells or ghosts (10^7) were transferred to foil-covered 5 ml tubes containing PBS with 0.15% BSA for a final volume of 990 μl. Stock MC540 (10 μl), was added to each sample and samples were incubated, covered, for 5 min at room temperature. Samples were washed in 4 ml of PBS with 0.15% BSA twice, resuspended in PBS and examined by flow cytometry. Cells and ghosts were filtered through a 58 μm nylon mesh and analyzed on an Coulter 751 flow cytofluorograph equipped with a Coherent argon excitation laser. The laser was tuned to 514 nm and fluorescence was monitored through a 570 nm long pass filter. Forward angle light scatter and side (90°) scatter were measured for each sample. Data acquisition and analysis was performed using the Cicero-cytomation analyzer system.

Modified Russell's viper venom (RVV) assay. Lyophilized RVV was resuspended in saline at a concentration of 1 mg/ml and stored at −20°C in aliquots for daily use. Stock solutions of RVV were further diluted to 1:10000 for use in the assay. Platelet-poor plasma was prepared daily from citrated whole blood centrifuged at 250 × *g* for 30 min at 4°C. Plasma was aspirated, carefully layered on an RBC cushion (1 ml washed, packed cells) and centrifuged at 1000 × *g* for 30 min at 4°C. Plasma was removed (leaving undisturbed and discarding the 5 ml above the RBC cushion) and placed on ice for use in the RVV assay. Platelet counts of the prepared platelet-poor plasma ranged from 1700 to 4200 platelets/μl. For assay of outer leaflet anionic phospholipid, an aliquot of RBCs/ghosts (75 μl) in saline was added to platelet-poor plasma (75 μl) and RVV 1:10000 (75 μl) and warmed to 37°C for 1 min. $CaCl_2$, 25 mM (75 μl) was added and the fibrometer (Becton Dickinson and Co., Lincoln Park, NJ) immediately started. The time to clot formation was recorded and all samples were run in duplicate. Total lipid phosphorus for each sample was determined from identical aliquots of RBCs/ghosts extracted by the method of Rose and Oklander [34] and analyzed by the method of Gerlach and Deuticke [36].

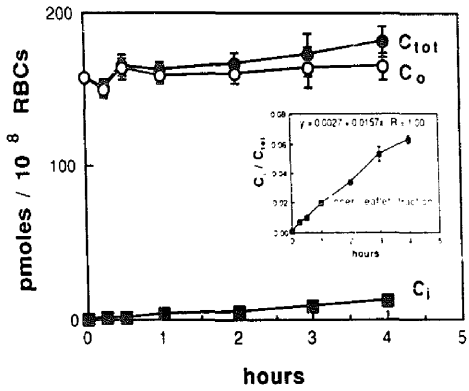

Fig. 1. Transbilayer distribution of PAF in native RBCs. Duplicate samples are used to measure the total concentration (C_{tot}) of cell-associated PAF and that remaining cell-associated after albumin extraction, the inner leaflet concentration (C_i). Outer leaflet PAF concentration (C_o) is determined by the equation $C_{tot} - C_i = C_o$. Inset, the fraction of PAF in the inner leaflet at any given time is shown by the ratio C_i/C_{tot}. Points represent mean ± S.E., $n = 3$–4.

Statistics. Either a linear or exponential function was selected to model the relationship between time and dependent variables. The model was then fit to the data set and the fitted model parameters were tested by ANOVA. Tukey's multiple comparison procedure was used to determine significance. For analysis of MC540 staining, cell/ghost populations appeared to be log normally distributed and mean channel fluorescence was compared by either paired *t*-test or two-sample *t*-test.

Results

Transbilayer movement of PAF in RBCs and RBC ghosts is enhanced by the loss of membrane asymmetry

The movement of PAF from the outer leaflet to the inner leaflet of the plasma membrane in the native human erythrocyte was examined previously by Schneider et al. [31] and found to proceed at an extremely slow rate of 1.2%/h, similar to 1.87%/h seen for the transbilayer movement of lyso-PC as studied by Mohandas et al. [33]. Using a modification of the method by Schneider et al. [31], we documented a similar rate of transbilayer movement of PAF in native erythrocytes (RBCs). As shown in Fig. 1, there was very rapid association of PAF with RBCs that was initially confined to the outer leaflet of the cell where it was almost entirely removed by albumin extraction. However, with time there was progressive internalization of PAF to the inner leaflet where it was inaccessible to

albumin extraction with transbilayer movement measured at a rate of 1.57%/h (inset, Fig. 1).

Previous investigation from various laboratories utilizing nonlytic enzymatic degradation of the plasma membrane outer leaflet has demonstrated that the RBC exhibits a defined phospholipid asymmetry with the following outer to inner leaflet composition: phosphatidylcholine (PC) 70 : 30; phosphatidylethanolamine (PE) 20 : 80; phosphatidylserine (PS) 0 : 100; sphingomyelin 100 : 0% (15,37). We hypothesized that treatment of the RBCs with agents known to result in loss of endogenous asymmetry would enhance transbilayer movement of exogenously added PAF. As demonstrated in Fig. 2, treatment with oxidative cross-linking agents, diamide (shown) or tetrathionate (not shown), which result in loss of membrane asymmetry [29,38], markedly enhanced the transbilayer movement of PAF to the inner leaflet. Enhanced transbilayer movement of an acyl-linked lyso-PC has been previously demonstrated following diamide treatment [28] and prompted us to ask whether differences in transbilayer movement could be demonstrated for the ether-linked PAF as compared to the ester-linked analogue. As demonstrated in Fig. 2, no differences were seen for the two compounds differing in the position 1 linkage. Another agent, gramicidin, a channel-forming antibiotic shown to enhance transbilayer movement of exogenously added acyl-linked lyso-PC in RBCs [30], resulted in identical enhancement of transbilayer movement of PAF (Fig. 3) and its ester analogue (not shown) in a concentration dependent manner. Treatment of RBCs with either the oxidative cross-linker (tetrathionate) or gramicidin resulted in enhanced MC540 staining (see below) demonstrating that such treatment results in enhanced hydrophobicity or looser packing of the plasma membrane phospholipids and suggesting that such treatment results in the loss of membrane asymmetry [22,23]. These studies demonstrate that creation of 'flip-sites' in the plasma membrane with a variety of agents, acting through different mechanisms, enhances the transbilayer movement of both phosphatidylcholines, PAF and its ester analogue identically, a finding consistent with others [39–41] who have demonstrated the importance of phospholipid head group as a major determinant of transbilayer 'flip' rate.

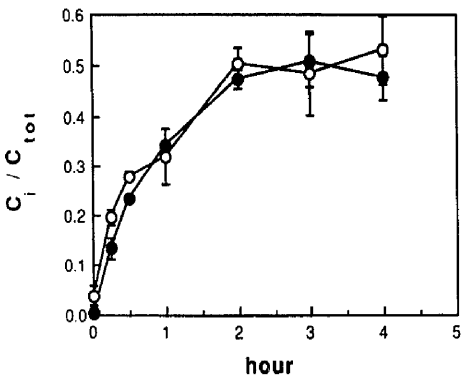

Fig. 2. Diamide treatment of RBCs results in similar enhancement of transbilayer movement of PAF (●) and its ester analogue (○) as shown by the inner leaflet fraction (C_i/C_{tot}). RBCs were incubated in 5 mM diamide for 2 h at 37°C prior to addition of phospholipid at time zero. Points represent mean ± S.E., $n = 3$.

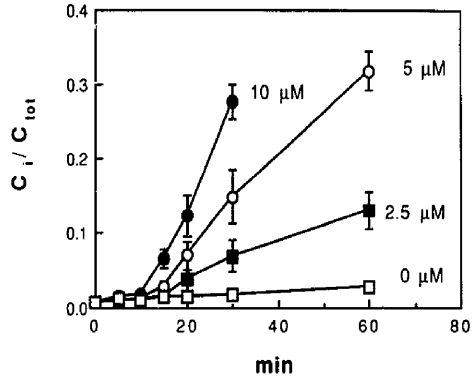

Fig. 3. Gramicidin treatment of RBCs results in concentration-dependent enhancement of PAF to the inner leaflet. RBCs were treated with the designated concentration of gramicidin for 1 h prior to the addition of PAF at time zero. Points represent mean ± S.E., $n = 3$.

Because a plasma membrane model that could be loaded with PAF with either the inner or outer leaflet was required for study of transbilayer movement in each direction (see below), we extended these observations to the RBC ghost. Previous investigation has shown that the RBC ghost can be prepared in the presence of magnesium as the only divalent cation, to retain native membrane asymmetry, or prepared in the presence of calcium to lose native asymmetry [22]. Loss of membrane phospholipid asymmetry in such ghosts prepared in the presence of calcium has been demonstrated by the appearance of PS and increased PE in the outer leaflet upon nonlytic enzymatic degradation by phospholipases, alterations which correlate with enhanced staining with MC540 [22]. Transbilayer movement of PAF in ghosts prepared by the method of Williamson et al. to maintain native asymmetry occurred at a rate of 1.66%/h (inset, Fig. 4) and as would be expected, was nearly identical to that seen for native RBC's (compare with Fig. 1). Conversely, the transbilayer movement of exogenously added PAF to the inner leaflet was markedly enhanced in 'symmetric' ghosts prepared with 1 mM calcium as shown in Fig. 5. Cytofluorographic examination of ghosts prepared in the presence or absence of calcium exhibited markedly different staining with MC540. Significant enhancement ($P < 0.005$) in MC540 staining of 'symmetric' ghosts (prepared in the presence of calcium) was demonstrated (compare Fig.

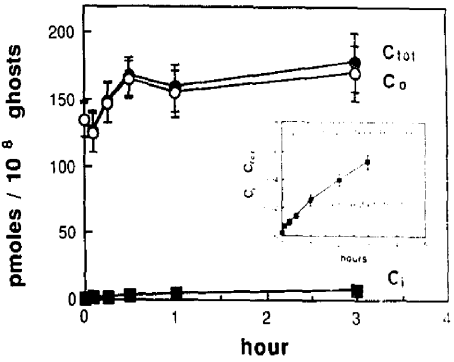

Fig. 4. Transbilayer distribution of PAF in asymmetric ghosts. Concentrations for total (C_{tot}), outer (C_0), and inner (C_i) leaflet, and inner leaflet fraction (C_i/C_{tot}) are determined as described in Materials and Methods and Fig. 1. Points represent mean ± S.E., n = 3–4.

6A to 6B) and served to verify the expected loss of membrane phospholipid asymmetry.

Further confirmation of the loss of membrane asymmetry in 'symmetric' ghosts was provided by the enhanced clotting times demonstrated in the modified Russell's viper venom (RVV) assay (Fig. 7). In this assay, the formation of prothrombinase complex, composed of factors Va and Xa, calcium ion and phospholipid, determines the rate of clot formation measured in a fibrometer. A marked acceleration of clot formation is demonstrated by RBCs exhibiting loss of membrane lipid asymmetry [19] and is thought to result from the presence of PS in the external leaflet [42].

The metabolic fate of PAF was examined in both asymmetric and 'symmetric' ghosts. Samples obtained

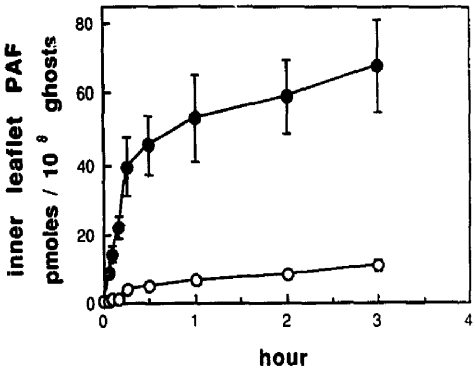

Fig. 5. The transbilayer movement of PAF in ghosts prepared so as to maintain and lose endogenous phospholipid asymmetry. Inner leaflet PAF (that remaining ghost-associated after albumin extraction) is shown for asymmetric (○) and 'symmetric' (●) ghosts. Points represent mean ± S.E., n = 8. Negative exponential curves described by $y = a(1 - e^{-b(time)})$ were fit to the data. Both the asymptote (parameter 'a') and rise of the curve (parameter 'b') were significantly different ($P < 0.0001$).

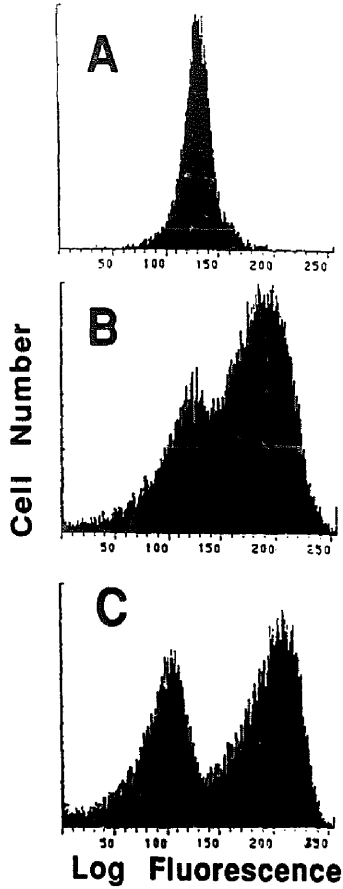

Fig. 6. Representative distribution of MC540 staining of different ghost populations. (A) Asymmetric ghosts (prepared in the presence of magnesium/EGTA) take up little stain. Mean channel fluorescence = 113 ± 25 (S.D., n = 4). (B) 'Symmetric' ghosts (prepared with calcium added) comprise a brightly staining population. Mean channel fluorescence = 174 ± 18 (S.D., n = 4). (C) Asymmetric ghosts with calcium addition after resealing are a mixed population of poorly staining ghosts (64% ± 8, mean ± S.D., n = 4) and brightly staining ghosts with mean channel fluorescence of 103 ± 29 and 183 ± 19 (S.D., n = 4), respectively. Paired t-test comparisons of asymmetric (A) and 'symmetric' (B) ghost staining was significantly different ($P < 0.002$) as were the two populations resulting from calcium addition after resealing (C), ($P < 0.01$). Two-sample t-test comparisons showed that asymmetric ghosts (A) were significantly different than the brightly staining population shown in C and 'symmetric' ghosts (B) different from the poorly staining population shown in C, ($P < 0.01$).

at 30 min, and 3 h were subjected to phospholipid extraction and thin layer chromatography. No detectable metabolic conversion to long-chain alkyl acyl-PC or hydrolysis to hexadecyl-lyso-PC was demonstrated in either asymmetric or 'symmetric' ghosts. These studies verify that the movement of PAF into a pool that is not

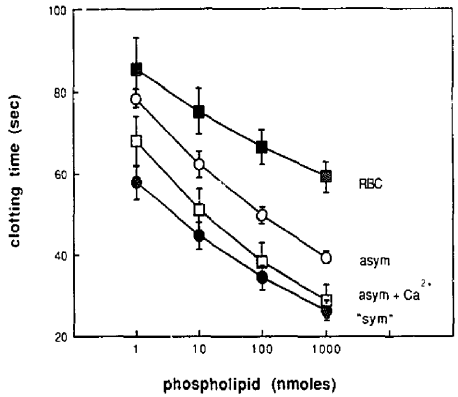

Fig. 7. Effect of different ghost populations on RVV-induced clotting time. The RVV clotting time is plotted against logarithm of phospholipid concentration. Points were determined by mean regression lines and bars represent 95% confidence intervals for each RBC/ghost population. The mean slopes for the three ghost populations were not significantly different, allowing comparison of intercepts by a repeated measure ANOVA. There was a significant difference ($P < 0.05$) in the clotting time for asymmetric ghosts (○), compared with asymmetric ghosts with calcium added after resealing (□) and 'symmetric' ghosts (●). The RVV clotting time for saline was approx. 95.2 ± 3.0 (mean ± S.E.) s. $n = 5$ experiments.

extractable by albumin is not due to metabolic conversion to long-chain alkylacyl-PC [43].

Enhanced transbilayer movement of PAF is bidirectional

Because enhanced transbilayer movement of PAF from outer to inner leaflet was demonstrated with loss of membrane asymmetry (Fig. 5), we hypothesized that enhanced transbilayer movement would function in either direction, and as such, could play a pivotal role in the movement of PAF out of the PAF-synthesizing inflammatory cell. To test this hypothesis, ghosts were required in which PAF could be loaded in the inner leaflet while maintaining native phospholipid asymmetry and then treated to lose plasma membrane asymmetry while monitoring transbilayer movement of PAF from inner to outer leaflet. To accomplish this, experiments were carried out in which calcium was added after loading the ghosts with PAF and after resealing. To this end, ghosts were prepared by hypotonic lysis as before in the presence of magnesium and EGTA to preserve native membrane asymmetry. While still at 4°C and in the open state, they were incubated with PAF so as to load both the inner and outer membrane leaflets. The ghosts were then resealed at 37°C for 30 min. Subsequent washing with 1% albumin was used to remove outer leaflet PAF (see Materials and Methods) and calcium was added at the time of assay. Confirmation that calcium addition after ghost resealing results in loss of plasma membrane asymmetry is supported by enhanced MC540 staining. As shown in Fig. 6C, ghosts prepared in this manner (calcium addition after resealing) demonstrated a brightly staining population of cells with the same fluorescence as seen in ghosts prepared in the presence of calcium prior to resealing ('symmetric' ghosts – Fig. 6B). In addition, a poorly staining population (comprised of 64 ± 80% (S.D.) of cells, $n = 4$) was demonstrated with fluorescence that corresponds quite closely to the staining of asymmetric ghosts (Fig. 6A). Similarly, calcium addition after ghost resealing likewise results in intermediate enhancement of clotting time when compared with asymmetric ghosts prepared in the absence of calcium and those prepared with calcium prior to resealing ('symmetric' ghosts), as shown in Fig. 7.

It was expected that asymmetric ghosts loaded with PAF in the inner leaflet, when subjected to treatment resulting in loss of endogenous membrane asymmetry, would demonstrate enhanced movement of the inner leaflet PAF to the outer leaflet where it would be available for albumin extraction. As shown in Fig. 8, the amount of PAF remaining associated with the ghosts (non-extractable) was significantly reduced following calcium addition ($P < 0.0001$), demonstrating that calcium added after ghost resealing can enhance transbilayer movement of PAF, in this case from the inner to outer leaflet. The addition of magnesium, demonstrated to stabilize membrane asymmetry [22], appeared to retard the outward movement of PAF loaded in the inner leaflet.

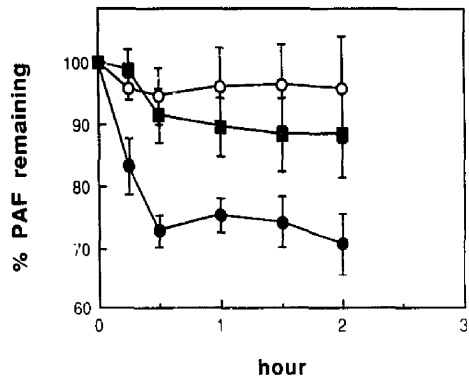

Fig. 8. The release of PAF from ghosts prepared to maintain and lose membrane asymmetry. Asymmetric ghosts were loaded with PAF, resealed, washed in albumin to extract outer leaflet PAF and then placed in media containing 1 mM Mg^{2+} (○), PBS (■), or 1 mM Ca^{2+} (●). At various time points samples were removed, albumin-extracted and the remaining ghost-associated PAF measured. As shown, in the presence of calcium, more PAF was 'releasable', and was extracted by albumin. Points represent mean ± S.E., $n = 12$. Release of PAF was significantly enhanced from ghosts treated with Ca^{2+} than from those treated with Mg^{2+} or PBS ($P < 0.0001$).

Furthermore, from these findings it is predicted that a mixed population of asymmetric and 'symmetric' ghosts prepared with the addition of calcium after resealing should also show intermediate enhancement of transbilayer movement of PAF from outer leaflet to inner leaflet. To demonstrate this, ghosts were prepared as above to maintain native asymmetry (in the presence of magnesium and EGTA), resealed, treated with calcium, incubated with PAF in the extracellular media and then examined from transbilayer movement of PAF to the inner leaflet. As expected, in ghosts treated with calcium after resealing, transbilayer movement of PAF to the inner leaflet was enhanced over that seen in asymmetric ghosts maintained in either PBS or with magnesium added ($P < 0.05$) (Fig. 9), but was not enhanced to the degree seen in ghosts treated with calcium prior to resealing (compare with 'symmetric' ghosts in Fig. 5) which constitute a more uniformly 'symmetric' population by MC540 staining (Fig. 6B). These studies would suggest then, that calcium addition either prior to resealing, or to a lesser extent, after resealing, promotes the rapid formation of membrane 'flip-sites' that result in loss of endogenous phospholipid asymmetry and enhance flipping in either direction of PAF added to one leaflet or the other.

The distribution of PAF depends on transbilayer movement and the presence of 'acceptor' molecules

Evidence would suggest that albumin can compete with biological membranes for PAF [44] and intracellular proteins may function similarly [25]. Removal of PAF from the plasma membrane by 'acceptor' molecules should therefore influence disposition of the mediator. We predicted that loading ghosts with albumin prior to resealing would create an intracellular trap for PAF and should result in the enhanced accumulation of PAF inside both asymmetric and 'symmetric' ghosts. As demonstrated in Fig. 10, the introduction of an intracellular 'acceptor' does enhance intracellular accumulation of PAF by likely retarding the recycling of PAF back to the outer leaflet where it is available for albumin extraction.

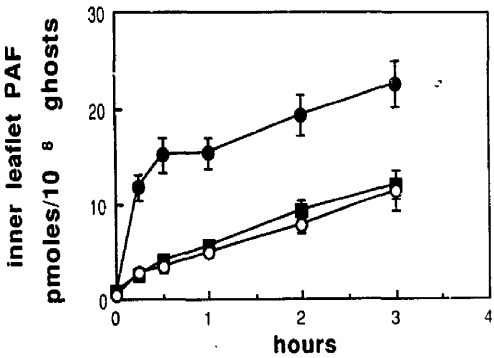

Fig. 9. Transbilayer movement of PAF in ghosts with calcium added, after resealing. Inner leaflet PAF (that remaining ghost-associated after albumin extraction) is shown for asymmetric ghosts, those prepared with Mg^{2+}/EGTA and maintained in PBS (○) or 1 mM Mg^{2+} buffer (■) and those following 1 mM Ca^{2+} addition (●). Points represent mean ± S.E., $n = 4$. Treatment with calcium resulted in significant ($P < 0.05$) enhancement of movement of PAF from outer to inner leaflet by ANOVA as in Fig. 5.

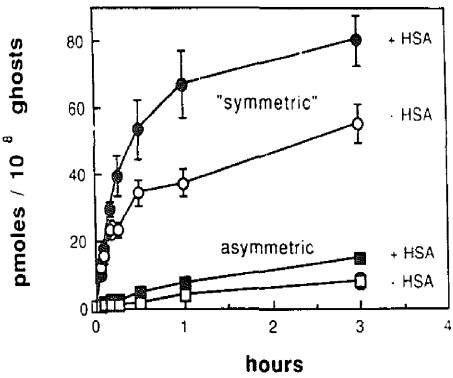

Fig. 10. Accumulation of PAF in 'empty' and albumin-loaded ghosts. Ghosts were prepared in Mg^{2+}/EGTA, to maintain asymmetry, (□, ■), or with Ca^{2+}, to be 'symmetric' (○, ●) and then resealed 'empty' (open symbols) or loaded with albumin prior to resealing (filled symbols). Transbilayer movement to the inner leaflet and intracellular accumulation of PAF (as measured by that PAF remaining after albumin extraction) is significantly ($P < 0.001$) enhanced for 'symmetric' ghosts when loaded with albumin as determined by the multiple comparisons procedure using the fitted model parameters 'a' and 'b' (see Fig. 5). Points represent mean ± S.E., $n = 4$.

Discussion

Several important observations in the literature have contributed to the proposed schema. Evidence from the work of Chap et al. [45] would indicate that the platelet exhibits qualitatively similar plasma membrane phospholipid asymmetry as that demonstrated for the native RBC [15], suggesting that such asymmetry is found more widely among circulating cells [17]. Enhanced MC540 staining appears to correlate with loss of plasma membrane asymmetry in the RBC and RBC ghost [21,22] and is seen in a variety of stimulating inflammatory cells, platelets [20], neutrophils, lymphocytes, monocytes [24], suggesting loss of asymmetry of the plasma membrane occurs with cellular activation. Additionally, previous investigation documents further evidence that loss of plasma membrane asymmetry occurs during platelet activation a shown by 'accessibility' of inner leaflet phospholipids to nonlytic enzymatic degradation [46,47], and the appearance of phosphatidylserine in the outer leaflet as measured by procoagulant activity [18,48]. Finally, the demonstration of enhanced uptake of exogenous phospholipid accompanying loss

of endogenous phospholipid asymmetry in RBCs [28,49,50], suggests that enhanced transbilayer movement of exogenously added phospholipid may reflect membrane events resulting in loss of endogenous asymmetry. These findings taken together, suggest the hypothesis that in a PAF-synthesizing inflammatory cell, cellular activation may enhance flip-flop of the mediator in the plasma membrane, making it available to 'acceptor' molecules such as albumin [5], other plasma proteins [25], or adjacent cellular membranes [51] and thereby in part govern its release.

In our hands, the addition of calcium to ghosts during hypotonic lysis did lead to enhanced clotting time (Fig. 7) and MC540 staining (Fig. 6B), strongly supporting loss of plasma membrane asymmetry. We extended the findings of Williamson et al. [22] by showing that calcium can also be added after ghost resealing and results in a mixed population of brightly staining ghosts (similar to 'symmetric' ghosts) and poorly staining ghosts (similar to asymmetric ghosts) (Fig. 6C). As would be predicted, such a mixed population also resulted in intermediate enhancement of clotting time (Fig. 7). Importantly, using these ghost populations, we demonstrated that in 'symmetric' ghosts made to lose plasma membrane asymmetry, enhanced transbilayer movement of PAF occurred. Furthermore, by adding exogenous PAF to either the inner or outer plasma membrane leaflet, we demonstrated that such enhanced transbilayer movement operates in either direction (Fig . 5 and 8). The data suggest that if such alterations occur in the plasma membranes of inflammatory cells synthesizing PAF as is suggested by MC540 staining, that release of the mediator may be governed in part by such enhanced transbilayer movement. Additionally, the data suggest that the process of uptake of exogenous PAF by an inflammatory cell will be greatly enhanced in the presence of such plasma membrane changes. Enhanced internalization of PAF by activation cells has recently been demonstrated and shown to be the rate-limiting step in the subsequent metabolic conversion of PAF [52].

The treatment of ghosts with calcium during preparation is thought to result in loss of membrane asymmetry by at least two possible mechanisms. Calcium entry into the cell likely results in (1) inhibition of the aminophospholipid translocase [53,54] which translocates aminophospholipids (but not phosphatidylcholines – see below) to the inner leaflet and (2) proteolysis and aggregation of cytoskeletal elements [22,55,56], which are though to normally exert a stabilizing influence on the plasma membrane. This latter mechanism likely involves spectrin, thought to be largely responsible for the maintenance of membrane asymmetry [29] and possibly other cytoskeletal proteins by disrupting their interaction with inner leaflet phosphatidylserine. By analogy, Zwaal and co-workers have shown in the platelet, that the appearance of phosphatidylserine in the outer leaflet during cellular activation is accompanied by calcium entry that results in degradation of the cytoskeleton [18,48].

As noted above, calcium entry may also inhibit the cells' aminophospholipid translocase [53], now characterized for the RBC [57], platelet [58] and lymphocyte [59], possibly by energy depletion following activation of the ATP-dependent calcium pump [54]. It is thought that while PE and PS are translocated to the inner leaflet by a calcium-inhibitable, magnesium and energy-dependent translocase (54,57,60], the transbilayer movement of phosphatidylcholines is relative slow [40,41], passive [41], energy-independent [33,41] and perhaps compensatory so as to maintain cell integrity [40]. Thus, it seems likely that these events, calcium entry, alteration in the cytoskeleton and inhibition of the aminotranslocase, likely play a role in the loss of plasma membrane phospholipid asymmetry that may occur in a variety of inflammatory cells with the potential of synthesizing PAF.

Our data demonstrate, as have the data of others [5,44], that the affinity of albumin for PAF can result in competition with biological membranes for PAF. We have also demonstrated that the addition of albumin as a trap on one side of the membrane (Fig. 10) can influence the ultimate disposition of PAF that has undergone transbilayer movement, further suggesting the role of 'acceptor' molecules as participants in the suggesting schema.

Additionally, these data demonstrate that the ester analogue of PAF, a substantial product of certain inflammatory cells [61] and a lipid which may have its own mediator role [62], undergoes transbilayer passage identically to PAF in treated RBCs (Fig. 2) and in ghosts (data not shown). Likewise, in data not shown, the enhanced transbilayer movement of lyso-PAF, the immediate and inactive precursor of PAF [35], was demonstrated. These data are consistent with previous demonstration that transbilayer movement (flip-flop) of phospholipids is largely determined by phospholipid head group [39–41]. Thus, we would hypothesize that if these choline-linked phospholipids, the ester analogues of PAF and lyso-PAF, are synthesized and localized to the plasma membrane, then they, too, would undergo enhanced transbilayer movement similar to PAF and be available for release to the extracellular environ. A physiologically important example of this latter may be the release of lyso-PAF, a major product of phospholipase A_2 activity in the macrophage [63], and platelet, [64], and its uptake by nearby neutrophils resulting in transcellular conversion to the potent mediator, PAF [65].

Of note, the PAF synthesized in a variety of inflammatory cells is largely, but variably retained intracellularly, suggested that, as yet, undefined mechanisms

operate in determining its release [1]. Data presented here suggest that the release of PAF from the cell in which it is synthesized would depend first on localization to the plasma membrane followed by transbilayer movement accompanying loss of membrane asymmetry. As our data suggest, disposition is also likely to depend on removal of PAF from the plasma membrane by 'acceptor' molecules on either side of the membrane.

In summary, the use of the erythrocyte and erythrocyte ghost has allowed us to model the transbilayer passage of PAF and show that it is enhanced with the loss of endogenous membrane asymmetry. Mounting evidence in the literature would suggest that some degree of membrane asymmetry may be present in circulating cells and that loss of such asymmetry accompanies the activated state and participates in adhesive interactions between cells [17]. We hypothesize that these membrane events likely occur in activated inflammatory cells that synthesize PAF and that such events may well govern the release of this potent inflammatory mediator.

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