PLA_2 promotes fusion between PMN-specific granules and complex liposomes

R. Alexander Blackwood, Amy T. Transue, Donna M. Harsh, Ruth C. Brower, Sima J. Zacharek, James E. Smolen,* and Ronald J. Hessler*

*Department of Pediatrics, *Division of Infectious Diseases and Hematology-Oncology, University of Michigan Medical Center, C.S. Mott Children's Hospital, Ann Arbor*

Abstract: Neutrophil stimulation results in the activation of a variety of phospholipases, including phospholipase A_2 (PLA_2), which releases arachidonic acid from the 2 position of membrane phospholipids, leaving a lysophospholipid. Because arachidonic acid is known to be a potent fusogen in vitro, we examined the effect of metabolism by PLA_2 on the fusion of complex liposomes (liposomes prepared with a phospholipid composition similar to that found in neutrophil plasma membrane). We observed that PLA_2 augmented the fusion of complex liposomes with each other as well as with specific granules isolated from human neutrophils, lowering the Ca^{2+} requirement for fusion by three orders of magnitude. Furthermore, although lysophospholipids inhibited fusion, the incorporation of arachidonic acid into liposome membranes overcame the inhibitory effects of the lysophospholipids. Thus with PLA_2 and annexins we were able to obtain fusion of complex liposomes at concentations of Ca^{2+} that are close to physiological. Our data suggest that the activation of PLA_2 and the generation of arachidonic acid may be the major fusion-promoting event mediating neutrophil degranulation. *J. Leukoc. Biol.* 59: 663–670; 1996.

Key Words: *neutrophils · arachidonic acid · degranulation · phosphatidylcholine*

INTRODUCTION

Stimuli that activate neutrophils and promote degranulation also activate phospholipase A_2 (PLA_2), an enzyme that mediates the release of arachidonic acid from membrane phospholipids. Most of the arachidonic acid enters the lipoxygenase and cyclooxygenase pathways, but intracellular concentrations of free arachidonic acid also rise. Although the activation of PLA_2 has not been shown to be a prerequisite for secretion in any system, there is strong evidence that it may potentiate degranulation. For example, both eosinophils [1] and mast cells [2] can be stimulated to degranulate in response to activators of PLA_2. Furthermore, the inhibition of PLA_2 inhibits degranulation in these cell systems. O'Rourke and co-workers [3] demonstrated that arachidonic acid and not its metabolites influence basophil degranulation. Inhibition of cyclooxygenase and lipoxygenase metabolism, with BW755c, completely blocks leukotriene and prostaglandin production, but not degranulation, following immunoglobulin E (IgE) receptor stimulation in a basophilic leukemia cell line (RBL-2H3). However, inhibitors of PLA_2 (quinacrine and quercetin) block the release of arachidonic acid and the production of leukotrienes and prostaglandins, as well as degranulation. The production of arachidonic acid has also been implicated in secretion by adrenal medullary cells, since inhibitors of PLA_2 also block the release of catecholamine [4].

The mechanism by which PLA_2 may influence degranulation is not well understood. However, in vitro studies suggest that both products of the metabolism of phospholipids by PLA_2 (free fatty acids and lysophopholipids) may augment membrane fusion. *Cis*-unsaturated fatty acids (arachidonic acid being the most potent) have been shown to decrease Ca^{2+} requirements for phospholipid vesicle fusion [5]. Furthermore, the pretreatment of adrenal chromaffin cell plasma membrane vesicles with PLA_2 primes them for fusion with secretory vesicles [6, 7] even in the absence of free arachidonic acid. This suggests that the formation of lysophospholipids may also increase the fusogenicity of some biological membranes.

Not only can membrane phospholipid composition affect membrane fusion, but a number of proteins such as the annexins have been implicated as potential fusogens during degranulation. The annexins are a family of Ca^{2+}-dependent phospholipid-binding proteins that share a common structural motif. They each contain at least four repeats of a 70-amino acid sequence [8]. Although the function of the annexins remains unclear, their ability to bind to phospholipids in the presence of Ca^{2+} suggests that they mediate the membrane fusion that occurs during exo-

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Abbreviations: PLA_2, phospholipase A_2; IgE, immunoglobulin E; LUV, large unilamellar vesicles; TLC, thin-layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; RBC, red blood cells; EGTA, ethyleneglycol-bis(β-aminoethylether)-*N*, *N*'-tetraacetic acid.

Reprint requests: R. Alexander Blackwood, MD, Ph.D., University of Michigan Medical School, Dept. of Pediatrics, 1500 E. Medical Center Dr., Box 0244, Ann Arbor, MI 48109-0244.

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cytosis [9, 10]. In support of this hypothesis, annexin I (lipocortin I), annexin II (calpactin I), and annexin VII (synexin) [11–16] have been found to be capable of promoting the aggregation and fusion of phospholipid vesicles in a Ca^{2+}-dependent manner. In addition, exocytosis in adrenal chromaffin cells permeabilized with digitonin can be reconstituted with annexin II [17, 18]. These studies indicate that both an annexin and arachidonic acid in conjunction may play a role in neutrophil degranulation.

In an attempt to understand the effect of PLA_2 metabolism on neutrophil degranulation, we prepared liposomes with a phospholipid head group composition similar to that found in the plasma membrane of human neutrophils. While these liposomes were highly resistant to fusion on their own, following hydrolysis by PLA_2 the Ca^{2+} concentration required for fusion was decreased by more than three orders of magnitude into the low micromolar range. PLA_2 had a similar effect on the fusion between complex liposomes and specific granules from human neutrophils. Furthermore, fusion between neutrophil-specific granules and liposomes, mediated by synexin, was found to be dependent on the presence of arachidonic acid [19]. Also, at subthreshold doses of PLA_2, annexin I augmented the fusogenicity of the vesicles treated with PLA_2, indicating a complementary effect between annexin I and PLA_2 with respect to membrane fusion.

MATERIALS AND METHODS

Chemicals

All phospholipids were obtained from Avanti Polar Lipids, Birmingham, AL. $CaCl_2$ hydrate (>99.99% pure) was obtained from Aldrich Chemical Co., Milwaukee, WI. Ethyleneglycol-bis(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA; puriss grade, >99%) was obtained from Fluka Chemical Co., Ronkonkoma, NY. High-performance liquid chromatography-grade H_2O was obtained from Fisher Scientific, Pittsburgh, PA. Phospholipase A_2 (*Naja naja atra*) and arachidonic acid were obtained from Calbiochem, San Diego, CA. L-3-Phosphatidylcholine, 1-stearoyl-2-[^{3}H]arachidonyl, was obtained from Amersham Life Sciences, Arlington Heights, IL. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO.

Annexin purification

Annexin I was purified from human placenta and bovine lung by use of a modification of the method of Haigler [20] as described previously [21].

Ca^{2+} buffers

A stock solution of Ca^{2+} was prepared in buffer A (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 100 mM KCl, 1 mM EGTA, pH 7.0) such that dilutions would give a range of final ionized [Ca^{2+}] (100 nM–14 mM) as predicted through using the system of Bers [22] and measured using a Ca^{2+}-sensitive electrode (Orion Research, Boston, MA).

Liposome preparation

Phospholipids were mixed, dried under argon, and redissolved in diethylether. Complex liposomes were prepared with a molar ratio of 44 phosphatidylcholine: 27 phosphatidylethanolamine:16 phosphatidylinositol:13 phosphatidylserine (PC:PE:PI:PS, respectively) with 50 mol% cholesterol. This phospholipid head group composition was chosen to resemble that of neutrophil plasma membrane [23] with the exception that the sphingomyelin was replaced with phosphatidylcholine. To prepare *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-*N*-(lissamine rhodamine B sulphonyl) (NBD-Rh)-labeled liposomes, 1% NBD-PE and 1% Rh-PE were added to the initial phospholipid mixture prior to drying. Phospholipids disolved in diethylether were emulsified with buffer A and large unilamellar vesicles (LUV) were prepared by reverse-phase evaporation [24, 25]. After evaporation, liposomes were extruded three times through a 0.1-μm filter to ensure uniform size.

Fusion assay

Fusion was measured by lipid dilution as previously described [21]. Briefly, LUV containing 1% NBD-PE and 1% Rh-PE were formed such that the distance between NBD and Rh was sufficiently close that Rh quenched fluorescence of NBD. Liposomes (300 μg total phospholipids, 9:1 unlabeled-to-labeled vesicles) and various amounts of PLA_2 were combined in a cuvette (final volume 1 mL), and fusion was initiated with the addition of Ca^{2+}. Fluorescence was measured continuously on an SLM 8000 spectrofluorometer for 90 s. To determine fusion between specific granules and complex liposomes, the unlabeled liposomes were replaced with specific granules. The concentration of specific granules was such that the OD_{450} equaled 0.17 to maintain consistency between experiments. Fusion between an unlabeled liposome or granule and a labeled vesicle resulted in an increase in the distance between NBD and Rh and a corresponding decrease in fluorescence quenching.

Quantitation of arachidonic acid release

Complex liposomes containing 25 μCi/mL 2-[^{3}H]arachidonyl PC were prepared by reverse-phase evaporation. Liposomes (75 μg phospholipids) were incubated with varying amounts of PLA_2 and Ca^{2+} for 90 s in buffer A (final volume 250 μL, final concentrations corresponded to those used in the fusion assay). The lipids were extracted with chloroform:methanol:acetic acid (75:45:3) and separated by thin-layer chromatography (TLC) using chloroform:methanol:acetic acid:water (25:15:4:2) [26]. PC and arachidonic acid spots were visualized with I_2 vapor, scraped from the plates, and counted in a Beckman scintillation counter. The percentage of PC hydrolyzed was determined by the following formula: 100 × (counts in arachidonic acid peak/ total counts). Verification of PLA_2 hydrolysis of PC was determined by the appearance of lyso-PC using TLC. Failure to visualize significant amounts of other lysophospholipids (PE, PI, PS) by TLC verified the relative selectivity of our PLA_2. Although arachidonic acid cannot be accurately expressed as mol% phospholipid, because it is not a phospholipid, we have chosen this representation because of the one-to-one molar correlation between PC metabolized and lyso-PC and arachidonic acid formed.

Isolation and preparation of specific granules

Neutrophils were isolated from fresh venous blood of healthy donors by use of the method of Boyum [27]. Briefly, acid-citrate-phosphate anticoagulated venous blood was separated on a ficoll-Hypaque cushion, followed by dextran sedimentation of red blood cells (RBC). Residual RBC were removed by hypotonic lysis. The resulting neutrophils were washed and suspended in buffer A (1×10^8/mL). Following treatment with DFP (2 mM) the neutrophils were disrupted by nitrogen cavitation [28] and the nuclei removed by low-speed centrifugation in a Beckman J6 centrifuge (1000 *g* for 10 min at 4°C). The resulting supernatant was layered onto a continuous sucrose gradient (15–60%) and the specific granules were separated by rate zonal centrifugation. The centrifuge tubes were punctured at the bottom and 1-mL fractions were collected. The fractions containing the specific granules were pooled, diluted, and centrifuged onto a 90% percoll cushion. Following three washes, the specific granules were resuspended in buffer A and used at an OD_{450} of 0.1704.

RESULTS

Effect of hydrolysis by PLA_2 on fusion of complex liposomes

We prepared complex liposomes with a phospholipid composition that resembled that of plasma membrane of human neutrophils and determined the effect of the actions of PLA_2 on Ca^{2+}-dependent fusion of liposomes. Complex liposomes were relatively resistant to fusion induced by Ca^{2+}. No detectible fusion was observed until 6 mM Ca^{2+} was used. Treatment with PLA_2 lowered the $[Ca^{2+}]$ required by more than three orders of magnitude, down to the micromolar range (**Fig. 1**). Increasing concentrations of PLA_2 resulted in an increase in both the initial rate of fusion as well as the total fusion of complex liposomes (**Fig. 2, A** and **B**). Furthermore, PLA_2 produced a concentration-dependent decrease in the $[Ca^{2+}]$ required for liposome fusion (Fig. 1). In control experiments, performed in the absence of fusion partners (unlabeled vesicles), only a minimal signal could be detected. Hence, fusion was dependent on the presence of target liposomes as well as Ca^{2+} and could not be explained by lysis of the labeled vesicles or release of the labeled fatty acid.

Because both annexin I and II are known to be fusogenic [21], we examined the combined effect of the annexins and PLA_2 on the fusion of complex liposomes. No fusion was observed with annexin I or II (140 nM) with 1 mM Ca^{2+} (data not shown). However, both annexin I and II augmented the effects of PLA_2 (**Fig. 3**). At subthreshold concentrations of PLA_2, annexins I and II increased both the initial rate and the total extent of the fusion of phospholipid vesicles. In the presence of 0.5 units of PLA_2, annexin I and II lowered the $[Ca^{2+}]$ required for the fusion of complex liposomes to 1.2 μM (Fig. 3). Thus, the Ca^{2+} requirements are close to physiological following this combination.

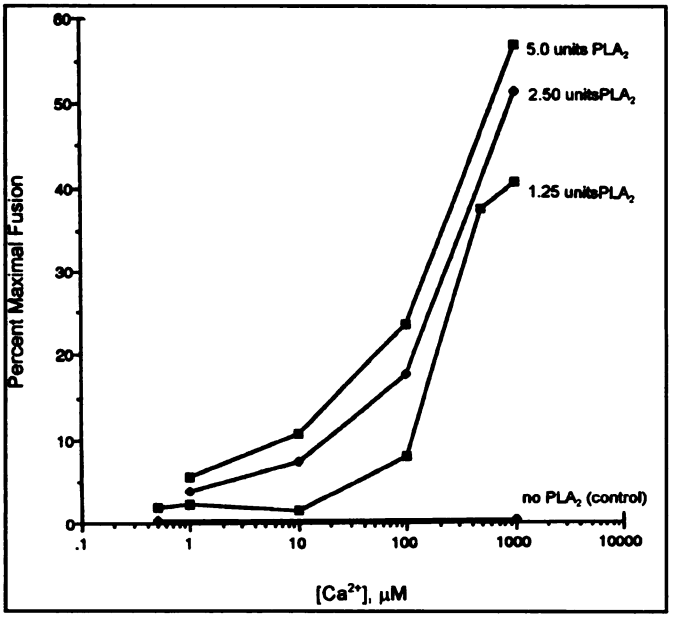

Fig. 1. PLA_2 Lowers the Ca^{2+} requirement for complex-liposome fusion. Indicated concentrations of PLA_2 were added to mixtures of labeled and unlabeled complex liposomes as detailed in Figure 2. Fusion was initiated with the addition of varying concentrations of Ca^{2+} and monitored on an SLM 8000 spectrofluorometer for 90 s. Results represent means of at least three experiments.

PLA_2 augmented fusion of neutrophil-specific granules with complex liposomes

Neutrophil-specific granules are highly resistant to fusion in vitro. To determine whether treatment with PLA_2 may affect the fusion of biological membranes, we examined the Ca^{2+} requirement for the fusion of complex liposomes with spe-

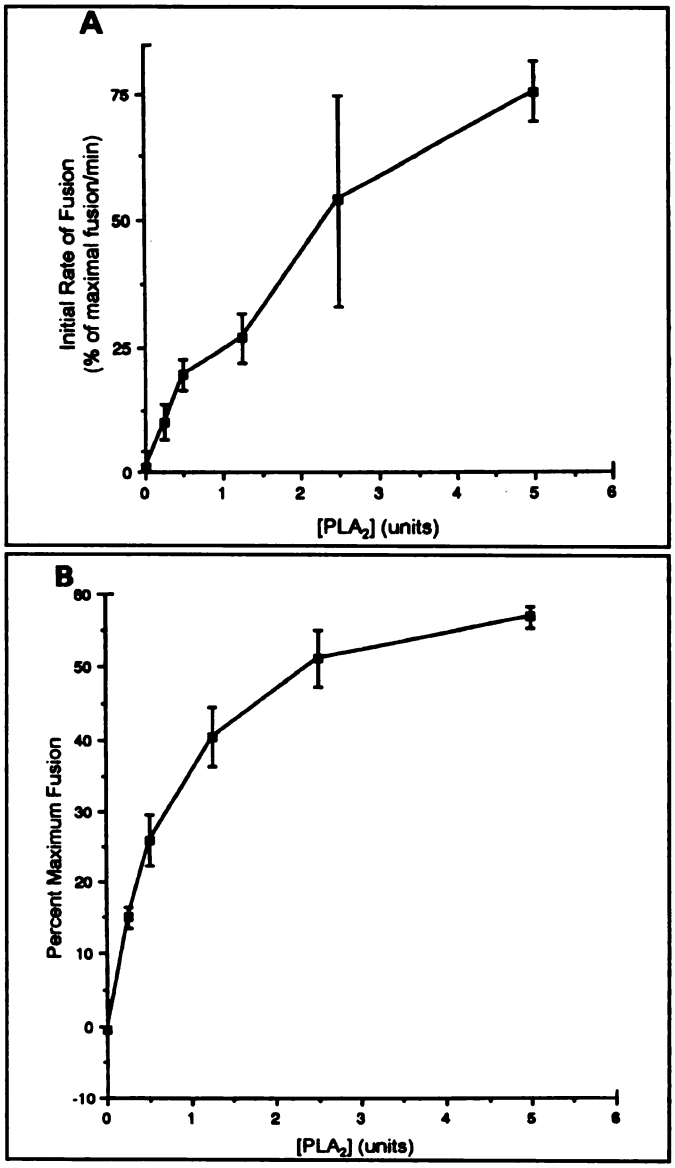

Fig. 2. Effect of PLA_2 on fusion of complex Liposomes. (A) Initial rate of fusion; (B) total fusion in 90 s. Complex liposomes were prepared with both NBD and Rh labels, and were then incubated with unlabeled complex liposomes and varying concentrations of PLA_2. Fusion was initiated with the addition of 1 mM Ca^{2+} and monitored for 90 s on an SLM spectrofluorometer. The initial rate of fusion is defined as percent of maximal signal per minute over the first 12 s. Results represent the means ± SD for at least three experiments.

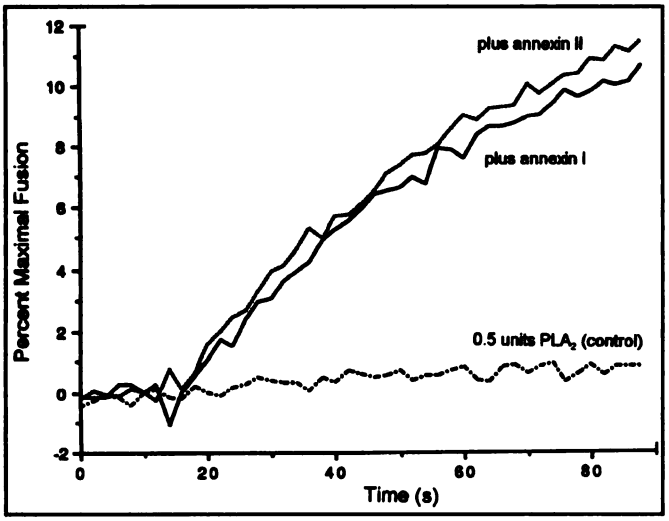

Fig. 3. Annexins augment PLA_2-induced liposome fusion at micromolar Ca^{2+} concentrations. Annexin I or II (140 nM) was added to labeled and unlabeled complex liposomes in the presence of 0.5 units of PLA_2. Fusion was initiated with the addition of 1.2 μM Ca^{2+}, and fusion was monitored continuously on an SLM 8000 spectrofluorometer for 90 s. Neither 0.5 units of PLA_2 alone nor the annexins alone promoted the fusion of complex liposomes. Representative curve from duplicate experiments performed on at least two independent granule preparations.

cific granules from human neutrophils. We confirmed that specific granules were extremely resistant, observing no fusion at 14 mM Ca^{2+} in the absence of PLA_2 (**Fig. 4**). As with the fusion of complex liposomes, treatment with PLA_2 lowered the Ca^{2+} requirement for fusion between complex liposomes and specific granules to as low as 10 μM (Fig. 4).

Relationship between arachidonic acid released and the extent of fusion

To determine the extent of phospholipid hydrolysis that occurs during our fusion assay, we prepared complex liposomes with 2-[^{3}H]arachidonyl phosphatidylcholine. Varying concentrations of PLA_2 were incubated with complex liposomes and 1 mM Ca^{2+} for 90 s. All conditions were designed to be parallel to those used in the fusion assay. The lipids were extracted and separated by TLC. As expected, PLA_2 produced a concentration-dependent release of arachidonic acid from the radiolabeled liposomes. Hydrolysis of PC by PLA_2 was concentration, time, and Ca^{2+}-dependent, with detectible activity at [Ca^{2+}] above 2 μM (data not shown). Combining our fusion and hydrolysis data, we determined the relationship between the amount of arachidonic acid released and the extent of fusion. Detectible fusion was observed when as little as 5% of the total PC of the liposome had been hydrolyzed (2.2 mol% of the total phospholipid for both arachidonic acid and lyso PC) (**Fig. 5**). Increased hydrolysis of PC resulted in a further increase in the extent of liposome fusion.

Isolated and combined effects of arachidonic acid and lysophospholipids

Phospholipid hydrolysis by PLA_2 results in two major products that may influence the fusion of phospholipid vesicles, lyso-PC, and arachidonic acid. To determine whether either or both of the products of PLA_2 were fusogenic, we prepared complex liposomes, replacing different amounts of the PC with lyso-PC. Liposomes containing lyso-PC were more resistant to fusion than the regular complex liposomes. Replacing as little as 5% of the PC with lyso-PC increased the Ca^{2+} threshold for fusion above 14 mM (our maximal calibrated [Ca^{2+}]) (**Fig. 6**). Although liposomes containing lyso-PC were highly resistant to fusion induced by Ca^{2+}, the addition of exogenous arachidonic acid potentiated fusion. The higher the concentration of lyso-PC contained in the complex liposomes, the smaller the effect a given concentration of arachidonic acid had on promoting Ca^{2+}-dependent fusion (data not shown).

Effect of dispersions of arachidonic acid

One means of supplementing our fusion mixture with arachidonic acid is by the addition of external sources of fatty acid. The exogenous addition of dispersions of arachidonic acid by itself was not fusogenic. However, dispersed arachidonic acid augmented the fusion of complex liposomes induced by Ca^{2+} in a dose-dependent manner (data not shown). Although liposomes containing lyso-PC fused in the presence of Ca^{2+} and arachidonic acid, they were less fusogenic than control complex liposomes, indicating that exogenously added arachidonic acid can only partially overcome inhibition by lysophospholipids. The total mass of dispersed arachidonic acid required to be effective was very large, rivaling the total amount of lipid in the fusion system.

Although PLA_2 will preferentially hydrolyze PC over other phospholipids, it is not completely specific. To determine the potential role that other lysophospholipids may be playing in our system, we examined the effects of lyso-PS, -PI, and -PE on the fusion of complex liposomes. We prepared complex liposomes replacing PS, PI, or PE with lyso-PS, -PI, or -PE, respectively. Although all three lysophospholipids inhibited complex liposome fusion, the effects of PS and PI were more dramatic, with no fusion

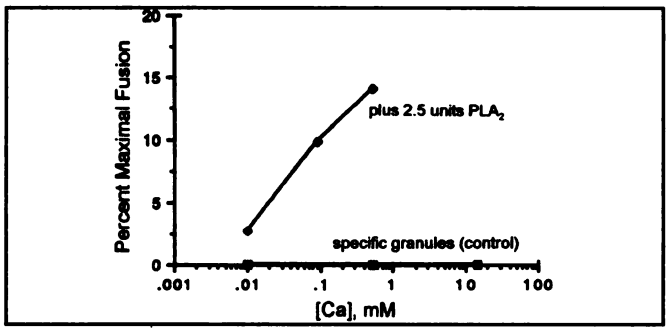

Fig. 4. PLA_2 augments the fusion of PMN-specific granules with complex liposomes. Specific granules from PMN were incubated with complex liposomes labeled with both NBD and Rh in the presence and absence of 2.5 units of PLA_2. Fusion was initiated with the addition of varying concentrations of Ca^{2+} and monitored on an SLM 8000 spectrofluorometer for 90 s. Representative curve from duplicate experiments performed on at least two independent granule preparations.

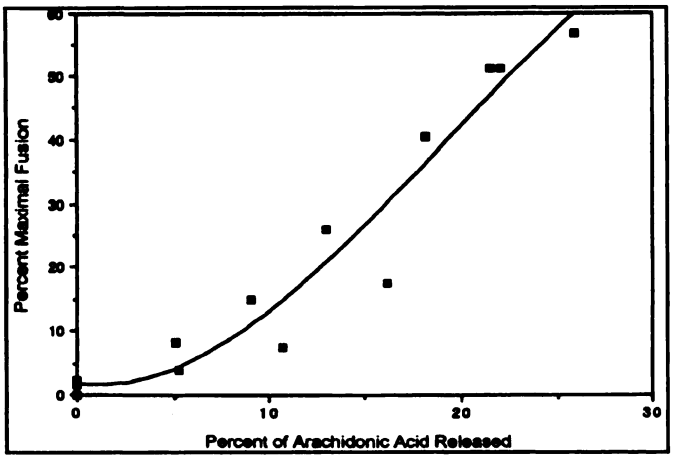

Fig. 5. Extent of liposome fusion is directly related to arachidonic acid release. Percent arachidonic acid released was extrapolated from PLA_2 concentration and Ca^{2+}-dependent fusion curves. Ten percent arachidonic acid release was equivalent to a 10% conversion of liposome PC to lyso-PC and arachidonic acid, or 4.4 mol% of the total phospholipid.

observed at 14 mM Ca^{2+} (data not shown). Lyso-PE increased threshold $[Ca^{2+}]$ to ~8 mM (data not shown). These results indicated that the augmentation in the fusion of complex liposomes observed following hydrolysis by PLA_2 could not be explained by the formation of these lysophospholipids.

Direct incorporation of arachidonic acid into phospholipid vesicles

Although dispersions of exogenous arachidonic acid augmented the fusion of complex liposomes and partially overcame the inhibitory effects of the lysophospholipids, the amount of arachidonic acid required was much more than could be accounted for by our PC hydrolysis data. If all of the PC contained arachidonic acid in the 2 position and all of this PC were hydrolyzed by PLA_2, then ~100 μM arachidonic acid would be generated. Thus, the data show that the arachidonic acid requirements to promote fusion are at least an order of magnitude greater when its fatty acid is added as a dispersion, rather than generated in situ (Fig. 5). Three potential explanations for our divergent results are as follows: (1) the concentrations of arachidonic acid at the liposome-liposome interface of fusing liposomes are locally much higher than the overall arachidonic acid concentration of the solution in the presence of PLA_2; (2) hydrolysis by PLA_2 results in the incorporation of at least some of the arachidonic acid into the membrane of phospholipid vesicles, and it is this arachidonic acid that plays a significant role in membrane-membrane fusion; or (3) the PLA_2 used in these experiments releases other fatty acids that may be more potent than arachidonic acid for promotion of membrane fusion.

To determine whether arachidonic acid directly incorporated into the membrane augmented fusion, we constructed complex liposomes with arachidonic acid alone (2.2 mol%), or lyso-PC plus arachidonic acid (both at 4.4 mol%). Complex liposomes containing 4.4 mol% of both lyso-PC and arachidonic acid would be representative of complex liposomes undergoing 10% conversion of membrane PC to lyso-PC, assuming that all of the generated arachidonic acid remained in the membrane. We found that liposomes prepared in this fashion were more fusogenic with complex liposome targets than unmodified complex liposomes themselves (**Fig. 7**), shifting the threshold Ca^{2+} requirement to the left and augmenting the extent of fusion. Complex liposomes containing 2.2 mol% arachidonic acid alone showed a further decrease in the $[Ca^{2+}]$ required for fusion (Fig. 7). These liposomes model the situation in which arachidonic acid remains incorporated in the membrane and lyso-PC is rapidly re-acylated or segregates to an area of the membrane (if incorporated directly into the liposomes) that is not involved in fusion. These results demonstrate that arachidonic acid can augment membrane fusion, both increasing the extent of fusion as well as lowering Ca^{2+} requirements. Furthermore, the actions of PLA_2 are better represented by a model that assumes that arachidonic acid remains associated with the membrane rather than being released in solution and then reincorporated from the medium.

We also wished to assess the potential role of free fatty acids other than arachidonic acid in the augmentation of phospholipid vesicle fusion by PLA_2. Consequently, we determined the Ca^{2+} requirements for fusion of complex liposomes in the presence of four other free fatty acids, using both dispersions and direct incorporation. When added as a dispersion, both oleic and linoleic acid were almost as potent fusogens as arachidonic acid (**Fig. 8A**). However, the concentrations required for their augmentation were still beyond levels that could have been achieved using PLA_2 (Fig. 1). Dispersions of palmitic and stearic acid had little effect on the fusion of complex liposomes (Fig. 8A). As we observed with arachidonic acid, the direct membrane incorporation of the other free fatty acids aug-

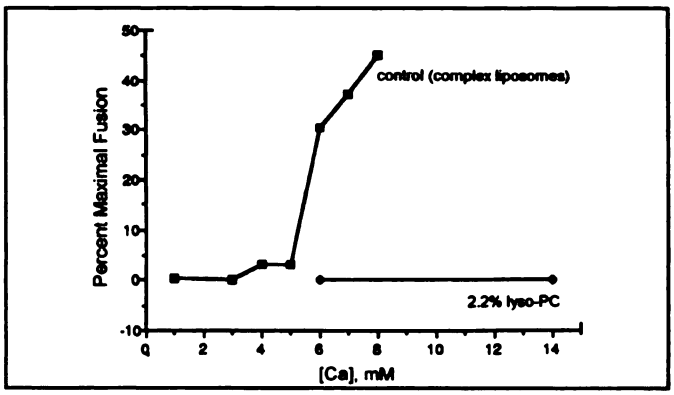

Fig. 6. Lysophospholipids inhibit fusion of complex liposomes. To determine which product of PLA_2 action (lyso-PC or arachidonic acid) augmented complex liposome fusion, we prepared labeled (NBD, Rh) and unlabeled complex liposomes replacing 5% of the PC with lyso- PC (final concentration 2.2% lyso-PC and 41.8% PC), thus modeling 5% hydrolysis by PLA_2. Fusion assays were performed in an identical manner as those in Figure 1. Lyso-PS and lyso-PI were also found to be inhibitory in our fusion system (data not shown). Results represent the mean of at least three independent experiments.

mented complex liposome fusion. However, none of the fatty acids examined were as potent as arachidonic acid (Fig. 8B).

CONCLUSIONS

The activation of PLA_2 following neutrophil stimulation is known to release arachidonic acid from membrane phospholipids. Although much of the released arachidonic acid enters the prostaglandin-leukotriene pathway, strong evidence exists that it may play a more direct role in the fusion of granules with the plasma membrane. In both eosinophils [1] and mast cells [2], degranulation can be stimulated by activators of PLA_2. In addition, the inhibition of PLA_2 (but not cyclooxygenase or lipoxygenase) inhibits degranulation by both neutrophils [29] and mast cell tumor lines [3].

In an attempt to better understand the potential role of PLA_2 and its products on the fusion in neutrophils, we prepared complex liposomes complex with the phospholipid head group composition of human neutrophil plasma membrane and examined the effect of treatment with PLA_2 on liposome fusion. We have shown that metabolism by PLA_2 augments the fusion of both complex liposomes with each other and complex liposomes with neutrophil-specific granules. In both cases, the Ca^{2+} requirement for fusion decreased by more than two orders of magnitude from the millimolar range to the low micromolar range. Although high rates of fusion could be achieved when PLA_2 was used in conjunction with millimolar levels of $[Ca^{2+}]$, the fusion rates with micromolar $[Ca^{2+}]$ were comparable to those achieved with untreated complex liposomes at millimolar $[Ca^{2+}]$. This is of particular significance because we have taken phospholipid vesicles that are highly resistant to fusion and have achieved membrane fusion at nearly physiological $[Ca^{2+}]$.

The annexins are a family of Ca^{2+}-dependent phospholipid-binding proteins that have been implicated as potential fusogens in exocytosis [11–14, 17, 18]. Because we have previously demonstrated that both annexin I and II can promote phospholipid vesicle fusion [21], we examined the combined effects of treatment with PLA_2 and annexin I and II. By themselves, neither annexin I nor II had an effect on the $[Ca^{2+}]$ required to promote the fusion of complex liposomes. However, at subthreshold concentrations of PLA_2 in the presence of 140 nM annexin I or II, significant fusion could be measured at 1.2 μM Ca^{2+}. This concentration is similar to that which has been reported locally at sites of synaptosome fusions [30]. Thus the annexins appear to augment the effect of PLA_2, decreasing the extent of phospholipid metabolism required for membrane fusion. Again it must be noted that complex liposomes are extremely resistant to fusion, but in the presence of PLA_2 and annexins, we have achieved fusion at truly physiological $[Ca^{2+}]$.

Although our results using liposomes were of interest, it was not clear whether the data were applicable to biological membranes. Neutrophil-specific granules are known to be relatively resistant to fusion [31]. Meers and co-workers [32] have been able to promote the fusion of neutrophil-specific granules with PA/PE (1:3) liposomes in the presence of annexin I. However, PA/PE liposomes are relatively fusogenic and are not good representatives of any known biological membrane (arguments for localized pooling of phospholipids notwithstanding). We found that following limited hydrolysis by PLA_2, highly resistant liposomes (complex liposomes) would fuse with specific granules isolated from human neutrophils. The Ca^{2+} concentrations required for fusion were comparable to those reported by Meers and co-workers (using PA/PE liposomes) [32]. Furthermore, the amount of arachidonic acid required to promote fusion was well within reported limits found following neutrophil stimulation [29].

PLA_2 generates two major products: lysophospholipids and arachidonic acid, both of which have been implicated as potential fusogens. Our data clearly demonstrate that lysophospholipids are not fusogenic, and in fact increase the Ca^{2+} requirements for fusion. Another research group has recently reported similar findings [33]. Alternately, we have verified the findings that arachidonic acid augments phospholipid vesicle fusion [5]. It has previously been suggested that lysophospholipids may potentiate fusion because PLA_2 had been shown to prime chromaffin granules for fusion in the absence of free arachidonic acid. Our data further suggest that this priming may be due to the incorporation of arachidonic acid into the membrane, rather than the formation of lysophospholipids. We have also examined four other free fatty acids, finding that, although others may promote the fusion of phospholipid vesicles, arachidonic acid was the most potent examined.

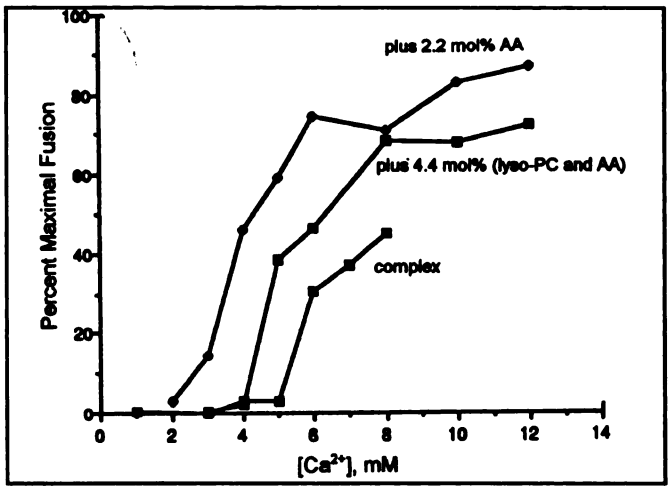

Fig. 7. Membrane incorporation of PLA_2 products (arachidonic acid and lyso-PC) augment Ca^{2+}-dependent liposome fusion. We prepared labeled (NBD, Rh) and unlabeled complex liposomes, by reverse-phase evaporation, replacing 10% of the PC with lyso-PC (4.4% of total phospholipid composition) and an equivalent molar amount of arachidonic acid. We also prepared complex liposomes that contained an extra 2.2 mol% arachidonic acid. Fusion assays were performed in an identical manner as previously described. Results are means of at least three experiments performed in duplicate.

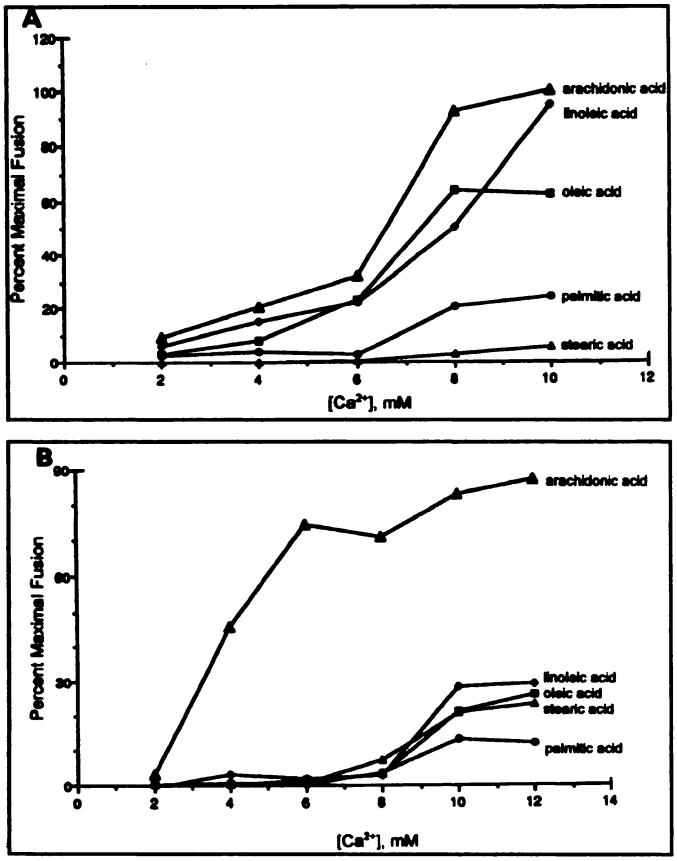

Fig. 8. Arachidonic acid is more fusogenic than other free fatty acids. Unlabeled and labeled (NBD, Rh) complex liposomes were fused with the indicated [Ca^{2+}] by: (A) the addition of dispersions of free fatty acids and (B) incorporation of free fatty acids (2.2 mol%) directly into both sets of liposome membranes. Fusion assays were performed as previously described. Results are means of two experiments performed in duplicate.

The role of phospholipid metabolism in membrane fusing events in neutrophil or other cell types is poorly understood. However, Goni et al. [34, 35] have demonstrated that metabolism by phospholipase C augments liposome fusion. Our data suggest a potential role for the generation of arachidonic acid during neutrophil activation. Furthermore, we believe that the local effects of phospholipid metabolism are far greater than might be observed with the products uniformly distributed throughout the membrane. Although liposomes artificially prepared to reflect PLA_2 metabolism mimicked the general trends obtained with the enzyme, the extent of augmentation could not be duplicated. Dispersions of exogenously added fatty acids proved to be the least effective and least physiological method of promoting membrane fusion. The direct incorporation of arachidonic acid into the liposome membrane during construction more closely approximated the results obtained with PLA_2. These data shed new light on the potential role of phospholipid metabolism in neutrophil degranulation as well as our approach to in vitro fusion models.

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REFERENCES

1. Henderson, W. R., Chi, E. Y., Jorg, A., Klebanoff, S. J. (1983) Horse eosinophil degranulation induced by ionophore A23187. Ultrastructure and role of phospholipase A2. *Am. J. Pathol.* **111**, 342–349.
2. Murakami, M., Kudo, I., Suwa, Y., Inoue, K. (1992) Release of a 14-kDa group-II phospholipase A2 from activated mast cells and its possible involvement in the regulation of the degranulation process. *Eur. J. Biochem.* **209**, 257–265.
3. O'Rourke, A. M., Mescher, M. F., Apgar, J. R. (1992) IgE receptor-mediated arachidonic acid release by rat basophilic leukemia (RBL-2H3) cells: possible role in activating degranulation. *Mol. Immunol.* **29**, 1299–1308.
4. Frye, R. A., Holz, R. W. (1983) Phospholipase A2 inhibitors block catecholamine secretion and calcium uptake in cultured bovine adrenal medullary cells. *Mol. Pharmacol.* **23, 547–550.**
5. Creutz, C. E. (1981) *cis*-Unsaturated fatty acids induce the fusion of chromaffin granules aggregated by synexin. *J. Cell Biol.* **91**, 247–256.
6. Morgan, A., Burgoyne, R. D. (1990) Relationship between arachidonic acid release and Ca^{2+}-dependent exocytosis in digitonin-permeabilized bovine adrenal chromaffin cells. *Biochem. J.* **271**, 571–574.
7. Karli, U. O., Schafer, T., Burger, M. M. (1990) Fusion of neurotransmitter vesicles with target membrane is calcium independent in a cell-free system. *Proc. Natl. Acad. Sci. USA* **87**, 5912–5915.
8. Pepinsky, R. B., Tizard, R., Mattaliano, R. J., Sinclair, L. K., Miller, G. T., Browning, J. L., Chow, E. P., Burne, C., Huang, K. S., Pratt, D. (1988) Five distinct calcium and phospholipid binding proteins share homology with lipocortin I. *J. Biol. Chem.* **263**, 10799–10811.
9. Kaplan, R., Jaye, M., Burgess, W. H., Schlaepfer, D. D., Haigler, H. T. (1988) Cloning and expression of cDNA for human endonexin II, a Ca^{2+} and phospholipid binding protein. *J. Biol. Chem.* **263**, 8037–8043.
10. Crumpton, M. J ., Dedman, J. R. (1990) Protein terminology tangle [letter] *Nature* **345**, 212.
11. Burgoyne, R. D. (1988) Calpactin in exocytosis [news] *Nature* **331**, 20.
12. Hong, K., Duzgunes, N., Ekerdt, R., Papahadjopoulos, D. (1982) Synexin facilitates fusion of specific phospholipid membranes at divalent cation concentrations found intracellularly. *Proc. Natl. Acad. Sci. USA* **79**, 4642–4644.
13. Ernst, J. D., Hoye, E., Blackwood, R. A., Jaye, D. (1990) Purification and characterization of an abundant cytosolic protein from human neutrophils that promotes Ca^{2+}-dependent aggregation of isolated specific granules. *J. Clin. Invest.* **85**, 1065–1071.
14. Creutz, C. E., Pazoles, C. J., Pollard, H. B. (1979) Self-association of synexin in the presence of calcium. Correlation with synexin-induced membrane fusion and examination of the structure of synexin aggregates. *J. Biol. Chem.* **254**, 553–558.
15. Creutz, C. E., Moss, S., Edwardson, J. M., Hide, I., Gomperts, B. (1992) Differential recognition of secretory vesicles by annexins. European Molecular Biology Organization Course "Advanced Techniques for Studying Secretion". *Biochem. Biophys. Res. Commun.* **184**, 347–352.
16. Meers, P., Ernst, J. D., Duzgunes, N., Hong, K. L., Fedor, J., Goldstein, I. M., Papahadjopoulos, D. (1987) Synexin-ike proteins from human polymorphonuclear leukocytes. Identification and characterization of granule-aggregating and membrane-fusing activities. *J. Biol. Chem.* **262**, 7850–7858.
17. Ali, S. M., Geisow, M. J., Burgoyne, R. D. (1989) A role for calpactin in calcium-dependent exocytosis in adrenal chromaffin cells. *Nature* **340**, 313–315.
18. Ali, S. M., Burgoyne, R. D. (1990) The stimulatory effect of calpactin (annexin II) on calcium-dependent exocytosis in chromaffin cells: requirement for both the N-terminal and core domains of p36 and ATP. *Cell Signal* **2**, 265–276.
19. Ernst, J. D., Meers, P., Hong, K., Duzgunes, N., Papahadjopoulos, D., Goldstein, I. M. (1986) Human polymorphonuclear leukocytes contain synexin, a calcium-binding protein that mediates membrane fusion. *Trans. Assoc. Am. Physicians* **99**, 58–66.
20. Haigler, H. T., Schlaepfer, D. D., Burgess, W. H. (1987) Characterization of lipocortin I and an immunologically unrelated 33-kDa protein as epidermal growth factor receptor/kinase substrates and phospholipase A2 inhibitors. *J. Biol. Chem.* **262**, 6921–6930.
21. Blackwood, R. A., Ernst, J. D. (1990) Characterization of Ca^{2+}-dependent phospholipid binding, vesicle aggregation and membrane fusion by annexins. *Biochem. J.* **266**, 195–200.
22. Bers, D. M. (1982) A simple method for the accurate determination of free [Ca] in Ca-EGTA solutions. *Am. J. Physiol.* **242**, C404–C408.
23. Woodin, A. M., Wieneke, A. A. (1966) Composition and properties of a cell-membrane fraction from the polymorphonuclear leukocyte. *Biochem. J.* **99**, 493–500.
24. Duzgunes, N., Wilschut, J., Fraley, R., Papahadjopoulos, D. (1981) Studies on the mechanism of membrane fusion. Role of head-group composition in calcium- and magnesium-induced fusion of mixed phospholipid vesicles. *Biochim. Biophys. Acta* **642**, 182–195.
25. Duzgunes, N., Allen, T. M., Fedor, J., Papahadjopoulos, D. (1987) Lipid mixing during membrane aggregation and fusion: why fusion assays disagree. *Biochemistry* **26**, 8435–8442.
26. Bligh, E. G., Dyer, W. J. (1959) A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**, 911–917.

27. Boyum, A. (1968) Isolation of leukocytes from human blood. A two-phase system for removal of red cells with methylcellulose as erythrocyte-aggregating agent. *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97), 9–29.

28. Evans, W. H. (1980) Preparation and characterization of mammalian plasma membranes. In *Laboratory Techniques in Biochemistry and Molecular Biology* (T. S. Work, E. Work, eds.), New York, North-Holland.

29. Forehand, J. R., Johnston, R. B., Bomalaski, J. S. (1993) Phospholipase A_2 activity in human neutrophils. Stimulation by lipopolysaccharide and possible involvement in priming for an enhanced respiratory burst. *J. Immunol.* **151**, 4918–4925.

30. Llinas, R. R., Sugimari, M., Silver, R. B. (1994) Localization of calcium concentration microdomains at the active zone in the squid giant synapse. *Adv. Second Messenger Phosphoprotein Res.* **29**, 133–138.

31. Francis, J. W., Balazovich, K. J., Smolen, J. E., Margolis, D. I., Boxer, L. A. (1992) Human neutrophil annexin I promotes granule aggregation and modulates Ca^{2+}-dependent membrane fusion. *J. Clin. Invest.* **90**, 537–544.

32. Meers, P., Mealy, T., Tauber, A. I. (1993) Annexin I interactions with human neutrophil specific granules: fusogenicity and coaggregation with plasma membrane vesicles. *Biochim. Biophys. Acta* **1147**, 177–184.

33. Yeagle, P. L., Smith, F. T., Young, J. E., Flanagan, T. D. (1994) Inhibition of membrane fusion by lysophosphatidylcholine. *Biochemistry* **33**, 1820–1827.

34. Goni, F. M., Nieva. J. L., Basanez, G., Fidelio, G., Alonso, A. (1994) Phospholipase-C-promoted liposome fusion. *Biochem. Soc. Trans.* **22**, 839–844.

35. Nieva, J. L., Goni, F. M., Alonso, A. (1993) Phospholipase C-promoted membrane fusion. Retroinhibition by the end-product diacylglycerol. *Biochemistry* **32**, 1054–1058.