A ROLE FOR DIACYLGLYCEROL IN ANNEXIN A7-MEDIATED FUSION OF LUNG LAMELLAR BODIES

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Abstract

Lung surfactant secretion in alveolar type II cells occurs following lamellar body fusion with plasma membrane. Annexin A7 is a Ca^{2+}-dependent membrane-binding protein that is postulated to promote membrane fusion during exocytosis in some cell types including type II cells. Since annexin A7 preferably binds to lamellar body membranes, we postulated that specific lipids could modify the mode of annexin A7 interaction with membranes and its membrane fusion activity. Initial studies with phospholipid vesicles containing phosphatidylserine and other lipids showed that certain lipids affected protein interaction with vesicle membranes as determined by change in protein tryptophan fluorescence, protein interaction with *trans* membranes, and by protein sensitivity to limited proteolysis. The presence of signaling lipids, diacylglycerol or phosphatidylinositol-4,5-bisphosphate, as minor components also modified the lipid vesicle effect on these characteristics and membrane fusion activity of annexin A7. In vitro incubation of lamellar bodies with diacylglycerol or phosphatidylinositol-4,5-bisphosphate caused their enrichment with either lipid, and increased the annexin A7 and Ca^{2+}-mediated fusion of lamellar bodies. Treatment of isolated lung lamellar bodies with phosphatidylinositol- or phosphatidylcholine phospholipase C to increase diacylglycerol, without or with preincubation with phosphatidylinositol-4,5-bisphosphate, augmented the fusion activity of annexin A7. Thus, increased diacylglycerol in lamellar bodies following cell stimulation with secretagogues may enhance membrane fusion activity of annexin A7.

Keywords

Membrane Fusion; surfactant secretion; protein fluorescence; proteolysis; membrane insertion; membrane binding; signaling lipids

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Lung surfactant is essential for normal lung function in air-breathing mammals because it is required for lowering surface tension at the air-liquid interface during end-expiration (reviewed in [1,2]). This lipoprotein-like complex of phospholipid and proteins is synthesized and secreted by the alveolar type II cells (reviewed in [3]). The phospholipid and some of the protein components are stored in lamellar bodies, the secretion organelles unique to type II cells. Surfactant secretion occurs through fusion pores that are formed following lamellar body fusion with plasma membrane (reviewed in [4,5]). Several studies have demonstrated that several agents increase surfactant secretion in isolated perfused lung and in isolated type II cells by increasing cell Ca^{2+}, cAMP, and protein kinase C (PKC) activity [4,5]. Some of these agents

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also increase the number of surface fusion pores suggesting that the membrane fusion activity is increased to allow elevated secretion of surfactant [6,7].

Previous studies have suggested a role for annexin proteins in membrane fusion during surfactant secretion [8–10]. Annexin A7 is postulated to promote membrane fusion during exocytic secretion in some cell types [8,11]. We have previously demonstrated that annexin A7 could promote membrane fusion between isolated lamellar bodies and lung plasma membrane fractions [9], or increase surfactant secretion in semi-intact type II cells [8]. Annexin A7 binding to lamellar bodies is higher than to the plasma membrane or the cytosol fraction [12] suggesting that the lamellar body membrane characteristics could contribute to the higher binding. Although annexin A7 binding protein was detected in both the lamellar bodies and the plasma membrane fractions [12], the lipids in the lamellar body membrane could also contribute to annexin A7 binding. This study also showed that the protein binding to lamellar bodies and plasma membrane can be further enhanced followed cell treatment with calcium ionophore or phorbol myristate acetate (a direct activator of PKC). Since intracellular membranes show differences in lipid composition [13–16], it is also likely that these differences contribute to specificity and membrane binding and fusion activities of annexin A7.

Annexin A7, like other annexin proteins, binds to phospholipid membranes in a Ca^{2+}-dependent manner through the highly homologous COOH- (core) domain (reviewed in [17–19]). The unique NH_2- (tail) terminus is short for most annexin proteins except for annexin A7 and A11 that have long tails. The unique nature of the NH_2-terminus is postulated to contribute to specificity of annexin function [20]. Several previous studies employing annexin A5 as a model annexin protein have demonstrated high affinity binding to acidic phospholipid like phosphatidylserine (PS) or phosphatidic acid (reviewed in [17]). This is also supported by the presence of postulated PS binding sequences in the annexin molecule [21]. Even though PS binding sites are present in the core domain, our studies with recombinant wild type and deletion mutant annexin A7 proteins suggest that the NH_2-terminus could modify the core domain and its Ca^{2+}-dependent interaction with phospholipid vesicle (PLV) membranes [22]. Although annexin proteins bind poorly to major membrane phospholipids like phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [17], a comparison of the Ca^{2+}-dependent binding of annexin A7 and its mutants to PC:PS (3:1) and PE:PS (3:1) PLV indicated that change in major lipid could influence annexin A7 interaction with membranes [23]. Further, annexin A7 preferably binds to specific biological membranes [12,24–26] alluding to the possibility that the membrane lipid composition could have a role in affecting protein interaction with these membranes. Others have demonstrated [27–29] preferred binding of annexin A2 to specific cellular membranes like plasma membrane or to phospholipid vesicles (PLV) enriched with phosphatidylinositol-4,5-bisphosphate (PIP_2). This important phospholipid is also implicated in the action of synaptotagmin, a Ca^{2+}-binding synaptic vesicle protein, which showed preference for PIP_2-containing domains in membranes [30]. These observations suggest that other phospholipid species (besides PS) could also control protein interaction with membranes. Such regulation of protein binding by specific lipid could contribute to site-specific physiological regulation of protein function [14,16], since significant differences in phospholipid composition of intracellular membranes have been reported [13–16]. Another possible implication of observations with annexin A2 and synaptotagmin lies in the involvement of PIP_2 in the signal transduction pathways (reviewed in [31–33]), since its hydrolysis by phospholipase C (PLC) would generate inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) that increase cell Ca2+and protein kinase C (PKC) activity, respectively. In alveolar type II cells, as in most other cell types, Ca^{2+} and PKC are implicated in stimulation of secretion by exocytosis [34–37]. Thus, modulation of annexin A7 properties by DAG or PIP_2 would suggest that stimulation of cells with appropriate secretagogues would regulate annexin A7 function for augmented secretion.

In this study, we tested the hypothesis that the PIP$_2$ and DAG levels would significantly modulate the properties of annexin A7, since increased membrane fusion activity would be needed in type II cells stimulated with surfactant secretagogues [34–37]. We utilized PLV prepared from various lipid mixtures to show that lipid composition, PIP$_2$ and DAG in particular, affected the molecular characteristics (protein fluorescence and protease sensitivity) and the membrane fusion function of annexin A7. In parallel, altering the PIP$_2$ or DAG content in isolated lung lamellar bodies modulated the membrane binding and membrane fusion activities of annexin A7. We propose that these signaling lipids in biological membranes could contribute to the specificity of annexin A7 action and regulation of membrane fusion during surfactant secretion.

MATERIALS AND METHODS

L-α-phosphatidylcholine (brain), L-α-phosphatidylserine (Brain, bovine), L-α-phosphatidylinositol-4,5-bisphosphate (brain, Porcine), 1,2,dioleoylglycerol, 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl) (dansylPE, DPE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine were obtained from Avanti Polar Lipids (Alabaster, AL) and were used without further purification. Recombinant trypsin (Promega, Madison, WI) was used to cleave recombinant fusion protein for the release of free annexin A7. Chymotrypsin, PC-PLC, PI-PLC, cholesterol and other fine chemicals were from Sigma Chemical Co. (St. Louis, MO). Polyclonal antibodies to PIP$_2$ were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All standard chemicals and glassware were from VWR Scientific (Rochester, NY) unless indicated otherwise. Silica gel G coated thin layer chromatography plates were by Whatman Inc and obtained from Fisher Scientific (Philadelphia, PA). Recombinant annexin A7 protein was expressed in E. coli as described [22]. The purified protein was stored in small aliquots at −70° C in 50mM Tris-Acetate-1mM EGTA (TAE) buffer (pH 8.3).

Preparation of Phospholipid Vesicles

Lipid mixtures of indicated composition in chlroform:methanol (20:1, v/v) were evaporated to dryness under a stream of N$_2$. The dried lipids were suspended by vortexing in TAE buffer and passed through a membrane extruder (LIPEX, Northern Lipids, Inc., Vancouver, Canada) fitted with a 100 nm filter to prepare lipid vesicles. Lipid suspensions were passed (x4) through a 200 nm filter and then (x4) through the 100 nm filter. The passage of lipid suspension through the filter causes formation of lipid vesicles of approximately 100 nm in diameter. The vesicle suspension was stored at 4° C and brought to experimental temperature before use. If any small unilamellar vesicles (<30 nm) were present, they would spontaneously fuse to form larger vesicles during storage at 4° C, which may be below the phase transition temperature of vesicle lipid mixtures.

Isolation of lamellar bodies

Lungs of anesthetized (Nembutal, 50mg/Kg, ip) and exsanguinated male Sprague-Dawley rats (~200g) were ventilated and cleared of residual blood by perfusion through the pulmonary artery with phosphate buffered saline containing 10 mM glucose. The visibly cleared lungs were instilled with sucrose (1M, unbuffered), harvested, and homogenized in 1M sucrose. Lamellar bodies from lung homogenate (10%) were isolated by upward flotation on a discontinuous sucrose density gradient, as described previously [38]. We routinely recover approximately 80–100 μg protein from one rat. We have previously shown in several studies that thus isolated lamellar bodies from lungs or type II cells show good homogeneity and purity as determined by electron microscopy and biochemical characterization [12,25,38,39]. The isolated lamellar bodies were used for the binding and membrane fusion studies.

Lamellar body enrichment with PIP$_2$ and DAG

Isolated lamellar bodies were incubated for 30 min at room temperature with either suspension of 100% PIP$_2$ or DAG, or PLV containing PC:PE:PS:Cholesterol and PIP$_2$ or DAG (42.5:17.5:25:10:5) and 10μM Ca^{2+} in 0.2 M sucrose in 50mM TAE buffer (pH 7.4). Following incubation, the lamellar bodies were separated by centrifugation for 10 min at 14,000rpm, washed twice with TAE-sucrose containing 10 μM Ca^{2+}, and evaluated for the membrane fusion activity. Aliquots of thus incubated lamellar bodies were analyzed for PIP$_2$ by dot blot analysis using a polyclonal PIP$_2$ antibody (1:1000 dilution). The lipid of lamellar bodies were extracted [40] and analyzed for DAG by thin layer chromatography on silica gel G plates. The lipids were identified after exposure to I$_2$ vapors and by co-migration with authentic lipids.

Tryptophan Fluorescence Studies

Protein tryptophan fluorescence of Annexin A7 was measured as described previously [22]. Triplicate fluorescence spectra of about 1 μM protein in TAE buffer (pH 8.3) were obtained in the absence or presence of 20 μM PLV. Fluorescence was measured first in the absence of Ca^{2+} and then in the presence of indicated concentrations of Ca^{2+} by sequential addition of 100 mM CaCl$_2$ to provide the calculated free Ca^{2+} [41]. Calcium electrode was used to verify 5 μM or higher (up to 100 μM) Ca^{2+}. All fluorescence results were corrected for dilutions, and emission due to buffer or other additions.

Proteolysis Studies

Trypsin or chymotrypsin proteolysis was performed for 1 or 4 h at 37° C. The reaction mixture (30 μl) contained 265.6 μg/ml annexin A7, and 6.56 μg/ml trypsin (15 units/μg protein) or 0.66 μg/ml chymotrypsin (81 units/mg protein), and indicated concentrations of PLV without or with 1mM Ca^{2+} in TAE buffer (pH 8.3). The protease reaction was stopped with the addition of appropriate amounts of loading buffer for SDS-PAGE. The proteins were resolved by electrophoresis, and quantified by photo imaging of Coomassie blue-stained gels using co-run recombinant annexin A7 as standard. In some experiments, proteins were transferred to PVDF membranes, stained with Coomassie blue and relevant protein bands were excised for N-terminal sequence analysis, which was performed by Edman degradation (Procise Protein Sequencer, Applied Biosystems, Inc.) at the Iowa State University Protein Sequence facility.

Lamellar Body Binding of Annexin A7

All binding studies were conducted by co-sedimentation assay as described previously [22]. Briefly, the lamellar bodies (7 μg protein) without or with incubation for enrichment with DAG or PIP$_2$ (see above) were washed and incubated for 10 min at room temperature with 10 μg annexin A7 and 10 μM Ca^{2+} in TAE buffer in 200 μl total volume. The binding reaction was stopped by centrifugation (10 min at 14,000 rpm), the lamellar body pellet was washed (x2, each with 200 μl followed by centrifugation as described above) with the incubation buffer containing appropriate amounts of Ca^{2+}, and the bound annexin A7 quantified after separation on SDS-PAGE and photo imaging of the Coomassie blue-stained gels. Protein binding to PLV containing DAG or PIP$_2$ was assayed by similar protocol in which 45 μM PLV was used in place of lamellar bodies.

Membrane Fusion

Change in transfer efficiency of resonance energy due to lipid mixing was used as a measure of fusion between PLV or lamellar bodies with labeled PLV containing NBD-PE and Rh-PE, the donor and acceptor molecules for resonance energy, as described previously [25]. The reaction mixture (1.5 ml) contained 2 μM labeled PLV (PC:PE:PS:NBD-PE:Rh-PE, 65:10:25:0.75:0.75) and unlabeled 40 μM PLV of indicated composition, or 30 μg lamellar body protein. Changes in fluorescence of the reaction mixture were measured as a function of

time after each addition. The fusion reaction was initiated with the addition of 10 μM Ca^{2+} and fluorescence monitored for 10 min. The reaction was terminated by adding 1% Triton X-100 to obtain maximum dilution of the probe. The fluorescence change during 10 min was expressed as percent of maximum (after addition of Triton X-100).

Other analyses

Proteins were quantified by the protein-dye binding assay according to Bradford [42] using bovine-γ-globulin as standard.

Statistical Analysis

Comparison between experimental and control groups were performed by Student's *t* test for paired or unpaired observations, as appropriate. ANOVA was performed for comparison within multiple groups, which was followed by Tukey's post-hoc test for comparison between two groups. P value <0.05 was considered significant.

RESULTS

Tryptophan Fluorescence Studies

Our initial studies were aimed to determine if membrane lipid composition would affect molecular organization of annexin A7. Our previous studies with annexin A7 and its deletion mutants showed that the presence of PLV significantly modified the Ca^{2+}-dependent increase in protein fluorescence, which occurs possibly due to molecular reorganization and the position of its tryptophan residue relative to other residues that can quench fluorescence [22,23]. In the present study, we evaluated if PLV composition would affect Ca^{2+}-dependent changes in protein fluorescence. The protein and phospholipid concentrations were kept low to ensure negligible inner filter effect on fluorescence. None of the PLV affected the protein fluorescence without calcium. The addition of 0.1mM Ca^{2+} without PLV caused a slight increase in fluorescence (not shown; [22]). However, in the presence of 20 μM PLV (PC:PS, 3:1), Ca^{2+} caused a large increase in protein fluorescence (Fig. 1A). The emission due to PLV was unaffected with Ca^{2+}. Substitution of PC with PE in these PLV caused greater increase in fluorescence suggesting that the two lipids differentially influenced molecular organization and the tryptophan residue in annexin A7. Figure 1B shows fluorescence changes upon addition of 0.1 mM Ca^{2+} in presence of different PLVs and figure 1C shows dependence of fluorescence change on Ca^{2+} concentrations achieved with sequential additions of 100 mM Ca^{2+}. The increase in fluorescence with PC:PE:PS (2:1:1) PLV, which was tested since biological membranes contain PC and PE in about 2:1 ratio, was intermediate between those seen with PC:PS (3:1) and PE:PS (3:1) (Fig. 1B and C). In this experiment, the protein concentration was half (20μg/ml, ~0.5 μM) of that used with other PLV. The fluorescence increases with Cholesterol-PLV (PC:PE:PS:cholesterol, 45:20:25:10) and PC:PS (3:1) were similar. Interestingly, the presence of 5% PIP_2 in cholesterol-PLV caused a large increase that was equivalent to that seen with PE:PS (3:1). Similar change in fluorescence was observed with cholesterol-PLV containing 5% DAG (not shown). In order to facilitate comparison amongst different PLV, the increase is expressed as percent of fluorescence without Ca^{2+} (Fig. 1D). The increase was maximum with PE:PS (3:1) and minimum with PC:PS (3:1) PLV. Thus, these studies clearly suggested that the lipid composition of membranes affected the molecular organization of the protein and that minor components like PIP_2 or DAG can significantly affect protein interaction with membranes.

Membrane domains enriched with PIP_2 are postulated as sites for interaction and membrane insertion for some proteins [27–30]. Modified lipids, like dansylPE (DPE), that act as acceptors of energy transfer and quench the fluorescence of reporter (tryptophan) residue in close vicinity have been used to demonstrate membrane insertion of the reporter into vesicle membranes

[30]. Next, we exploited the protein fluorescence quenching with DPE as an indicator of change in molecular organization. In these studies, the Ca^{2+}-dependent change in protein fluorescence was measured in the presence of one PLV (20 μM) and then after addition of similar concentration of second PLV. The cholesterol-PLV composition for these studies was PC:PE:PS:Cholesterol (45:20:25:10). The composition of PLV containing 5% lipid X (PIP_2, DAG, or DPE) was PC:PE:PS:Cholesterol:X (42.5:17.5:25:10:5). In presence of cholesterol-PLV, Ca^{2+} (0.1mM) increased the protein fluorescence by 97% (Fig. 2A), which was reduced by only 3% with DPE-PLV. Similar experiments with PIP_2-PLV (Fig. 2B) and DAG-PLV (Fig. 2C) showed respective increases of 272% and 140% with Ca^{2+} that were decreased by 7% and 13%, respectively, after addition of DPE-PLV. In comparison, Ca^{2+} increased the fluorescence only by 39% with DPE-PLV, which was further increased to 64% with PIP_2-PLV (Fig. 2D). These observations suggest that 1) annexin A7 could form bridge between *trans* membranes of different lipid compositions, and 2) the protein could insert into membranes of specific lipid composition. The presence of DAG in membranes could facilitate such membrane insertion of the protein.

Proteolysis of Annexin A7 – Protease-specific Protection by Phospholipid

Annexin A7 contains several potential sites for trypsin and chymotrypsin enzymes. We have previously shown that the presence of PLV protects against trypsin proteolysis possibly by restricting access to the enzyme sites by masking them or by modifying the protein molecular organization [22]. Here we evaluated if the lipid composition of PLV affected the protection against trypsin and chymotrypsin. In the presence of PC:PS (3:1), the presence of 1mM Ca^{2+} increased trypsin proteolysis of annexin A7 during 1h incubation at 20 μM PLV (Fig 3A). At higher concentrations of PLV and during both 1 and 4h incubations, trypsin proteolysis was lower in presence of Ca^{2+} (not shown). During the 4h proteolysis in presence of 0.2 mM PC:PS (3:1), two major products at 28, and 33kDa were detected in the absence of Ca^{2+} (Fig. 3B, arrowheads). In comparison, trypsin proteolysis was lower with 0.2 mM PE:PS (3:1) and showed greatly diminished formation of 28kDa peptide. While Ca^{2+} did not provide additional protection with PE:PS (3:1), except at high trypsin concentration, its additional protective effect was apparent with PC:PS (3:1) at all annexin A7 to trypsin ratios and was near maximum at a ratio of 40 (Fig. 3C). Subsequent studies were undertaken at annexin A7 to trypsin ratio of 40 and at 0.2 mM PLV with varying compositions (Fig. 3D). Trypsin proteolysis in the presence of DAG-PLV was lower than that with cholesterol-PLV or PIP_2-PLV and was similar to that with PE:PS (3:1). In all cases, the Ca^{2+}-dependent protection against trypsin was reflected in decreased appearance of the 33kDa and 28kDa peptides (Fig. 3D) suggesting that the effect was due to lipid interaction with the parent protein and not with its degradation product. Examination of the proteolysis pattern did not reveal any new major peptide, suggesting that all types of PLV protected against the same site. The only notable difference was in the amounts of 28kDa peptide since its relative proportion was much lower when proteolysis was performed in presence of DAG-PLV or PE:PS (3:1). Thus, cell stimulation and degradation of PIP_2 to generate IP_3 (for increased Ca^{2+}) and DAG would cause decreased accessibility of trypsin sites suggesting further modification of the annexin A7 molecule. These results suggest that protein interaction with specific phospholipid domains in biological membranes would modify annexin A7 conformation and possibly regulate protein function.

The chymotrypsin proteolysis generated two major peptides of 37 and 30 kDa in presence of PC:PS (3:1) or PE:PS (3:1) PLV. At 20 μM PLV, the proteolysis in presence of Ca^{2+} was greater (not shown). At higher PLV concentrations, however, the degradation in presence of Ca^{2+} was lower (Fig. 3E). Better protection was observed in presence of PE:PS (3:1) in comparison to the PC:PS (3:1) PLV. The degradation was similar in presence of 0.2 mM of either PLV and in the absence or presence of Ca^{2+} at varying ratios of annexin A7/enzyme (not shown). None of the three different PLVs that were evaluated for these studies showed

significant differences in the proteolysis products (Fig. 3F). The major effect of PLV was on degradation of the 30 kDa peptide, which was found to increase in presence of Ca^{2+}. To determine the possible trypsin and chymotrypsin sites, we obtained the N-terminal sequence for the major peptides at 33 and 28 kDa from trypsin and 37 and 30 kDa from chymotrypsin proteolysis (Table 1). Since the N-terminal sequence for trypsin generated peptides matched the N-terminal sequence of recombinant annexin A7, we conclude that the two potential trypsin sites are present in the COOH-terminus. The sequences of chymotrypsin generated peptides were compared with the potential site sequences according to the PeptideCutter program (ExPASY Proteomics Server, Swiss Institute of Bioinformatics, Geneva, Switzerland). Such comparison indicated that the potential sites were at Y110 and F160 for the 37 kDa and the 33 kDa peptides, respectively, both of which are in the NH_2-terminus of annexin A7 [22].

Membrane Binding and Fusion Studies with Lamellar bodies

Because the presence of PIP_2 or DAG in PLV affected protein fluorescence of annexin A7, we investigated if lamellar body enrichment with PIP_2 or DAG would affect binding and membrane fusion activities of annexin A7. In initial studies, we investigated membrane binding activity using PIP_2 or DAG containing PLV. Co-sedimentation analysis showed that the Ca^{2+}-independent protein binding was higher for DAG-PLV in comparison to that for PIP_2-PLV (not shown). The Ca2+-dependent membrane association of protein was similar for Cholesterol-PLV without or with DAG, but lower for PIP_2-PLV. Next, we evaluated protein binding to lamellar bodies that were pre-incubated without or with DAG or PIP_2. Such treatment caused lamellar body enrichment with either lipid as confirmed by TLC analysis for DAG and visualization after exposure to I_2, or by dot blot analysis for PIP_2 with a commercially available antibody (Fig. 4A and B). Annexin A7 binding was higher for lamellar bodies that were incubated with DAG, but not for those incubated with PIP_2 (Fig. 4C).

Initial studies with PLV containing various amounts of DAG or PIP_2 and 2 μM labeled PLV (the fluorescence probe) showed that the membrane fusion activity of annexin A7 increased with increasing DAG concentration reaching a maximum at 60 μM DAG and then declined at higher concentrations (Fig. 5A). In comparison, the membrane fusion activity of annexin A7 increased with increasing concentration of PIP_2. In some experiments, lamellar bodies were added along with DAG (or PIP_2) and the Ca2+-dependent fusion activity was assayed. In this mixed assay system, the fusion activity of annexin A7 was higher in the presence than in the absence of lamellar bodies. Increased fusion in presence of lamellar bodies and DAG is likely because of rapid incorporation of DAG in lamellar bodies. In some experiments, as discussed above for the protein binding studies, the lamellar bodies were incubated with PIP_2 or DAG and separated by centrifugation before evaluation for the membrane fusion activity of annexin A7. The Ca^{2+}-dependent membrane fusion activity was also higher with DAG-enriched lamellar bodies in comparison to those not enriched (control) or enriched with PIP_2 (Fig. 5B). However, the membrane fusion activity of annexin A7 was augmented if the lamellar bodies were pre-incubated with high concentration of PIP_2 (0.2 mM). In another series of experiments, we incubated lamellar bodies without or with 0.2 mM PIP_2 and treated them with PC-PLC or PI-PLC before evaluation for annexin A7-mediated membrane fusion. The commercial preparations of these enzymes were first evaluated for the formation of DAG in PLV containing zero or 5% PIP_2 without or with 10 μM Ca^{2+}. The PI-PLC formed DAG with PIP_2-PLV but not with Cholesterol-PLV suggesting that it was specific for PIP_2. We did not observe any additional effect of Ca2+ on DAG formation. Treatment of lamellar bodies with either PLC increased the membrane fusion activity of annexin A7 (Fig. 5C). Although the membrane fusion activity was higher with PIP_2-enriched lamellar bodies, their treatment with PI-PLC further increased the fusion activity of annexin A7. Thus, several protocols for increasing DAG content of lamellar bodies increased the membrane fusion activity of annexin A7.

DISCUSSION

In this study, we have presented evidence for a novel role for DAG in annexin A7-mediated fusion of lamellar bodies that is postulated to occur during surfactant secretion. Although a role for DAG and other lipids in signal transduction has long been recognized [31,33], it is only during the last decade that a role for phosphoinositides in membrane-associated functions of proteins has been investigated (reviewed in [43]). Some protein motifs show high affinity and specificity towards inositol lipids [44,45]. Several investigators over the last few years have postulated that specific regions of membranes containing cholesterol [46] or phosphoinositide molecules [28,30] could be preferred sites for interactions with certain proteins that facilitate or participate in the membrane fusion process. Annexin A2 [27–29] and synaptotagmin [47] show affinity towards membranes containing PIP_2, which forms microdomains in syntaxin clusters in the membranes [48]. Since phosphoinositide turnover is rapid and involves phosphorylation of inositol phosphatides and degradation through phospholipase and phosphohydrolase enzymes [43], it raises the question of a role for DAG in membrane binding and membrane-associated functions of a protein. In many cell types including type II cells, DAG levels increase following cell stimulation with agents that stimulate PI-PLC and cause hydrolysis of PIP_2 [31]. Our current study shows that DAG in membranes increases annexin A7 binding and activity and supports the concept of membrane lipids modifying protein (annexin A7) interaction and function. Such interaction between DAG and protein could alter the molecular organization of the protein with important consequence for the protein function. In type II cells, purinergic and other agents increase cell DAG levels and surfactant secretion. Thus, the DAG regulation of annexin A7 function could have physiological relevance during fusion of lamellar bodies (with plasma membrane) for increasing surfactant secretion.

The Ca^{2+}-dependent membrane binding characteristics have led to several postulated functions for annexin proteins [20]. These proteins demonstrate selectivity in binding to membranes [25,26,46,49,50], suggesting that the differences in lipid composition of intracellular membranes could contribute to the site-specific actions of these proteins. Since the tryptophan residue (W239) in annexin A7 is present in the second homologous repeat in the core domain of the molecule, changes in protein tryptophan fluorescence [51,52] would be indicative of molecular reorganization involving this repeat and the various amino acid residues in close vicinity of the tryptophan residue (embedded or surface-exposed). A large change in protein fluorescence with substitution of PC with PE (Fig. 1B), as major phospholipid, suggests change in protein conformation and in the mode of annexin A7 interaction with membranes. In our studies, the increase in protein fluorescence in presence of Cholesterol-PLV (with lipid composition closer to biological membranes) was similar to that seen with PC:PS (3:1). However, inclusion of low levels of signaling lipids, PIP_2 or DAG, caused large increases in protein fluorescence (Fig. 1C and 2B and C) suggesting that these lipids could have a role in regulating the protein function by affecting the annexin A7 molecule. Previously, interaction between annexin and Ca^{2+} was postulated to alter the conformation of the protein core domain in order to explain annexin interactions with membranes [53]. We now propose that interaction with specific membrane lipids would cause further change in the protein molecule and accessibility of specific protein domains for relevant protein functions. Since Ca^{2+} is required to affect these changes, such reorganization of annexin A7 molecule probably occurs upon cell stimulation.

Given the large variety of physiological functions assigned to annexin proteins and the Ca^{2+}-dependence of protein binding to membranes, several studies employing atomic force microscopy and electron microscopy techniques have showed that, at least in the binary lipid mixture containing PLV, annexin A2 and A5, assemble as 2-dimensional structures along the membrane without penetrating the membranes [54–57]. Our studies demonstrating DPE

quenching of tryptophan fluorescence, however, suggest that tryptophan-containing domain of annexin A7 can penetrate into membranes of appropriate lipid composition. Similar conclusion can be drawn from smaller increase in protein fluorescence with DPE-PLV (39%, Fig. 2D) in comparison to that with cholesterol-PLV (97%, Fig. 2A). Although our protocol differed from that used for synaptotagmin [30], annexin A7 appears to interact with *trans* membranes and penetrate DPE-containing membranes provided the *cis* membranes contained DAG. Thus, activation of PLC-mediated hydrolysis of PIP_2 and formation of DAG would cause further reorganization of annexin A7 bound or in close vicinity of membrane and its penetration into the membrane. Possibly such change in molecular organization facilitates the membrane binding and fusion activity of annexin A7 in vivo.

The proteolysis studies also suggest that lipid composition of membranes could affect protein molecule and, consequently, the accessibility of protease sites. Previous studies have suggested that stable or transient interaction with Ca^{2+} could expose unique protein domains for the protein function [53,58,59]. Annexin proteins show high affinity binding to PS (reviewed in [17]) and have specific PS binding sequences in their core domains [21]. One previous study investigated PS interaction with annexin A4 and A6 by measuring protein effects on the surface pressure of PS monolayer and by evaluating PS protection against proteolysis [60]. The authors suggested that electrostatic interaction between PS and Ca^{2+}-annexin complex could alter the conformation of annexin core domain. However, protein immobilization at surface could also contribute to the inhibition of proteolysis. Thus, additional factors could modify the protein conformation, although partial membrane penetration of protein(s) could still be one underlying cause of protection against proteolysis. Our study demonstrates that other lipids, in addition to PS, also modulate molecular characteristics of protein, which in some cases, results in partial membrane penetration, possibly to facilitate annexin A7 function. However, we cannot exclude that these effects of other lipids (including DAG) require PS, which was present in all PLV, in light of several studies suggesting that annexin proteins do not exhibit significant binding to lipids like PE or PC (reviewed in [17]). Further, since the DAG effect on proteolysis was distinct from PIP_2, we postulate that additional molecular organization of annexin A7 occurs following PIP_2 hydrolysis to DAG.

Molecular changes during Ca^{2+}-bridging [58] and membrane interactions could promote stable protein binding to membranes as observed with DAG containing membranes including lamellar bodies (Fig. 4C). However, in contrast to higher binding of annexin A2 [28], the binding of annexin A7 to PIP_2 containing membranes was lower (Fig. 5) suggesting different modes of membrane interaction of these two annexin proteins that are postulated to promote membrane fusion during lamellar body exocytosis. Certain proteins can bind to PIP_2 through well-defined motifs like the plextrin homology domain [43], while other proteins like annexin A2 and actin binding proteins (gelsolin, vilin and MARCKS proteins) could bind through a motif containing cationic residues [28]. Given the high degree of homology in the core domain of annexin proteins, it is likely that the unique NH_2-termini of these proteins contribute to this difference in binding to PIP_2-membranes. Although PIP_2 increases membrane-protein interactions as suggested by a large increase in fluorescence (Figs. 1 and 2), it decreased annexin A7 binding to membranes (Fig. 4C). The underlying cause for the decreased binding is unclear, but we speculate that PIP_2 enrichment causes a change in the mode of protein binding to membranes, membrane insertion or electrostatic binding, as previously suggested for annexin A5 [61] and annexin A2 [62] to explain pH effect on protein-membrane interaction. Nevertheless, lamellar bodies appear to bind more annexin A7 than required for membrane fusion, since decreased binding (Fig. 4C) did not reflect in decreased membrane fusion (Fig. 5B). Thus, only a part of the bound annexin A7 may be involved in membrane fusion. The membrane fusion activity of annexin A7 was higher for DAG-enriched PLV or lamellar bodies suggesting that annexin A7 activity may be primarily regulated by DAG. The biphasic effects of DAG concentration, increased fusion at low and decreased fusion at high concentrations, are possibly due to

membrane destabilization with high concentrations of DAG [63] or due to alterations in membrane characteristics necessary for supporting fusion. On the other hand, membrane fusion increased with PIP_2 concentration and also in lamellar bodies that were preincubated with 0.2 mM PIP_2, which possibly increases the PIP_2 content of lamellar bodies to sufficiently high levels.

In intact cells, the facilitating effects of DAG on annexin A7-mediated membrane fusion are possibly mediated through multiple mechanisms because of DAG activation of PKC, which causes increased secretion of lung surfactant [34,35,37,64]. Annexin A7 binding to lamellar bodies or plasma membrane from calcium ionophore- or PMA-treated type II cells – both agents activate PKC – is increased [12]. Annexin A7 can also be phosphorylated with PKC, which is associated with increased membrane fusion activity, and the level of phosphorylated annexin A7 correlates well with increased secretion in chromaffin cells [11]. Because of the new evidence supporting a membrane fusion modifying role for DAG, we speculate that by forming annexin A7-binding domains within fusing membranes and modifying the conformation of the bound protein, which may be amenable to PKC-mediated phosphorylation, DAG contributes to promoting annexin A7-mediated membrane fusion during secretion in some cell types.

In conclusion, our studies demonstrate that the annexin A7 interactions with membranes are regulated by lipids other than PS, notably PIP_2 and DAG that undergo changes upon cell stimulation. The regulation of annexin A7 activity is a novel function for DAG that occurs at the level of the protein-membrane interaction and affects the binding and membrane fusion activities of this protein. The postulated role for DAG fits well in the overall scheme of surfactant secretion in alveolar type II cells, which upon stimulation with several agents show increased DAG formation (concomitant with PIP_2 and PC hydrolysis) and increased secretion.

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Abbreviations

DAG

diacylglycerol

DPE

dansyl phosphatidylethanolamine

F_{345}

fluorescence at 345 nm

PC

phosphatidylcholine

PE

phosphatidylethanolamine

PIP_2

phosphatidylinositol-4,5-bisphosphate

PI

phosphatidylinositol

PS

phosphatidylserine
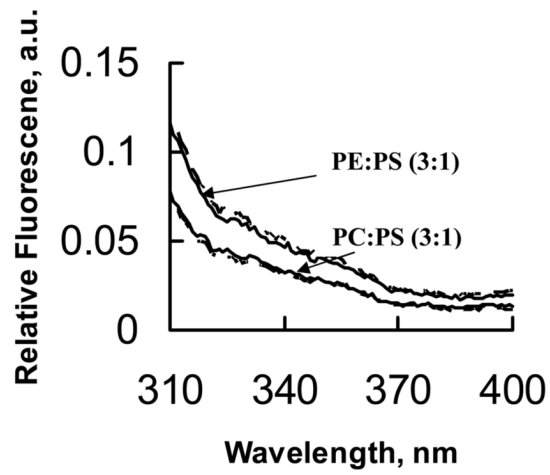
PKC

protein kinase C

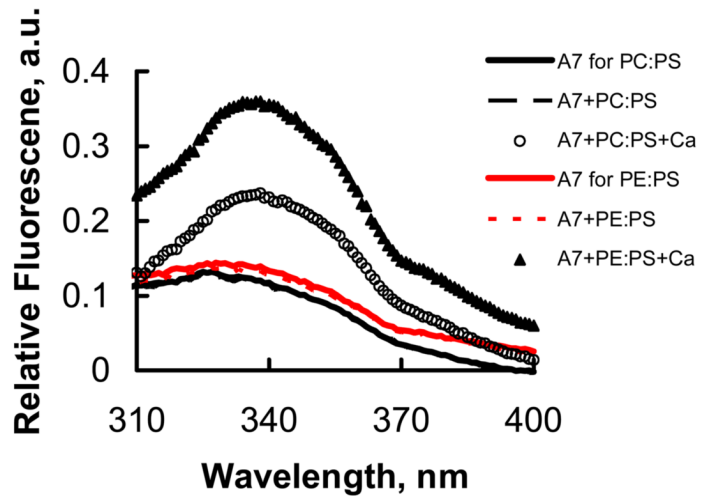**TAE**

Tris-acetate-EGTA

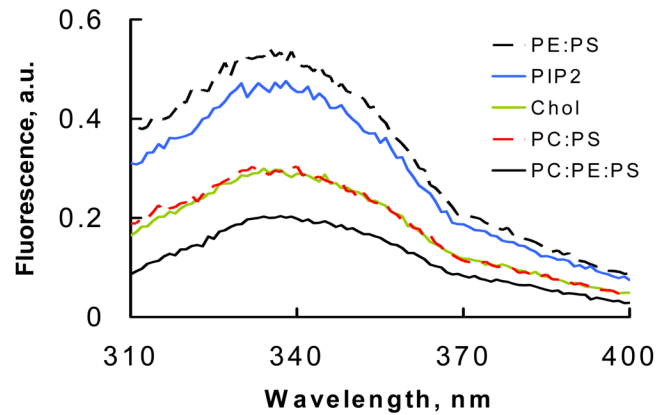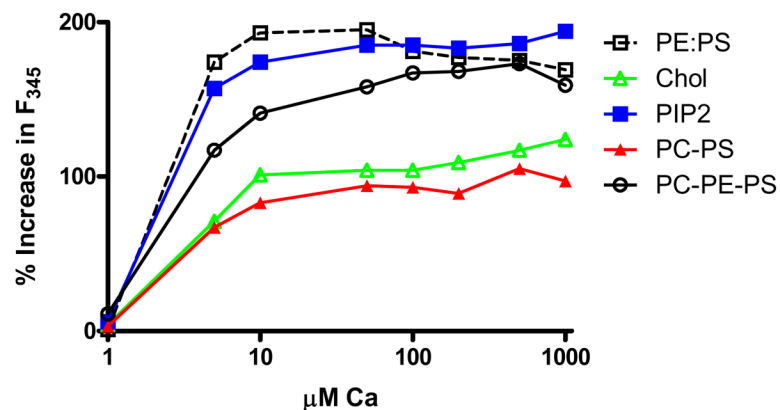Figure 1A

Figure 1B

Figure 1C

Figure 1D

Figure 1.
PLV composition affects annexin A7 protein fluorescence. **A.** Emission spectra for indicated PLV (20 μM) without (solid line) or with (broken line) 0.1 mM Ca^{2+}. **B**. Fluorescence spectra are shown for ~ 1 μM annexin A7 with indicated additions. The spectra were obtained before and after addition of 20 μM PLV and then after addition of 0.1 mM Ca^{2+}. The Ca^{2+}–dependent increase in protein fluorescence was greater with PE:PS (◇) in comparison to that with PC:PS (▲). **C.** Fluorescence spectra of annexin A7 (40 μg/ml) in presence of indicated PLV (20 μM) and 0.1 mM Ca^{2+} are shown. The spectra were unaffected by PLV without Ca^{2+}. In experiment with PC:PE:PS, the annexin A7 concentration was ~0.5 μM. **D**. The change in annexin A7 fluorescence at 345 nm (F_{345}) with 20 μM vesicles and various concentrations of Ca^{2+} is expressed as percent of F_{345} without Ca^{2+}. For all studies, the PLV composition was PC:PS, 3:1; PE:PS, 3:1; PC:PE:PS, 2:1:1;PC:PE:PS:Cholesterol, 45:20:25:10; and PC:PE:PS:Cholesterol:PIP_2, 42.5:17.5:25:10:5. These experiments were replicated at least

once with similar results. All results were corrected for emission changes due to buffer, PLV, or dilutions.

Figure 2A
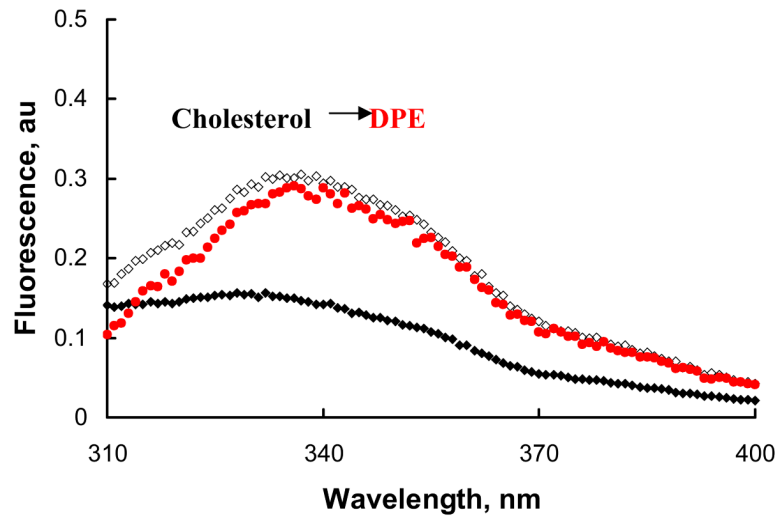
Figure 2B
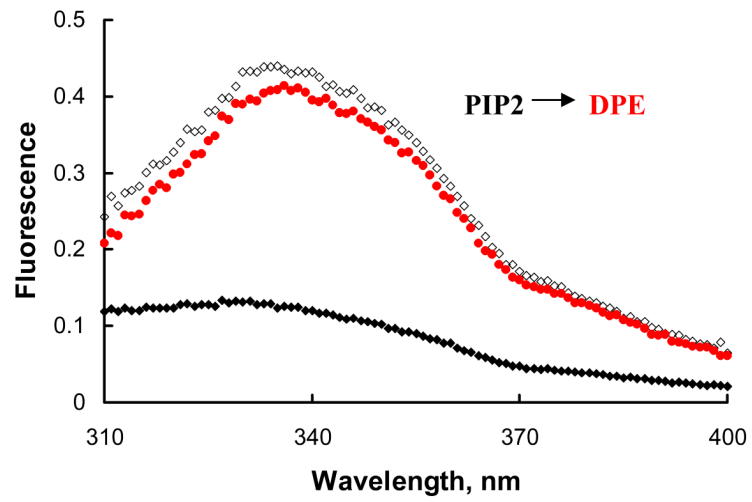
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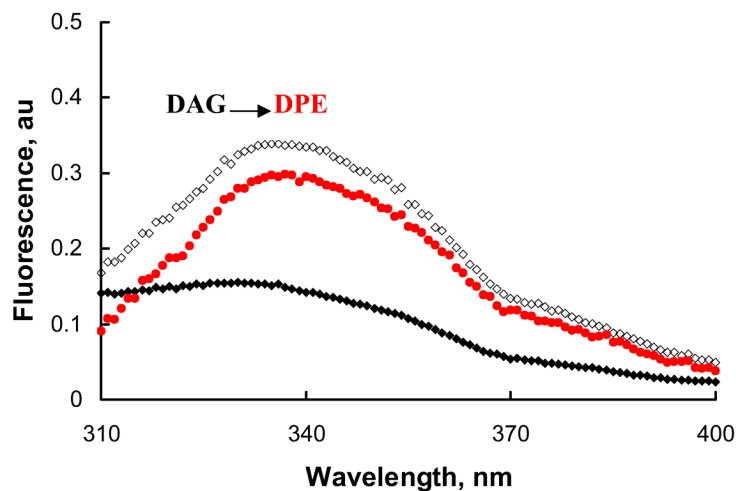
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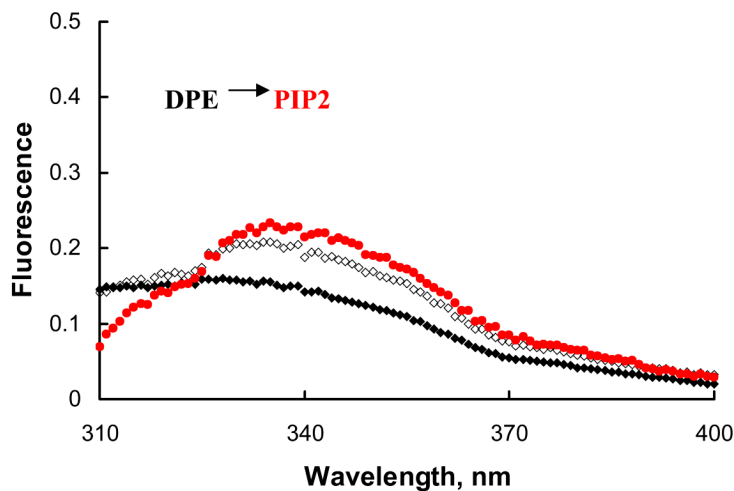
Figure 2.
Fluorescence quenching studies with dansyl phosphatidylethanolamine (DPE) demonstrate annexin A7 interaction with *trans* membranes. Corrected fluorescence spectra for annexin A7 (~1 μM) are shown before (filled symbol) and after the addition of 0.1 mM Ca^{2+} (open symbols) in the presence of 0.02 mM PLV of indicated composition (black). The second spectrum (red) was acquired after addition of the second PLV in the same fluorescence cuvette. The basic composition for all PLV was PC:PE:PS:Cholesterol (45:20:25:10) indicated as Chol. The other PLV contained PC:PE:PS:Cholesterol:X (42.5:17.5:20:10:5), where X was PIP_2, DAG, or DPE. All experiments were repeated at least once with similar results. **A**. Cholesterol-PLV

followed by DPE-PLV, **B**. PIP2-PLV followed by DPE-PLV, **C**. DAG-PLV followed by DPE-PLV, and **D.** DPE-PLV followed by PIP$_2$-PLV.

Figure 3A
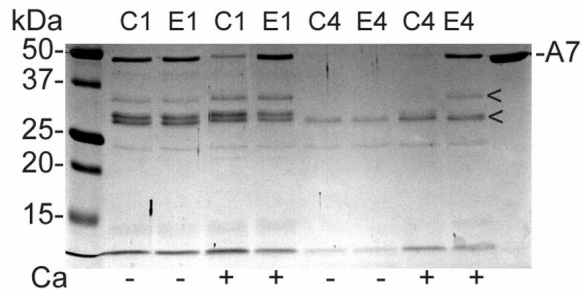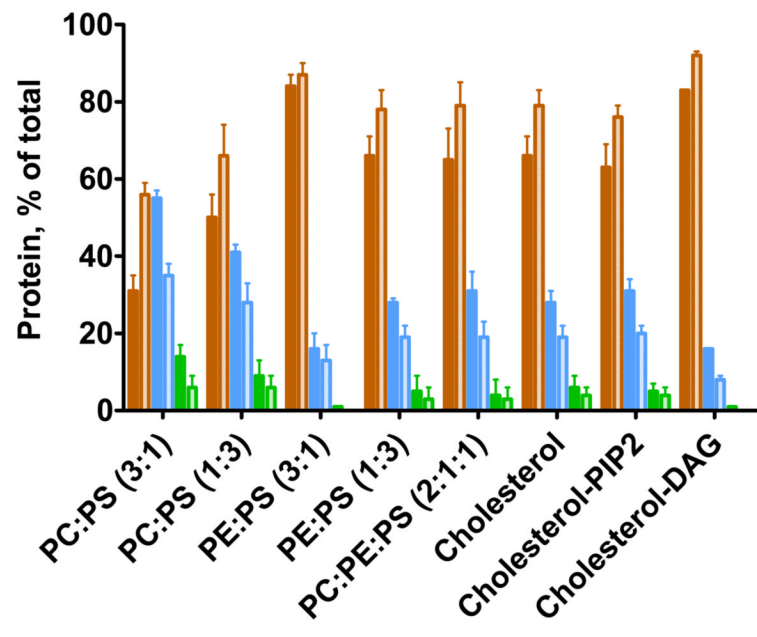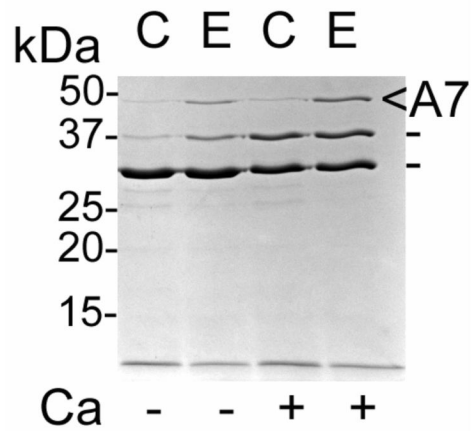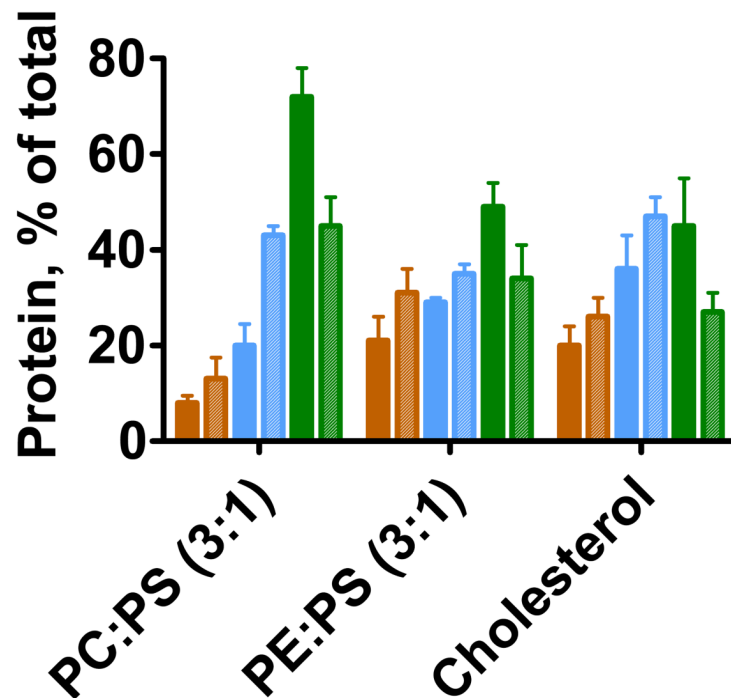

Figure 3B
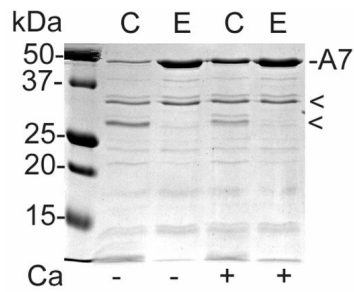
Figure 3C
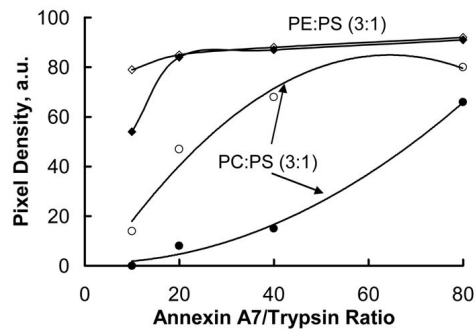
Figure 3D

Figure 3E

Figure 3F

Figure 3.
Membrane lipid composition modifies limited proteolysis of annexin A7. **A**. Trypsin proteolysis of annexin A7 in the absence or presence of 1 mM Ca^{2+} and in the presence of 20 μM PLV with either C (PC:PS, 3:1) or E (PE:PS, 3:1) composition. Incubations were conducted for 1 (C1 and E1) or 4 h (C4 or E4) at 37° C. **B**. Ca^{2+}-dependence of annexin A7 proteolysis with trypsin (4 h, 37° C) in presence of 200 μM PLV of C (PC:PS, 3:1) or E (PE:PS, 3:1) composition without or with 1 mM Ca^{2+} and at annexin A7/trypsin ratio of 40 showed higher levels of annexin A7 in comparison to that in presence of 20 μM PLV (see **A** above). **C.** Ca^{2+} effect on annexin A7 remaining after trypsin proteolysis for 4h in the presence of 200μM PLV and without (●, ◆) or with (○, ◇) 1mM Ca^{2+}. The reaction was conducted using equal amounts of annexin A7 but variable amounts of trypsin. Note that the Ca^{2+} effect in presence of PC:PS (3:1) PLV was observed at all concentrations of trypsin. **D.** Limited proteolysis of annexin A7 with trypsin was performed for 4 h in the presence of 0.2 mM PLV of indicated composition and without (filled bar) or with 1 mM Ca^{2+} (hatched bar). The proteins were quantified after SDS-PAGE and photo imaging against annexin A7 standard. Cholesterol-PLV (PC:PE:PS:cholesterol, 45:20:25:10) contained zero or 5% PIP_2 (Cholesterol-PIP2) or DAG (Cholesterol-DAG) with concomitant reduction in PC and PE to 42.5 and 17.5%, respectively. Note that annexin A7 protection against trypsin was mostly by blocking formation of 33 kDa peptide. The presence of DAG, but not PIP_2, provides further protection against tryspin. Annexin A7, 47 kDa (■); Peptide 33kDa (■): Peptide 28kDa (■). Results are mean ± SE of 3–5 experiments. **E**. Limited proteolysis of annexin A7 with chymotrypsin (4 h, 37° C) in presence of 0.2 mM PLV with compositions described in **A** above and in the absence or presence of 1 mM Ca^{2+}. Major proteolysis products at 37 and 33 kDa are indicated by – in the right margin relative to the position of annexin A7 (47 kDa): **F**. Comparison of PLV with

different compositions showed smaller effects of Ca^{2+} with no preference for any of the three compositions evaluated. Annexin A7 ( ), Peptide 37 kDa ( ) and Peptide 33 kDa ( ). The proteolysis was performed without (filled bars) or with 1 mM Ca^{2+} (hatched bars). Results are mean ± SE of three experiments.
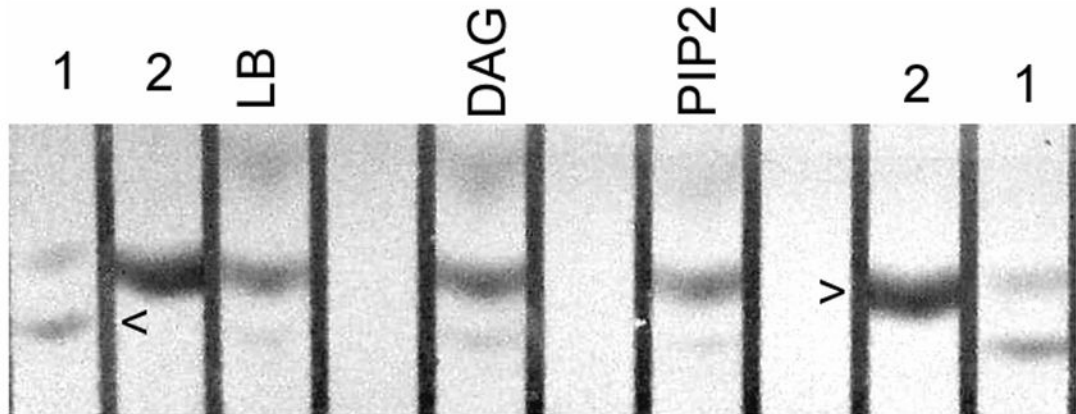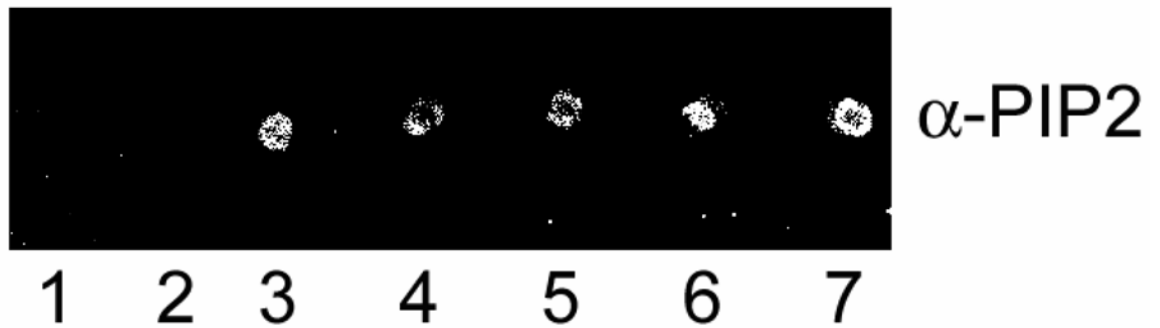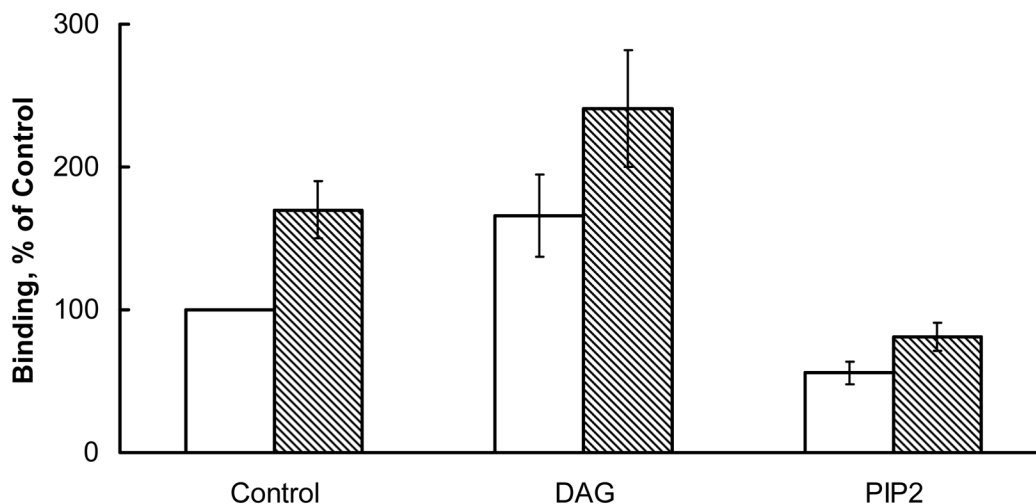
Figure 4A

Figure 4B

Figure 4C

Figure 4.
Annexin A7 binding to lamellar bodies is augmented with increased diacylglycerol content.
Incubation of lamellar bodies with DAG or PIP$_2$ increased the respective lipid content of
lamellar bodies. **A**. Lamellar bodies (LB) were incubated at room temperature for 30 min with
60 μM DAG or PIP$_2$ and 10 μM Ca^{2+}. The LB were separated by centrifugation, washed, and
their lipids analyzed for DAG by TLC. Authentic standards (arrowheads) for DAG (1) and
cholesterol (2) were co-chromatographed. **B**. Dot blot analysis of lamellar bodies incubated
with DAG or PIP$_2$. Lamellar bodies were incubated without (3) or with 1mM PLV containing
5% DAG (4) or PIP$_2$ (5), or with 0.2 mM suspension of DAG (6) or PIP$_2$ (7). Equal aliquots
were spotted for dot blot analysis. Controls with buffer (1) or BSA (2) did not show any
reactivity. PLV composition was PC:PE:PS:Cholesterol (42.5,17.5:25:10 and 5% DAG or
PIP$_2$). **C**. Annexin A7 binding to DAG and PIP$_2$-enriched lamellar bodies. Isolated lung
lamellar bodies were incubated for 10 min without or with 60 μM DAG or PIP$_2$, separated by
centrifugation, washed, and evaluated for binding of annexin A7 in the absence (open bar) or
presence (hatched bar) of 10 μM Ca^{2+}. Results are expressed relative to binding in the absence
of Ca^{2+} and are mean ± SE of 3–5 experiments. The presence of Ca^{2+} increased protein binding
in all cases. In comparison to others, the DAG-enriched lamellar bodies showed higher annexin
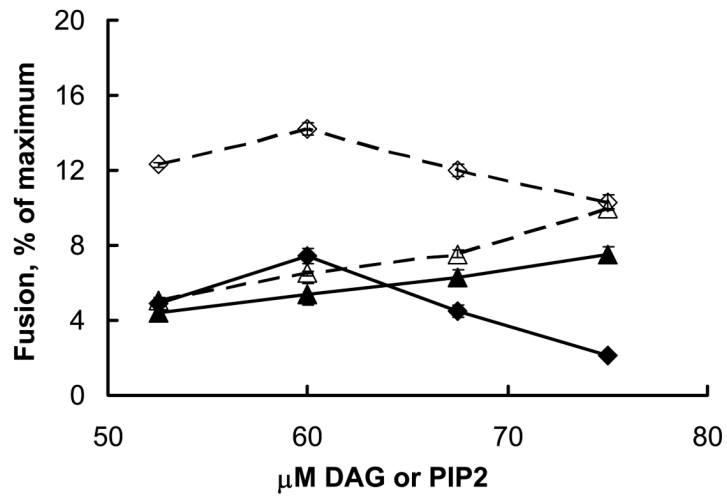A7 binding without or with Ca^{2+}.
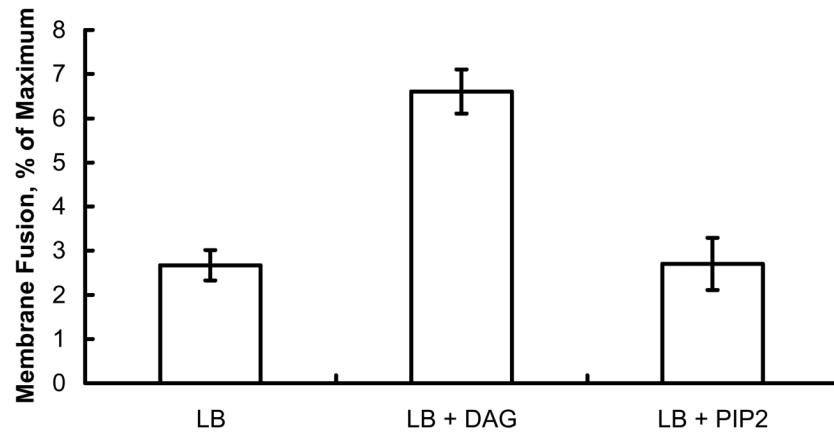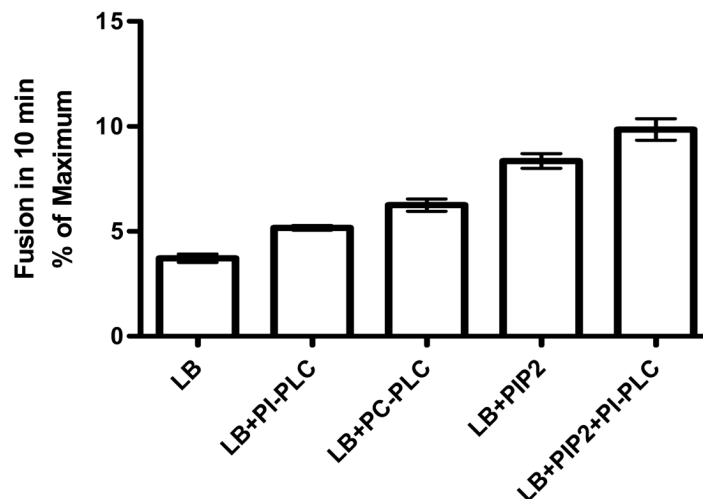
Figure 5A

Figure 5B

Figure 5C

Figure 5.
Annexin A7 mediated membrane fusion was measured by lipid mixing technique by measuring change in the transfer efficiency of resonance energy. All assay mixtures contained 10 μg/ml annexin A7 and 2 μM of labeled PLV containing NBD-PE and Rh-PEand suspension of indicated concentrations of DAG, PIP_2, or lamellar bodies (20 μg/ml). The fusion reaction was initiated with 10 μM Ca^{2+}. Fusion is expressed as fluorescence increase during 10 min as percent of maximum increase with 1% Triton X-100. **A**. Concentration-dependent effects of DAG (◆ , ◇) or PIP_2 (▲ , Δ) in the absence (closed symbols) or presence (open symbols) of lamellar bodies. The assay mixture contained a fixed concentration of labeled PLV. The presence of lamellar bodies caused consistently greater fusion at each concentration of DAG suggesting rapid incorporation of DAG into lamellar bodies, which enhanced the membrane fusion activity. Mean ± SE of 3–4 reactions. In case of 75 μM PIP_2 + lamellar bodies, the fusion was measured only once. **B**. Lamellar body fusion with PLV following incubation with DAG or PIP_2 (60 μM). The membrane fusion was enhanced with lamellar bodies preincubated with DAG. Mean ± SE of 3–4 experiments in each case. **C**. Lamellar bodies were pre-incubated for 30 min without or with 0.2 mM PIP_2 and 10 μM Ca^{2+}, pelleted by centrifugation, washed twice with buffer containing 10 μM Ca^{2+} and treated for 10 min without or with 1 unit/ml of PI-PLC or PC-PLC. Thus treated lamellar bodies were washed twice and evaluated for membrane fusion with annexin A7 and 10 μM Ca^{2+}. Results are mean ± SE of three separate observations. PLC pretreatment increased membrane fusion in each case.

Table 1

N-terminal sequence of major peptides obtained after limited proteolysis of annexin A7 with trypsin or chymotrypsin.

	Trypsin			Chymotrypsin	
	Site	N-terminal Sequence	Site	N-terminal Sequence	
Peptide 1	U	G S ? Y P (33 kDa)	Y110	– G – P A	(37 kDa)
Peptide 2	U	G S ? Y P (28 kDa)	F160	D A – R D	(33 kDa)

The proteolysis products were separated by SDS-PAGE, transferred to PVDF membranes and stained with Coomassie blue. The relevant bands were excised and processed for N-terminal sequencing. The N-terminal sequence of recombinant annexin A7 was verified to be GSSYP. The potential sequences were GSSYP for both trypsin generated and GGGPA and DAMRD for the chymotrypsin generated 37 and 33 kDa peptides, respectively. U – unknown, but possibly in the COOH-terminus. The undetermined sequence is indicated by ? and – indicates a non-matching residue.