Fusion of Lamellar Body with Plasma Membrane Is Driven by the Dual Action of Annexin II Tetramer and Arachidonic Acid

Received for publication, December 10, 2002, and in revised form, August 1, 2003
Published, JBC Papers in Press, August 5, 2003, DOI 10.1074/jbc.M212594200

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Annexin II has been implicated in membrane fusion during the exocytosis of lamellar bodies from alveolar epithelial type II cells. Most previous studies were based on the fusion assays by using model membranes. In the present study, we investigated annexin II-mediated membrane fusion by using isolated lamellar bodies and plasma membrane as determined by the release of octadecyl rhodamine B (R18) self-quenching. Immunodepletion of annexin II from type II cell cytosol reduced its fusion activity. Purified annexin II tetramer (AlII) induced the fusion of lamellar bodies with the plasma membrane in a dose-dependent manner. This fusion is Ca2+-dependent and is highly specific to AlII because other annexins (I and II monomer, III, IV, V, and VI) were unable to induce the fusion. Modification of the different functional residues of AlII by N-ethylmaleimide, nitric oxide, or peroxynitrite abolished AlII-mediated fusion. Arachidonic acid enhanced AlII-mediated fusion and reduced its Ca2+ requirement to an intracellularly achievable level. This effect is due to membrane-bound arachidonic acid, not free arachidonic acid. Other fatty acids including linolenic acid, palmitoleic acid, myristoleic acid, stearic acid, palmitic acid, and myristic acid had little effect. AlII-mediated fusion was suppressed by the removal of arachidonic acid from lamellar body and plasma membrane using bovine serum albumin. The addition of arachidonic acid back to the arachidonic acid-depleted membranes restored its fusion activity. Our results suggest that the fusion between lamellar bodies with the plasma membrane is driven by the synergistic action of AlII and arachidonic acid.

Lung surfactant is a surface active material synthesized and secreted by cuboidal alveolar type II cells (1–3). It is composed of phospholipids, mainly dipalmitoylphosphatidylcholine, and surfactant proteins A–D. It minimizes the surface tension at the air-liquid interface by inserting phospholipids between water molecules. As a result, surfactant disrupts the cohesive hydrogen bonds and prevents them from exerting high molecular forces at the alveolar surface. Deficiency of dipalmi-
tophosphatidylcholine in the alveolar surface has been associated with infant respiratory distress syndrome.

The secretion of surfactant by type II cells involves the translocation, docking, and fusion of lamellar bodies to the plasma membrane. For the lamellar body content to reach its extracellular destination, a continuity of the vesicular lumen and extracellular fluid must be established. This continuity is maintained through the formation of a narrow pore similar to membrane channels (4–6). The formation of this pore is a complex event involving physical and chemical factors and is regulated by intracellular Ca2+ and proteins (7). Studies on mast cells and chromaffin cells revealed that the opening of fusion pore and extrusion of granule content is influenced by an osmotic force depending upon the osmolarity of the surrounding solution (8).

Annexin II plays multidimensional roles in different aspects of vesicular trafficking and exocytosis (9–11). It has been implicated in Ca2+-dependent membrane fusion during exocytosis in a variety of cells including chromaffin cells and alveolar type II cells (12–14). Annexin II binds to negatively charged phospholipids and mediates the aggregation and fusion of liposomes in the presence of Ca2+ (13, 15). Annexin II exists as a monomer (AlI, 36-kDa) or heterotetramer (AlII, two AlI monomers plus two 11-kDa subunits) (16). Formation of AlII reduces its Ca2+ requirement for membrane aggregation (16). Other members of the annexin family have been studied regarding their membrane fusion activity in different cells, for example annexin I in neutrophils (17) and annexin VII in chromaffin cells and alveolar type II cells (18–20). However, these annexins require higher than normal physiological Ca2+ concentrations.

The activity of annexin II is regulated by post-translational modifications and other cofactors. Serine or tyrosine residues near the N terminus of annexin II can be phosphorylated by protein kinase C and tyrosine kinase pp60(Src), respectively. In vitro phosphorylation of these sites reduces the ability of this protein to aggregate vesicles without affecting its membrane binding capacity (21, 22). We have shown recently (23, 24) that the modification of cysteine or tyrosine residues of annexin II by nitric oxide or peroxynitrite leads to the loss of its liposome aggregation activity. Addition of cholesterol to liposome enhances the ability of AlII to bind with and aggregate phosphatidylserine vesicles at submicromolar Ca2+ levels. Cholesterol also alters the subcellular localization of AlII at low Ca2+ concentrations (25, 26). Arachidonic acid enhances AlII-mediated fusion in model membranes (13). The mechanisms are not clear, but arachidonic acid itself acts as a fusogen (27–29).

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1 The abbreviations used are: AlI, annexin II monomer; AlII, annexin II tetramer; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; DTPA, diethylenetriaminepentaaacetic acid; BSA, bovine serum albumin; AA, arachidonic acid; GSNO, S-nitroso-L-glutathione; LB, lamellar body; PM, plasma membrane.
Annexin II and Membrane Fusion

Fig. 1. Depletion of annexin II from alveolar type II cell cytosol reduces its membrane fusion activity. Annexin II was removed from alveolar type II cell cytosol (100 μg) by immunoprecipitation using 2 μg of affinity-purified anti-annexin II antibodies (TII + Ab). Controls were treated the same way except that 2 μl of pre-serum (TII + pre-serum) or no antibodies (TII + beads) were added. These fractions were tested for the presence of annexin II by Western blot (A) and membrane fusion activity (B). Fusion was assayed by using unlabeled lamellar body membranes (5 μg/ml) in Ca2⁺-EGTA buffer containing 1 mM free Ca2⁺. In the add-back experiments, the fusion was initiated by the annexin II-depleted type II cytosol at 100 s, and 5 μg of purified AIIt was added back at 120 s or at 300 s. Fusion was expressed as a percentage of the maximal fluorescence. LB + PM only represents the control that no AIIt was added.

We have shown previously that AIIt can promote the fusion of lamellar bodies with liposomes, and this fusion is dependent on the composition of liposomes (13). Other groups also used liposomes to mimic granule membrane or plasma membrane (10, 15, 16, 21, 22, 25). However, a direct biochemical analysis of the ability of AIIt to fuse plasma membrane with lamellar bodies or other granule membranes has not been reported. In this report, we established a reproducible and sensitive in vitro fusion assay of lamellar bodies and the plasma membrane. By using this assay, we performed a detailed analysis for AIIt-mediated fusion of lamellar bodies with the plasma membrane.

EXPERIMENTAL PROCEDURES

Materials—Octadecyl rhodamine B chloride (R18) was obtained from Molecular Probes (Eugene, OR). DEAE-Sepharose CL 6B, Sephacryl S-300, Mono S, Mono Q, and enhanced chemiluminescence (ECL) reagents were from Amersham Biosciences. Maclura pumifera agglutinin gel was from EY Laboratories (San Mateo, CA). Protein G Plus-agarose was from Santa Cruz Biotechnology (Santa Cruz, CA). Arachidonic acid (AA, C20:4), linolenic acid (C18:3), palmitoleic acid (C16:1), myristoleic acid (C14:1), steraric acid (C18:0), palmitic acid (C16:0), and myristic acid (C14:0) were from Matreya (Pleasant Gap, PA), and eicosanoic acid (C14:1), palmitoleic acid (C16:1), myristoleic acid (C14:0) were from Matreya (Pleasant Gap, PA), and eicosanoic acid was from Aldrich (Deerfield, IL). N-Ethylmaleimide (NEM), phenylmethylsulfonyl fluoride (PMSF), and diethylenetriaminepentaacetic acid (DTPA) were obtained from Sigma. S-Nitroso-l-gluthathione (GSNO) was from Cayman Chemicals (Ann Arbor, MI). Bovine serum albumin (BSA, fatty acid-free) was purchased from JRH Biosciences (Lenexa, KS). Peroxynitrite was synthesized as described previously (23). Annexin II antibodies were generated using purified bovine annexin II and affinity-purified (30).

Preparation of Plasma Membrane—Sprague-Dawley rat lung was perfused with phosphate-buffered saline to remove blood. The lung tissue was then blotted with filter paper and homogenized with 10 ml of buffer B (0.32 M sucrose plus buffer A) (10 mM Na-Pi, pH 7.4, 30 mM NaCl, 1 mM MgCl₂, 5 μM PMSF, 0.02% NaN₃, and 10 μg/ml DNAse). Homogenate was loaded on the top of a discontinuous sucrose gradient (0.5, 0.7, 0.9, and 1.2 M) and centrifuged at 95,000 × g for 60 min. After collecting the band from the interface between 1.2 and 0.9 M sucrose gradient, sucrose concentration was measured by a refractometer (model 334610, Spectronic Instruments) and diluted to 0.32 M with cold buffer A. The plasma membrane fraction was centrifuged at 120,000 × g for 30 min, and the pellet was resuspended in buffer B. To separate the inside-out vesicles from the outside-out vesicles, 1 mg of plasma membrane in 300 μl of buffer B was incubated with 300 μl of M. pumifera agglutinin beads at 4 °C for 4 h by end-to-end rotation. The outside-out vesicles bound to the M. pumifera agglutinin beads were removed by centrifugation at 2,500 rpm for 5 min using an Eppendorf microcentrifuge. The inside-out plasma membrane vesicles in the supernatant were used for the membrane fusion assay.

Isolation of Lamellar Body—Lamellar bodies were isolated from rat lung tissue by upward flotation on a discontinuous sucrose gradient as described by Chandler et al. (31). A perfused rat lung was homogenized with 10 ml of 1.0 M sucrose and loaded at the bottom of a sucrose gradient (0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 M). After centrifugation at 80,000 × g for 3 h at 4 °C, the lamellar body fraction was collected from the interface between 0.4 and 0.5 M sucrose gradient. This fraction was diluted to 0.24 M sucrose with cold water and centrifuged at 20,000 × g for 30 min. The pellet was resuspended in 0.24 M sucrose containing 10 mM Tris, 50 mM Hepes (pH 7.0).

Preparation of Tissue and Cell Cytosol—Lung, kidney, liver, pancreas, spleen, and brain were dissected from Sprague-Dawley rats. These organs were homogenized in homogenization buffer (20 mM Tris-HCl (pH 7.4), 2 mM EGTA, and 150 mM NaCl) containing protease inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotanin, and 1 mM benzamidine). The homogenate was centrifuged at 100,000 × g for 1 h at 4 °C, and the supernatant was collected as cytosol. Type II cells were isolated from 180 to 200 × g Sprague-Dawley rats according to the method of Dobbs et al. (32) as described previously (33). Type II cells were lysed in homogenization buffer by sonication on ice for 20 s and subjected to centrifugation in the same way to collect tissue cytosol.

Purification of Annexins—Annexins were purified from bovine lung tissue through sequential column chromatography using DEAE-Sepharose CL6B, Sephacryl S300, and Mono S columns by the method of Khanna et al. (34) as described previously (33).

Immunodepletion of Annexin II from Type II Cell Cytosol—One hundred micrograms of type II cell cytosol were incubated with 2 μg of affinity-purified polyclonal anti-annexin II antibody at 4 °C for 2 h. 20 μl of protein G Plus-agarose beads was added to the mixture and incubated overnight at 4 °C with gentle shaking. Protein G beads (20 μl) were pre-treated with 1.0 mg of BSA at 4 °C for 1 h to reduce nonspecific binding before use. Antibody-bound beads were washed...
three times with the homogenization buffer. The controls were treated the same way with pre-serum or without any addition of antibodies. The annexin II-depleted cytosol was tested for the presence of annexin II by Western blot and for its fusogenic activity.

Isolation of Mitochondria and Microsomes—Sprague-Dawley rat lung was homogenized in 10 ml of 0.32 M sucrose containing 10 mM Tris-HCl (pH 7.4), 0.15 mM NaCl, 1 mM MgSO₄, 2 mM EGTA, and 1 mM CaCl₂ along with a protease inhibitor mixture (1 mM PMSF, 10 mM aprotinin, and 1 mM benzamidine). The homogenate was centrifuged at 300,000 g for 10 min. The supernatant was further centrifuged at 8,000 g for 10 min, and the pellet was collected as mitochondrial. The remaining supernatant was spun down at 20,000 × g for 20 min, and the pellet was then discarded to remove the other subcellular organelles (other than microsomes). The supernatant was centrifuged at 80,000 × g for 3 h, and the microsomal fractions were collected from the pellet (35).

Incorporation of R18 into Plasma Membrane—A 20 mM stock solution of R18 was prepared in ethanol, and 1 µl of this stock solution was injected using a 10-µl syringe to 300 µl of plasma membrane in 300 µl of buffer B (the same buffer used in plasma membrane preparation) while vortexing. The mixture was incubated in the dark at room temperature for 1 h by end-to-end rotation. To remove the excess R18, the labeled plasma membrane was dialyzed against 3 liters of buffer B overnight at 4 °C in the dark.

Membrane Fusion Assay—Membrane fusion between lamellar bodies with the plasma membrane was measured according to the method of Hoekstra et al. (36). In this method, plasma membrane was labeled with the fluorescent dye, R18, at a self-quenching concentration. Fusion of lamellar bodies with the plasma membrane resulted in the relief of self-quenching of R18 and thus an increase in the R18 fluorescence. R18-labeled plasma membrane was mixed with unlabeled lamellar bodies in 1 ml of Ca²⁺-EGTA buffer at 37 °C under a continuous stirring. A base line was recorded for 2 min, and annexins or cytosolic fractions were added. Fluorescence was recorded for an additional 6 min at excitation and emission wavelengths of 560 and 590 nm, respectively. The maximal fluorescence was achieved by the addition of 0.1% (v/v) Triton X-100. Fusion was calculated as a percentage of the maximal fluorescence and corrected for sample dilution when appropriate.

Western Blot—Protein samples were separated on 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane at 100 mA for 2 h. The blot was blocked with Tris-buffered saline (TBS) containing 2% (w/v) gelatin overnight. After blocking, the blot was incubated with polyclonal anti-annexin II antibodies (1:1000 dilution) in TTBS (TBS plus 0.05%, v/v, Tween 20) containing 1% (w/v) gelatin for 2 h. The blot was washed three times with TTBS and incubated with goat horseradish peroxidase-conjugated anti-rabbit IgG (1:5000 dilution) for 2 h. Finally, the blot was visualized by developing it in ECL reagents. Signals on the blot were quantified by densitometric analysis using Bio-Rad Quantity One 4.0.3 software.

Extraction and Gas Chromatographic Analysis of Free Fatty Acids in Plasma Membrane and Lamellar Body—Methyl esterification and isolation of free fatty acids was performed according to the method of Liebich et al. (37). 5 ml of high pressure liquid chromatography grade methanol was added to 150 µg of plasma membrane or lamellar body in presence of 3 µg of eicosanoic acids as an internal standard. This mixture was placed inside screw-capped vials. 100 µl of acetyl chloride was added and continuously shaken at 25 °C for 45 min using a shaking incubator. The solution was neutralized with 2 ml of 6% (w/v) K₂CO₃.
followed by the addition of 500 μl of n-hexane. The mixture was shaken and centrifuged at 1,800 × g for 10 min, and the supernatants were collected for GC analysis. Separation of fatty acid methyl esters was performed on a PerkinElmer Life Sciences Autosystem XL gas chromatograph using a 30 m × 0.53 mm (inner diameter) DB-FFAP column from Agilent J&W Scientific (Palo Alto, CA). Helium was used as a carrier gas at a flow rate of 9.3 ml/min, and the flame ionization detector was supplied with hydrogen (40 ml/min) and air (450 ml/min). Free fatty acids were quantified by comparing their peak area with the corresponding peak area of pure standards, which were subjected to the same procedures.

Other Procedures—Protein concentration was determined by the method of Bradford (38) using bovine γ-globulin as standard. Phospholipid content in plasma membrane and lamellar body was directly measured colorimetrically, based on the formation of a complex between phospholipids and ammonium ferriochromate according to the method of Stewart (39). Absorbance of the unknown solution was read at 488 nm and compared with known amounts of a standard phospholipid solution.

RESULTS

Type II Cell Cytosol-mediated Fusion—We first established an in vitro assay for membrane fusion of lamellar bodies with the plasma membrane using the fluorescent probe, octadecyl rhodamine (R18) (36). To increase the sensitivity of the fusion assay, outside-out plasma membrane vesicles, which cannot participate in the fusion, were separated from inside-out vesicles using M. pomifera agglutinin-conjugated beads. M. pomifera agglutinin has been shown to bind specifically to the luminal surface of alveolar type II cells (40, 41), and thus inside-out vesicles cannot bind to M. pomifera agglutinin. Type II cell cytosol caused a significant fusion of lamellar bodies with the plasma membrane (Fig. 1A). Annexin II exists in alveolar type II cells and is involved in lung surfactant secretion (13, 33). To test directly whether annexin II is the protein responsible for the type II cell cytosol-mediated fusion, we removed annexin II from type II cell cytosol by immunoprecipitation using affinity-purified anti-annexin II antibodies. One fraction of the annexin II-immunodepleted cytosol was tested for the presence of annexin II, and the other was subjected to the membrane fusion assay. Western blot showed that most of annexin II was removed from the immunodepleted type II cell cytosol (Fig. 1A). The fusion activity of the same fraction was markedly reduced

Fig. 3. Allt and lamellar body dose dependence of Allt-mediated membrane fusion. A and B, unlabeled lamellar body (10 μg/ml) was mixed with R18-labeled plasma membrane (5 μg/ml) in 1 ml of Ca²⁺-EGTA buffer containing 1 mM free Ca²⁺. Membrane fusion was initiated by the addition of various amounts of Allt. C and D, unlabeled lamellar body (5–30 μg/ml) was mixed with R18-labeled plasma membrane (5 μg/ml) in 1 ml of Ca²⁺-EGTA buffer containing 1 mM free Ca²⁺. Membrane fusion was initiated by the addition of 10 μg to Allt. The fusion is expressed as a percentage of the maximal fluorescence. A and C show the time course and B and D show the fusion content at 6 min.
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**Figure 4.** Specificity of AIIt-mediated fusion of lamellar bodies with the plasma membrane. **A**, annexin specificity. Unlabeled lamellar body (10 µg/ml) was mixed with R18-labeled plasma membrane (5 µg/ml) in 1 ml of Ca\(^{2+}\)-EGTA buffer containing 1 mM free Ca\(^{2+}\). Membrane fusion was initiated by the addition of 10 µg of annexins I–VI and is expressed as a percentage of the maximal fluorescence at 6 min. Data shown are mean ± S.E. from three separate experiments. **B**, membrane specificity. R18-labeled plasma membrane (5 µg/ml) was mixed with lamellar body, mitochondria or microsome (10 µg/ml each) in 1 ml of Ca\(^{2+}\)-EGTA buffer containing 1 mM free Ca\(^{2+}\). Membrane fusion was initiated by the addition of 10 µg AIIt and is expressed as a percentage of the maximal fluorescence.

(Fig. 1B), indicating that annexin II is the major protein to mediate the fusion of lamellar bodies with the plasma membrane in type II cells. The addition of 5 µg of AIIt to the immunodepleted type II cell cytosol resulted in a partial recovery in the membrane fusion.

**Membrane Fusion with Different Tissue Cytosolic Fractions**—We next tested the effect of cytosols prepared from different rat tissues on the fusion of lamellar bodies with the plasma membrane. As shown in Fig. 2A, all cytosols including lung, kidney, liver, pancreas, spleen, and brain show significant fusion activities. The kidney cytosol had the highest capability to induce this heterotypic fusion. To test whether annexin II in the cytosols is responsible for the fusion activity observed, we determined annexin II levels in the cytosol preparations using Western blot and we quantitated by densitometry. The cytosols from lung and spleen showed the highest level of annexin II, whereas the kidney showed the lowest (Fig. 2, A and B). The result suggests that the proteins mediating membrane fusion may vary among different tissues. It is worthwhile to note that type II cell cytosol had a much higher fusion activity than lung cytosol (compare Fig. 1 and Fig. 2 where 100 µg of type II cell cytosol and 1 mg of lung cytosol were used).

**AIIt-dependent Fusion of Lamellar Bodies with the Plasma Membrane**—We purified AIIt and tested to see whether it causes the fusion of lamellar bodies with the plasma membrane by using the assay described above. In the presence of 1 mM Ca\(^{2+}\), AIIt causes a dose-dependent fusion (Fig. 3, A and B). At 40 µg of AIIt ~40% fusion was observed at 6 min. AIIt-mediated fusion of lamellar bodies with the plasma membrane was also lamellar body dose-dependent (Fig. 3, C and D). We chose 10 µg of lamellar body, 5 µg of plasma membrane, and 10 µg of AIIt as our standard conditions for subsequent experiments unless otherwise noted.

**Specificity of AIIt**—We further assessed the capability of different annexins in the induction of fusion between lamellar bodies with the plasma membrane. Except for AIIt, none of annexins tested (annexins I and II monomer, III–VI) had significant fusion activities (Fig. 4A). This result suggests that the fusogenic event of lamellar bodies with the plasma membrane is highly specific to AIIt. Furthermore, AIIt-mediated fusion is specific to lamellar bodies because the substitution of lamellar bodies with mitochondria or microsome resulted in no fusion with the plasma membrane (Fig. 4B).

**Inhibition of AIIt-mediated Fusion by AIIt Inhibitors**—NEM is known to modify cysteine residues of AIIt (42). Incubation of AIIt with 1 mM NEM resulted in a significant inhibition of AIIt-mediated fusion of lamellar bodies with the plasma membrane. AIIt can be also modified by protein nitration and S-nitrosylation (23, 24). The treatment of AIIt with GSNO (a nitric oxide donor) or peroxynitrite (a nitrating agent) led to a suppression of its fusion activity (Table I). Peroxynitrite is extremely unstable and decomposed rapidly in a buffer at physiological pH. The decomposed peroxynitrite in 0.1 mM phosphate buffer (15 min at room temperature) did not affect AIIt-mediated fusion (data not shown).

**Arachidonic Acid Enhances AIIt-mediated Fusion**—Al fusion assays above were performed in the assay buffer containing 1 mM Ca\(^{2+}\). We further tested the Ca\(^{2+}\) requirement for AIIt-mediated fusion of lamellar bodies with the plasma membrane. Fig. 5A shows that AIIt only caused the fusion at over 100 µM Ca\(^{2+}\). Because intracellular Ca\(^{2+}\) concentration is much lower than this, there must be other factors regulating the activity of AIIt in cells. Arachidonic acid has a stimulatory effect on type II cell secretory process (43) and is a potential

| Inhibitors | Fusion (mean ± S.E.) | % inhibition |
|-----------|----------------------|--------------|
| None      | 16.16 ± 1.80         | 0            |
| NEM       | 4.01 ± 0.12          | 75           |
| ONOO−     | 3.73 ± 1.25          | 77           |
| GSNO      | 3.31 ± 0.55          | 80           |
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A lamellar bodies with the plasma membrane. Arachidonic acid markedly increased AIIt-mediated fusion. At 10–100 μM arachidonic acid and AIIt produces a bigger synergistic effect on the fusion. Arachidonic acid enhanced AIIt-mediated fusion in a concentration-dependent manner (Fig. 5B). The maximum fusion occurred at 45 μM arachidonic acid.

We also examined the effects of other free fatty acids on AIIt-mediated fusion. Results indicated that other fatty acids have little or no effect on the fusion between lamellar bodies and the plasma membrane in the presence or absence of AIIt (Fig. 5C).

The experiments above were to add arachidonic acid directly to the membrane fusion assay buffer containing lamellar bodies and plasma membrane. To determine whether membrane-bound arachidonic acid or free arachidonic acid contributes to the arachidonic acid stimulation, plasma membrane and lamellar body were separately pre-incubated for 30 min with arachidonic acid, and free arachidonic acid was removed by centrifugation. In the presence of AIIt, the fusion was not different whether free arachidonic acid was removed or not (Fig. 6A), indicating that membrane-bound arachidonic acid is responsible for the stimulatory effect. Plasma membrane-bound arachidonic acid (AA-PM) appears to increase the fusion more than lamellar body-bound arachidonic acid (AA-LB) when assayed with untreated lamellar bodies or untreated plasma membrane, respectively. The different effects of AA-LB and AA-PM on the fusion are not due to the lipid concentrations because we used approximately the same concentrations of lipids for lamellar body limited membrane and plasma membrane in this experiment. Our plasma membrane preparation used for the fusion assay contained 1.6 μg of lipid/μg of proteins as determined by the method of Stewart (39). The lipid/protein ratio of lamellar body limiting membrane is 1.5 (w/w) (13). Interestingly, a robust increase was observed in the absence of AIIt when AA-LB and AA-PM were used for membrane fusion assay. This is not due to pre-incubation time of AIIt with arachidonic acid because we did not observe any differences when we pre-incubated AIIt with arachidonic acid for 1 or 30 min. However, we observed that this robust increase varied among the membrane preparations. If the basal fusion between untreated LB and untreated PM in the absence of AIIt is low, the fusion between AA-LB and AA-PM in the absence of AIIt is smaller. If the basal fusion is high, the fusion between AA-LB and AA-PM is bigger (data not shown). This is likely due to variations of endogenous annexin II associated with membrane preparations, although precise mechanisms remain to be determined.

Depletion of Arachidonic Acid Reduces AIIt-mediated Fusion—We further examined whether the removal of arachidonic acid from natural membranes influences AIIt-mediated fusion. We incubated lamellar bodies or plasma membrane

Fig. 5. Effect of arachidonic acid on AIIt-mediated fusion of lamellar bodies with the plasma membrane. A, Ca²⁺ dose dependence. Arachidonic acid (45 μM) was incubated with unlabeled lamellar body (10 μg/ml) and R18-labeled plasma membrane (5 μg/ml) in 1 ml of Ca²⁺-EGTA buffer containing 1–1000 μM free Ca²⁺ for 5 min at 37°C, and AIIt was then added to induce fusion. ●, AIIt; ○, arachidonic acid; ▼, AIIt + arachidonic acid. B, arachidonic acid dose dependence. Various concentrations of arachidonic acid were incubated with the same amount of lamellar body and plasma membrane as described above in 1 ml of Ca²⁺-EGTA buffer containing 10 μM free Ca²⁺ for 5 min at 37°C. AIIt (10 μg) was added to evoke the fusion. Fusion was expressed as a percentage of the maximal fluorescence at 6 min. Data shown are mean ± S.E. from three experiments. C, effect of free fatty acids on AIIt-mediated membrane fusion of lamellar bodies with the plasma membrane. Fusion assay was done in the same way as described in the B except 45 μM different fatty acids were used. Assay buffer, Ca²⁺-EGTA buffer containing 10 μM free Ca²⁺; AIIt, 10 μg. Data shown are mean ± S.E. from three separate experiments.

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\caption{Effect of arachidonic acid on AIIt-mediated fusion of lamellar bodies with the plasma membrane. A, Ca²⁺ dose dependence. Arachidonic acid (45 μM) was incubated with unlabeled lamellar body (10 μg/ml) and R18-labeled plasma membrane (5 μg/ml) in 1 ml of Ca²⁺-EGTA buffer containing 1–1000 μM free Ca²⁺ for 5 min at 37°C, and AIIt was then added to induce fusion. ●, AIIt; ○, arachidonic acid; ▼, AIIt + arachidonic acid. B, arachidonic acid dose dependence. Various concentrations of arachidonic acid were incubated with the same amount of lamellar body and plasma membrane as described above in 1 ml of Ca²⁺-EGTA buffer containing 10 μM free Ca²⁺ for 5 min at 37°C. AIIt (10 μg) was added to evoke the fusion. Fusion was expressed as a percentage of the maximal fluorescence at 6 min. Data shown are mean ± S.E. from three experiments. C, effect of free fatty acids on AIIt-mediated membrane fusion of lamellar bodies with the plasma membrane. Fusion assay was done in the same way as described in the B except 45 μM different fatty acids were used. Assay buffer, Ca²⁺-EGTA buffer containing 10 μM free Ca²⁺; AIIt, 10 μg. Data shown are mean ± S.E. from three separate experiments.}
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inhibition (~70%). The addition of arachidonic acid back to the BSA-treated membrane restored the membrane fusion at various degrees compared with the untreated membrane.

**Free Fatty Acid Content in Plasma Membrane and Lamellar Body**—Arachidonic acid and other free fatty acids were determined in plasma membrane and lamellar body by gas chromatography. We observed that lamellar body contains almost all the free fatty acids in a greater amount than plasma membrane (Table II), likely due to the lipid content inside the lamellar bodies. Linolenic acid was undetected in plasma membrane. A major loss in arachidonic acid and other free fatty acids, except palmitoleic acid, was evident when lamellar body and plasma membrane were treated with BSA. The removal of fatty acids by BSA was more effective for lamellar body than plasma membrane.

**DISCUSSION**

Lamellar body, the specialized organelle in lung type II cells, enables the secretion of surfactant by a process of Ca\(^{2+}\)-regulated exocytotic mechanism. Involvement of the cytoplasmic proteins, such as annexin, in the exocytotic process of alveolar cells and other cells has been studied intensely (9–11, 44). However, precise mechanisms of AIIt-mediated fusion of plasma membrane and lamellar body in this exocytotic process are far from being elucidated. The majority of the evidence for a role of AIIt in membrane fusion is based on model membrane (13, 15, 16, 22). In this study we established a sensitive in vitro assay for the fusion of lamellar bodies with the plasma membrane using a fluorescent probe R18, and we demonstrated a critical role of AIIt and arachidonic acid in this process.

R18 dequenching has been used to monitor biological membrane fusion including virus-cell fusion (36, 45), chromaffin granule-plasma membrane fusion (27), pancreatic zymogen granule-plasma membrane fusion (46), Saccharomyces cerevisiae secretory vesicle-plasma membrane fusion (47), and Golgi membrane fusion (48). The main concern for this assay is that R18 dequenching may result from the nonspecific probe transfer without fusion and probe transfer due to hemifusion (49). In virus-cell fusion, several studies (35, 50–52) were performed to validate the R18 dequenching assay using labeled viral proteins, different dyes, and PCR assay. By comparison with viral surface protein dilution assay, inhibition of the membrane fusion event by lysophospholipid or by inactivating virus and kinetic analysis, the complete membrane fusion appears to contribute to the major part of R18 dequenching under the conditions we used in the present study (37 °C, 6 min) (50, 52). The contributions of nonspecific probe transfer or hemifusion were estimated to be ~4% of maximal dequenching at 37 °C in 10 min (50), which corresponds to the basal rate without AIIt in the present study. Mathematical analysis was also used to separate a true fusion and a nonspecific probe transfer (53).

Independent methods to monitor membrane fusion such as content mixing assay should greatly complement the lipid mixing R18 assay used in this study. However, to our knowledge, no assays are available for measuring the mixing of fluid phase markers between secretory granules and plasma membrane vesicles, although various markers have been used to determine content mixing in liposomes (54–56) or endosomes/lysosomes (57). The main difficulty is to load markers into biological membranes, in particular secretory granules.

We reported previously the presence of AIIt in alveolar epithelial type II cells and its participation in the fusion of lamellar body exocytosis (13, 33). In the present investigation immunodepletion of annexin II from type II cell cytosol abolished its ability to fuse lamellar bodies and the plasma membrane, supporting the notion that annexin II is required for the fusion. The addition of AIIt to the immunodepleted type II cell cytosol

with BSA, followed by centrifugation, to remove free BSA. BSA-treated lamellar body or plasma membrane was then tested for AIIt-mediated fusion. BSA treatment of lamellar bodies inhibited the fusion by 57%, whereas the same treatment for plasma membrane only produced 15% inhibition (Fig. 6B). The treatment of both the membranes leads to further
means centrifugation. 150 II. Several groups (59 amount of AIIt, possibly because of the abundant amount of other fusogenic factors in its cytosolic fraction. Previous studies pancreas cytosols. Surprisingly, kidney cytosol had the highest of different cytosolic fractions to the annexin II level. Although fusion of lamellar bodies with the plasma membrane. The microsomal or mitochondrial membranes cannot annexin I, IIm, III, IV, V, and VI had no effect. Furthermore, AIIt is able to bind to phospholipids membrane. The microsomal or mitochondrial membranes cannot AIIt to several membrane proteins and has been implicated in zymogen granules exocytosis (62, 63).

Annexin-mediated fusion of lamellar bodies and the plasma membrane is specific to AIIt, because other annexins including annexin I, IIm, III, IV, V, and VI had no effect. Furthermore, this process is specific to lamellar bodies and plasma membrane. The microsomal or mitochondrial membranes cannot replace lamellar bodies to fuse with the plasma membrane. AIIt is able to bind to phospholipids in vitro. However, we have shown previously that AIIt binds to several membrane proteins (64). Furthermore, annexin II is concentrated in lipid microdomains (65). It is possible that the specificity may be due to its binding proteins on the membranes or to specific lipid microdomains such as rafts.

Seven cysteine residues are found in an AIIt molecule of which five are distributed to both the N and C termini of monomeric AIIt, and the remaining two are at p11. Blockage of the thiol groups of cysteine residues by NEM led to a 75% inhibition in fusion, suggesting that the cysteine residues of AIIt play a critical role in the membrane fusion. The possible mechanism behind this inhibition may be due to either a conformational change or a steric hindrance of the active sites of AIIt (42). Because AIItm was unable to mediate the fusion, another possibility is that NEM modifies p11 and therefore dissociates p11 from AIIt. The importance of cysteine residues of AIIt is also demonstrated by the fact that AIIt-mediated fusion was inhibited by nitric oxide, which modifies AIIt by S-nitrosylation (24). Tyrosine modification of AIIt by peroxynitrite led to the formation of nitrotyrosine in AIIt (23), and this modification also reduced its fusion activity, indicating that tyrosine residues are also critical for this process.

From the above discussion it is clear that AIIt is required for the fusion of lamellar body and plasma membrane. However, AIIt alone only caused the fusion at high Ca\(^{2+}\) concentrations. Inclusion of exogenous arachidonic acid significantly increased AIIt-mediated fusion and reduced its Ca\(^{2+}\) requirement. This effect is due to membrane-bound arachidonic acid and not free arachidonic acid. Insertion of exogenous arachidonic acid to plasma membrane increased fusion more than the lamellar body. This is probably due to the lower amount of endogenous arachidonic acid in plasma membrane than in the lamellar body. As a result plasma membrane has a higher capacity to accept exogenous arachidonic acid. This is consistent with the observation that the removal of arachidonic acid from plasma membrane produced less inhibition compared with its removal from lamellar bodies.

The mechanisms of action of arachidonic acid may relate to the disturbance of membranes. X-ray diffraction study revealed that arachidonic acid may destabilize membrane by converting closely associated lipid bilayers into hexagonal structure to promote membrane fusion (66). Another study also suggested that arachidonic acid favors the transition of membrane bilayers by a lipid arrangement to an HII phase, which is important for membrane fusion (67). Arachidonic acid may also promote the “stalk” formation (68). Another possibility for arachidonic acid action is to directly interact with AIIt. Some of annexins have been shown to bind with fatty acids in a Ca\(^{2+}\)-dependent manner (69).

Acknowledgment—We thank Candice Marsh for secretarial assistance.

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### TABLE II

**Free fatty acids in lamellar body and plasma membrane**

| Free fatty acid          | Plasma membrane | Lamellar body |
|-------------------------|-----------------|---------------|
|                        | −BSA (μg/mg protein) | +BSA (μg/mg protein) | % Loss | −BSA (μg/mg protein) | +BSA (μg/mg protein) | % Loss |
| Arachidonic acid (C20:4) | 2.73 ± 0.09     | 2.02 ± 0.07   | 26     | 9.83 ± 0.65       | 2.02 ± 0.07       | 90     |
| Linolenic acid (C18:3)  | UD              | UD            |        | UD                | UD                |        |
| Stearic acid (C18:0)    | 5.39 ± 0.33     | 3.09 ± 0.19   | 43     | 15.00 ± 0.95      | 2.69 ± 0.17       | 82     |
| Palmitoleic acid (C16:1) | 0.67 ± 0.35     | 0.67 ± 0.35   | 0      | 0.11 ± 0.10       | 0.11 ± 0.10       | 0      |
| Palmitic acid (C16:0)   | 10.08 ± 0.96    | 0.73 ± 0.42   | 56     | 112.63 ± 21.02    | 16.88 ± 3.15      | 85     |
| Myristoleic acid (C14:1) | 0.74 ± 0.33     | 0.41 ± 0.18   | 44     | 2.39 ± 0.52       | 0.52 ± 0.12       | 78     |
| Myristic acid (C14:0)   | 1.76 ± 0.77     | 1.30 ± 1.53   | 8      | 7.30 ± 0.45       | 1.45 ± 0.09       | 80     |

Concentration (g/mg protein). Free BSA was removed by centrifugation. 150 μg of plasma membrane or lamellar body was used for gas chromatographic analysis of free fatty acids. Data shown are means ± S.E. from three separate experiments. UD, undetected.
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