F-actin scaffold stabilizes lamellar bodies during surfactant secretion

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PULMONARY SURFACTANT IS CRITICAL for immune defense and blood oxygenation (6, 32). Secreted as a complex of phospholipids and proteins by alveolar type 2 (AT2) cells, surfactant forms the alveolar epithelial lining that maintains alveolar patency and protects the alveolar epithelium from inhaled pathogens (6). Loss of surfactant secretion promotes lung injury (11, 25).

Lamellar bodies (LBs) are vesicles that store surfactant phospholipids in complex with surfactant proteins B (SPB) and C (5). Physiological stimuli such as lung hyperinflation induce surfactant secretion, which occurs by LB exocytosis (1, 11). A characteristic, but inadequately understood, feature of surfactant secretion is its prolonged time course. Some reports indicate that F-actin disruption promotes secretion (3, 27, 29). Mikkalve et al. propose that F-actin associated with PM-docked LBs provides the contractile force for LB exocytosis (17, 18). Understanding of these issues in intact alveoli has been hampered by the lack of real-time studies of F-actin dynamics.

Here, we addressed this hypothesis through live detection of LB exocytosis in alveoli of isolated perfused lungs (IPLs). We carried out the F-actin studies in conjunction with studies of LB exocytosis using our previously described approach of staining LBs with water-soluble Lysotracker dyes (1, 11). Organic anion transporters sequester these dyes in LBs. During LB exocytosis, loss of Lysotracker fluorescence reflects loss of the aqueous phase in LBs. To concomitantly determine exocytosis of surfactant phospholipids, we loaded AT2 cells with fluorescent surfactant protein B (f-SPB) that localizes to the phospholipid phase of LBs (22, 31). As in our previous studies (1), we induced LB exocytosis by a 15-s lung hyperinflation. Our findings indicate that LBs remained immobile during secretion in an F-actin stabilized network, forming serial linkages with a PM-proximal vesicle instead of individually moving to the PM. These findings are, to our knowledge, the first instance of a secretory process in which the secretory vesicles remain stationary while the secretory material flows through an intervesicular pathway to the cell exterior.

METHODS

Reagents. Fluorophores used were the LB marking dyes lysotracker red (LTR) or lysotracker green (LTG) (100 nM), the lipid membrane marker phospholipid-BODIPY (4.4-difluoro 5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid), and a succinimidyl ester marker phospholipid-BODIPY (4,4-difluoro 5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid), and a succinimidyl ester form of BODIPY that was used for SPB binding. The dyes were purchased from Molecular Probes (Eugene, OR). A rabbit polyclonal IgG was used for detection of SPB (Seven Hills Bioreagents, Cincinnati, OH). Other reagents were purchased from Sigma (St. Louis, MO). The rat anti-AT2 antibody was a gift of Dr. Leland Dobbs (University of California, San Francisco).

SPB tagging with BODIPY. Purified human SPB was a gift of Dr. Timothy Weaver (University of Cincinnati). To obtain BODIPY-SPB (f-SPB), we dissolved SPB (100 µg) in a chloroform-methanol mixture (1:1 vol/vol, 50 µl, pH 9.0) to which we added a solution of BODIPY in anhydrous dimethyl formamide (30 µl, 10 mg/ml). We ran the mixture on a Sephadex LH-20 column to remove unbound SPB from f-SPB. We determined optical densities (280 nm) of the eluted column fractions to quantify protein concentration. For experiments, we used the fraction with the highest SPB concentration.

IPL preparation. Our animal methods were approved by the Institutional Animal Care and Use Committee of Columbia University Medical Center. We established the IPL as we previously described (1, 11, 21). Briefly, we anesthetized Sprague-Dawley rats (400 g) or Swiss Webster mice (25 g) with 4% isoflurane followed by 80 mg/kg
ketamine and 3 mg/kg xyazine intraperitoneally. We removed the lungs by sternotomy; cannulated the pulmonary artery, left atrium and trachea; and then pump-perfused the lungs at constant pulmonary artery, left atrial pressures, and alveolar (Palv) pressures of 10, 3, and 5 cmH₂O, respectively. To hyperinflate the IPL, we increased Palv from 5 to 15 cmH₂O for 15 s.

**Alveolar microinfusion.** To load the alveolar epithelium with fluorescent dyes, we microinfused the dyes through glass micropipettes inserted in the alveolar space (1, 11, 24). Each microinfusion filled 8–10 alveoli, but then quickly drained leaving the alveolar air space free of the injected fluid (30). We carried out studies in alveoli that were not directly micropunctured. After f-SPB loading, we microinfused alveoli with buffer to wash out free f-SPB from the alveolar space.

**Microscopy.** We imaged alveoli by laser scanning confocal microscopy (LSM 510; Carl Zeiss Microscopy, Heidelberg, Germany) using a ×40 water immersion objective (numerical aperture 0.80, Achroplan; Carl Zeiss Microscopy). We used light excitation at 488 nm for f-SPB and at 543 nm for LTR and BODIPY-PL. We imaged 2-µm-thick optical sections at vertical intervals of 1 µm from the pleural surface.

**Alveolar microlavage.** For alveolar microlavage (14), we introduced two micropipettes, each in a separate alveolus in a cluster of 8–10 alveoli. Through one micropipette we infused ATP assay buffer (14). After 2 min, we collected ~0.5 µl of the infused solution in the second micropipette. We viewed micropipette tips by confocal microscopy to determine fluorescence of collected samples.

**In vivo F-actin microscopy.** To determine F-actin fluorescence in alveolar epithelium in situ, we transfected alveoli with the probe Lifeact. The plasmid (Lifeact-RFP) was a gift of Dr. Roland Wedlich-Soldner (Max Planck Institute, Martinsried, Germany) (26). We prepared complexes of the plasmid (75 µg) with liposomes (20 µg/µl, 100 nm pore size, DOTAP; Avanti Lipids, Alabaster, AL) in sterile RPMI media. We gave the plasmid-liposome mixture (1 µl of the infused solution in the second micropipette. We viewed micropipette tips by confocal microscopy to determine fluorescence of collected samples.

**SPB immunoblot.** We used antibodies specific for the PLD1 phosphorylated form of SPB (SPB immunoblot) (11, 12). The gel was viewed for fluorescence detection (Kodak Image Station 4000MM). The proteins from the gel were transferred to nitrocellulose membrane (overnight, 4°C) and blotted with antibody against SPB (1:200 dilution; Seven Hills).

**Statistics.** Paired comparisons were carried out by paired t-test, and multiple comparisons by ANOVA with Bonferroni’s post hoc test. All data are means ± SE. Significance was accepted at P < 0.05.

**RESULTS**

**BODIPY-SPB (f-SPB).** We identified AT2 cells using a specific cell-surface recognizing antibody (24). Previous reports indicate that AT2 cells take up and localize exogenous SPB to LB phospholipids (4). We fluorescently labeled purified SPB with the succinimidyl ester of BODIPY (15). To determine efficiency of fluorophore binding, we eluted equal amounts of f-SPB and nonlabeled SPB by gel filtration. In eluted fractions, SPB concentration related directly to BODIPY fluorescence (Fig. 1A). Protein-free fractions lacked fluorescence (Fig. 1A). f-SPB and unlabeled SPB formed identical bands on SDS gels (Fig. 1B). These findings indicated that the fluorescent label on SPB was stable, that the label did not leach, and that the labeling did not cause major protein alterations.

To label LB phospholipids in AT2 cells in situ, we microinfused intact alveoli with f-SPB. Consistent with previous findings (2, 4), uptake of f-SPB in AT2 cells occurred in a time-dependent manner and was facilitated by inclusion of exogenous surfactant in the microinfusion (Fig. 2, A–D). Cooling inhibited the uptake (Fig. 2E). A single 15-s hyperinflation, an inducer of surfactant secretion (1), progressively decreased f-SPB in AT2 cells (Fig. 3A), indicating that the stimulus induced surfactant secretion. In absence of hyperinflation, f-SPB fluorescence did not change, thereby ruling out nonspecific photobleaching as a cause of the f-SPB decrease. We detected BODIPY fluorescence in alveolar microlavage (14) carried out 30 min after hyperinflation (Fig. 3B). Following the initial f-SPB microinfusion we microinfused alveoli with buffer to wash out free f-SPB, and we confirmed that microlavage obtained subsequently did not contain any fluorescence. These findings rule out the possibility that the microlavage obtained after hyperinflation contained free f-SPB left over from the initial microinjection, and indicate that the fluorescence in the microlavage reflected presence of secreted surfactant.

To determine LB movements during surfactant secretion, we comicroinfused alveoli with the lipophilic analog of BODIPY, BODIPY-PL, which binds membrane lipids (12). In alveoli loaded with these dyes, LBs were evident in AT2 cells as fluorescent 2-µm-diameter structures that organized in a linear chain-like configuration (Fig. 3A). The lipid dye enabled clear visualization of LB perimeters such that inter-LB distances could be determined using the line tool feature of the imaging program. These determinations indicated that, despite f-SPB loss after hyperinflation, there were no changes in inter-LB distances (Fig. 3C). Thus, the LBs appeared to retain their positions, suggesting that they were immobile during the secretion.

To determine f-SPB turnover in the immobile LBs, we applied fluorescence recovery after photobleaching (FRAP) to single LBs. By means of optical images taken at multiple focal planes, we confirmed that the targeted LBs were photobleached throughout the Z-axis. Our findings indicate that, within 4 min of hyperinflation, the rate of fluorescence recovery increased.
flow between LBs occurred in a Ca²⁺ nonbleached LBs. In alveoli infused with the Ca²⁺ recovery was not due to replacement of photobleached LBs by immobile during surfactant secretion and that fluorescence place. These determinations further affirmed that the LBs were inhibited (Fig. 3, BAPTA-AM, the hyperinflation-induced FRAP response was recovery took 4 min for f-SPB (Fig. 4, the aqueous marker (Fig. 5, that fluorescence recovery to 80% of initial occurred in 30 s for more rapidly. This finding was supported by FRAP studies in f-SPB, both fluorophores were detectable in LBs, and both accumulate in LBs. In alveoli coinfused with LTR and soluble, anionic Lysotracker dyes, LTG (green) and LTR (red), that fluorescence decreases commenced simultane-
fused cells, the probe did not interfere with surfactant secretion; hence, to this extent we agree with others (7, 8, 28) that Lifeact reports F-actin dynamics without interfering with cellular function.

The actin depolymerizer Latrinculin B (LatB) induced surfactant secretion, as indicated by bulk fluorescence loss from AT2 cells and by FRAP, at rates similar to that for hyperinflation (Fig. 6, A and B), indicating that F-actin stabilized LBs. Consistent with this notion, in alveoli expressing Lifeact, F-actin fluorescence colocalized with LBs (Fig. 6, C and E), and the fluorescence was higher in AT2 than AT1 cells (Fig. 6D). Hyperinflation transiently decreased F-actin fluorescence (Fig. 6, E and F). The fluorescence decrease was not due to threefold above baseline (Fig. 3, D and E). FRAP indicated the presence of convective surfactant flow from nonbleached LBs to the photobleached LB. Because SPB localizes selectively to LBs, the flow took place between LBs. Because SPB forms linkages with anionic surfactant phospholipids (23), the FRAP reflects phospholipid flow. By simultaneously imaging f-SPB and BODIPY-PL after hyperinflation, we confirmed that photo-
bleaching decreased SPB but not BODIPY-PL fluorescence (Fig. 3F), indicating that the phototargeted LBs remained in place. These determinations further affirmed that the LBs were immobile during surfactant secretion and that fluorescence recovery was not due to replacement of photobleached LBs by nonbleached LBs. In alveoli infused with the Ca²⁺ chelator BAPTA-AM, the hyperinflation-induced FRAP response was inhibited (Fig. 3, G–I), affirming that the induced surfactant flow between LBs occurred in a Ca²⁺-dependent manner. Thus, we interpret that hyperinflation induced Ca²⁺-dependent surfactant flow between LBs that remained stationary throughout.

LBs contain an aqueous phase that is marked by the water-soluble, anionic Lysotracker dyes, LTG (green) and LTR (red), that accumulate in LBs. In alveoli coinfused with LTR and f-SPB, both fluorophores were detectable in LBs, and both decreased following hyperinflation (Fig. 4, A and B). However, although the fluorescence decreases commenced simultaneously, the overall decrease was faster for LTR than f-SPB (Fig. 4, B and C), indicating that the aqueous phase was released more rapidly. This finding was supported by FRAP studies in that fluorescence recovery to 80% of initial occurred in 30 s for the aqueous marker (Fig. 5, B and C), whereas a similar recovery took 4 min for f-SPB (Fig. 4B). Taking the FRAP and bulk fluorescence data together, we conclude that during surfactant secretion the aqueous phase secretes much more rapidly than the phospholipid phase. Because surfactant secretion occurs through an enlarging fusion pore (9), the difference in secretion kinetics might be attributable to the higher viscosity, hence slower release of f-SPB. The aqueous flow might facilitate the convective passage of LB phospholipids across the expanded fusion pore.

To evaluate the role of alveolar actin in surfactant secretion, we transfected alveoli with the F-actin probe Lifeact (26). Studies by Riedl et al. indicate that Lifeact specifically reports F-actin fluorescence (26). The probe encodes a 17-amino acid sequence of the actin binding protein ABP140 and it colocalizes with the F-actin binding agent phalloidin. The 30-fold higher dissociation constant for F- than G-actin makes the probe highly selective for F-actin. There is no known cytotoxicity in transfected cells and no interference in F-actin interactions with other proteins such as myosin II and α-actinin. In our studies, the probe did not interfere with surfactant secretion; hence, to this extent we agree with others (7, 8, 28) that Lifeact reports F-actin dynamics without interfering with cellular function.

Lung Actin Scaffold Stabilizes LBs During Surfactant Secretion
Fig. 3. Surfactant secretion from immobile LBs determined by the fluorescence recovery after photobleaching (FRAP). A: confocal images show an AT2 cell with LBs stained with f-SPB (top) and phospholipid membranes stained with BODIPY-PL (bottom). White lines mark inter-LB distance. Scale bar, 5 μm. B: images show micropipette tips containing alveolar microlavage fluid obtained after the indicated microinfusions. Dotted lines delineate the margins of the micropipette tip. C: group data are changes in inter-LB distance determined after 30 min of imaging. Data are means ± SE, n = 40 LBs. D–G: confocal images show single AT2 cells with LBs stained with f-SPB (D, E, and G) or membranes stained with BODIPY-PL (F). A select region of the AT2 cells (rectangles on left) was magnified in images on right. Dotted lines delineate the photobleached region. Scale bars, 5 μm. H: tracings show the time course of FRAP after indicated treatments. I: group data are means ± SE, n = 3 lungs each bar. *P < 0.05 vs. without inflation.
nonspecific loss of fluorophore, since the fluorescence spontaneously returned to baseline (Fig. 6, E–G). Moreover, the fluorescence increased rapidly, as expected, after alveolar treatment with the actin-polymerizing agent jasplakinolide (Fig. 6, E and G). The hyperinflation-induced decrease of F-actin fluorescence was blocked by alveolar pretreatment with BAPTA-AM (Fig. 6, F and G). Together, these findings indicate that LBs are immobilized in an F-actin scaffold and that Ca\(^{2+}\)-induced decrease of F-actin was critical for surfactant secretion.

Fig. 4. Surfactant secretion from intact alveolus. A: confocal images show a single AT2 cell. Red and green renditions show fluorescence of LTR (top) and f-SPB (bottom). Note, inflation-induced surfactant secretion is denoted by loss of fluorescence. White line denotes the distance between two LBs. Scale bar, 5 μm. B and C: exponential decay curves (B) and the slopes of the decay curves (C) show, respectively, the time course and rate constants for decrease of LTR and f-SPB fluorescence. Data are means ± SE, n = 4 lungs each bar. *P < 0.05 compared with bar on left.

Fig. 5. Secretion of the aqueous phase. A: left, confocal image shows AT2 cells stained with LB-localizing aqueous dye lysotracker green (LTG). Right, high-power image of a select AT2 cell (arrow in left). Scale bar, 25 μm. B: images show a single AT2 cell with LBs stained with LTG. A select region of the left image (rectangle) is magnified in three right images. Dotted lines mark the region where LTG fluorescence was bleached. Scale bar, 4 μm. C: tracing shows the time course for LTG fluorescence recovery.
Fig. 6. F-actin depolymerization regulates alveolar surfactant secretion. A: tracings show time course of f-SPB and LTR fluorescence loss after LatB microinfusion. B: group data show f-SPB FRAP after indicated treatments. Data are means ± SE, n = 3 lungs each bar. C: confocal images show f-SPB-stained AT2 cells (left) and alveolar fluorescence of the expressed Lifeact probe (right). Scale bar, 30 μm. D: group data show alveolar actin in AT1 and AT2 cells. Data are means ± SE, n = 40 cells each bar. *P < 0.05 compared with AT1 cells. E: magnified images (arrow in C) show the merged image (left) and Lifeact fluorescence (2nd to 6th images from the left). JP, Jasplakinolide. Scale bar, 4 μm. F and G: tracings (F) and group data (G) show effect of inflation on alveolar F-actin after indicated treatments. INF, inflation; BL, baseline. Data are means ± SE, n = 40 AT2 cells from 4 lungs. *P < 0.05 compared with corresponding baseline.
DISCUSSION

We show here for the first time that in the AT2 cell in situ, surfactant secretion occurs by a unique process in which secretory vesicles remain stationary in an actin scaffold during the course of secretion. As expected (4), f-SPB given by intra-alveolar microinjection was readily taken up by AT2 cells from the extracellular space and then well retained in LBs. The resulting fluorescence profile permitted a clear and unequivocal definition of individual LBs in AT2 cells imaged in situ. The stationary profile was revealed by determinations of intervesicular distances that remained unchanged during secretion, indicating that LBs form an immobile chain of vesicles that convectively convey surfactant through intervesicular channels to the secretion site. These findings indicate that, contrary to the model proposed for compound exocytosis, during surfactant secretion LBs do not move from the cytosol to the PM.

Our ability to detect surfactant flow in single LB vesicles was attributable to a novel application of FRAP for detection of secretion in situ. Thus, FRAP was present only after the inflation stimulus, but not at baseline. The FRAP rate equaled the rate of loss of bulk SPB fluorescence from the AT2 cell, indicating that the convective flow detected by FRAP corresponded to the surfactant secretion rate. The BAPTA experiments indicated that inhibition of Ca\(^{2+}\), a major secretory stimulus, also blocked FRAP. These findings indicate that a steady flow of SPB takes place between the stationary LB vesicles throughout the secretion period.

A potential concern was that FRAP could result from SPB diffusion within the photobleached LBs. However, images taken at several focal planes across the LB diameter confirmed that the LBs were fully bleached before FRAP assessment, ruling out intra-LB diffusion. f-SPB fluorescence was not evident in the cytosol, ruling out the possibility that free SPB entered the photobleached LB directly from the cytosol. We conclude that the fluorescence recovery in the photobleached LB was entirely attributable to entry of SPB from adjoining LBs across communication channels. Structural evidence for inter-LB channels has been obtained through electron microscopy studies (personal communication, Dr. Matthias Ochs, Hannover Medical School, Germany). Following secretion, SPB could be recovered by alveolar microlavage, confirming that SPB was not only internalized in LBs but that it was also released in the alveolar lumen. Taking these findings together we interpret that, although inter-LB communication was absent under nonsecretory conditions, the secretory stimulus induced formation of intervesicular channels for the flow of LBs to the extracellular space.

We report here the first studies of F-actin dynamics in live alveoli. We detected F-actin fluorescence by means of the Lifeact probe that has been widely used in cultured cells (8, 28). Our findings indicate that, concomitant with induction of surfactant secretion, the inflation stimulus decreased F-actin in AT2 cells, suggesting that loss of the F-actin barrier is essential for the secretion to take place. Furthermore, actin depolymerization by LatB induced surfactant secretion. Based on these findings, we propose that F-actin stabilizes LBs and orders regulated surfactant flow between vesicles. This is consistent with findings that latrunculin A and cytochalasin D induce neurotransmitter release from neural synapses (16, 19) without affecting the refilling rate of vesicles (19), indicating that actin depolymerization facilitates exocytosis from fused vesicles.

The physiological significance of stationary LB vesicles might lie in the regulation of the surfactant secretion rate. It is well understood that the stretch stimulus caused by a single lung expansion induces surfactant secretion for durations of up to an hour (1, 10). Although it is known that the secretion is initiated and sustained by prolonged Ca\(^{2+}\) oscillations between AT1 and AT2 cells in the alveolar epithelium (1), the mechanisms responsible for the slow secretion rate are not understood. We suggest that, under stimulated conditions, the induced interconnectivity among LB vesicles provides a conduit for continuous secretion of surfactant across an extended time scale. Accordingly, sufficient time is afforded for unraveling the externalized LB particle and establishing phospholipid insertion in the air-liquid interface. Studies are required to clarify the role of the LB stabilizing F-actin scaffold in the regulation of surfactant secretion under disease conditions. We recently showed that endotoxin exposure for 24 h blocks alveolar surfactant secretion (11). Understanding of how disease processes destabilize LB organization in the F-actin scaffold, and thereby cause impaired surfactant release, might lead to novel therapy for lung disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHOR CONTRIBUTIONS


REFERENCES


