Reorganization of cytoskeleton during surfactant secretion in lung type II cells: a role of annexin II

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Abstract

The secretion of lung surfactant requires the movement of lamellar bodies to the plasma membrane through cytoskeletal barrier at the cell cortex. We hypothesized that the cortical cytoskeleton undergoes a transient disassembly/reassembly in the stimulated type II cells, therefore allowing lamellar bodies access to the plasma membrane. Stabilization of cytoskeleton with Jasplakinolinde (JAS), a cell permeable actin microfilament stabilizer, caused a dose-dependent inhibition of lung surfactant secretion stimulated by terbutaline. This inhibition was also observed in ATP-, phorbol 12-myristate 13-acetate (PMA)- or Ca^{2+} ionophore A23187-stimulated surfactant secretion. Stimulation of type II cells with terbutaline exhibited a transient disassembly of filamentous actin (F-actin) as determined by staining with Oregon Green 488 Phalloidin. The protein kinase A inhibitor, H89, abolished the terbutaline-induced F-actin disassembly. Western blot analysis using anti-actin and anti-annexin II antibodies showed a transient increase of G-actin and annexin II in the Triton X-100 soluble fraction of terbutaline-stimulated type II cells. Furthermore, introduction of exogenous annexin II tetramer (AIIt) into permeabilized type II cells caused a disruption in the cortical actin. Treatment of type II cells with *N*-ethylmaleimide (NEM) resulted in a disruption of the cortical actin. NEM also inhibited annexin II's abilities to bundle F-actin. The results suggest that cytoskeleton undergoes reorganization in the stimulated type II cells, and annexin II tetramer plays a role in this process.
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1. Introduction

Alveolar epithelial type II cells synthesize pulmonary surfactant and store it in lamellar bodies. The binding of agonists to the receptors on the plasma membrane initiates signal transduction leading to changes in second messengers and eventually an increase of lung surfactant secretion. For example, β-agonists such as isopoterenol or terbutaline raise intracellular cAMP levels via stimulation of adenylate cyclase, activate protein kinase A and thus increase the secretion of surfactant [1–5]. Two essential steps involved in the later stage of secretory process include the translocation of lamellar bodies to the apical plasma membrane and the fusion of lamellar body with the plasma membrane. The

movement of lamellar bodies may require the reorganization of cytoskeleton. In intact lungs, cytochalasin D treatment abolishes surfactant secretion [6]. However, in primary culture, cytochalasins A, B, C, and D enhance the release of surfactant [7].

The cytoskeleton serves as several functions in regulating secretory processes, including a barrier to regulate the release of secretory vesicles, a track for motor-mediated translocation of secretory vesicles, and a scaffold to buffer the reserve pool and the rapidly releasable pool of secretory vesicles [8]. Orci et al. [9] was the first to propose that a layer of cortical actin serves as a barrier to granule docking and the exocytic release of these secretory granules. In many secretory cells, actin network underneath the plasma membrane undergoes a transient depolymerization during exocytosis. A rapid depolymerization of filamentous actin (F-actin) occurs in toad bladder epithelial cells treated with either ADH or 8-bromo-cAMP [10]. In stimulated chromaffin cells, catecholamine secretion is preceded by disassembly of cortical actin [11]. The role of actin fila-

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ments in exocytosis has been studied by many other groups [12–16].

Some annexins have exhibited a Ca^{2+}-dependent binding to F-actin. However, only annexin II tetramer (AIIt) causes a significant actin filament bundling [17,18]. This bundling activity requires micromolar Ca^{2+} and is reversed by the addition of excess of EGTA [19]. A synthetic nonapeptide corresponding to residues 286–294 of annexin II inhibits its Ca^{2+}-dependent activation of actin filament bundling [20]. The C-terminus of annexin II is responsible for the binding to F-actin [21]. In addition to the bundling activity, annexin II has also been reported to sever actin filament [22]. Besides, annexin II has the ability to bind to other cytoskeletal proteins like non-erythroid spectrin (fodrin) [23,24]. These in vitro studies suggest that annexin II may be involved in the organization of cytoskeleton. Much less work has been done in cells regarding interaction between annexin II and cytoskeleton. In the present study, we investigated the relationship between cytoskeleton reorganization and surfactant secretion as well as a role of annexin II in this process.

2. Materials and methods

2.1. Materials

N-ethylmaleimide (NEM), phenylmethanesulfonyl fluoride (PMSF), β-escin, anti-actin antibody, bovine serum albumin (BSA), terbutaline, H89 and 8-bromo-cAMP (8-Br-cAMP) were purchased from Sigma (St. Louis, MO). A549 cell line was from American Type Culture Collection (Rockville, MD). Oregon Green 488 Phalloidin and Jasplakinolinde (JAS) were from Molecular Probes (Eugene, OR). Chamber slides were from Nalge Nunc International (Naperville, IL). Bio-Spin Chromatography Columns, D_C Protein Assay Kit, horseradish peroxidase (HRP) Color Development reagent, and pre-stained molecular mass standards were from Bio-Rad (Melville, NY). Nitrocellulose membrane was from Schleicher and Schell (Keene, NH). Anti-annexin II polyclonal antibody was raised in rabbit and affinity-purified in our laboratory [25]. Elastase and actin were from Worthington Biochemical (Freehold, NJ). Eagle's minimal essential medium (MEM) was from ICN (Costa Mesa, CA). Fetal bovine serum (FBS), trypsin-EDTA, L-glutamine, HEPES buffer and Penicillin/Streptomycin were from GIBCO (Grand Island, NY).

2.2. Type II cell isolation and culture

Type II cells were isolated from perfused rat lungs. Rat (Sprague–Dawley, male, 180–200 g) was anesthetized intraperitoneally with ketamine and xylazine. The trachea was cannulated and lungs were mechanically ventilated. After the thoracic cavity was exposed surgically, the lungs were perfused via a cannula inserted in the main pulmonary artery with a Solution II (10 mM HEPES, pH 7.4, 2.6 mM Na_2HPO_4/NaH_2PO_4, 154 mM NaCl, 3.9 mM KCl, 1.7 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 mM glucose, 100 μg/ml streptomycin and 60 μg/ml penicillin) until lungs were free of blood. Eight milliliters of Solution I (Ca^{2+}- and Mg^{2+}-free solution II plus 0.16 mM EGTA) was instilled through the trachea with a 10-cc syringe. Then the lungs were removed and placed in a beaker containing 15 ml of cold Solution II. The rest of the procedure was done inside the cell culture hood. The lungs were lavaged eight times with 7 ml of cold Solution I and twice with 7 ml of warm Solution II. The lungs were filled with 6–7 ml of elastase solution (3 units/ml in Solution II) and incubated in a water bath at 37 °C for 10–12 min. This step was repeated three times. Thereafter, the lobes were removed and placed into a beaker containing 3 ml FBS. Each lobe was minced three to four times using a tissue chopper and placed in a flask containing 10 ml Solution II and DNase. After mincing all the lobes, the 3-ml of FBS from the beaker was added into the flask. The flask was shaken by hand rapidly for 2 min at 37 °C. The minced material was filtered sequentially through 160-, 37-, and 15-μm filters. This step was done on ice. The resulting cell suspension was centrifuged at 1100 rpm for 10 min. Cell pellet was resuspended in 10 ml MEM, and placed in an IgG-coated dish (3 mg IgG/5 ml 50 mM Tris–HCl and 0.05% Tween 20) and incubated for an hour to remove alveolar macrophages. The unattached cells were removed (panning) with a sterile pipette and centrifuged at 1100 rpm for 15 min. The cells were resuspended in MEM containing 10% FBS, counted, and replated onto 35-mm cell culture dishes.

2.3. Surfactant secretion assay

Lung surfactant secretion was assayed by monitoring the release of PC as previously described [26]. Briefly, isolated type II cells were plated in 35-mm cell culture dishes in 1.5 ml MEM containing 1×10^6 cells and 1 μCi [^{3}H] choline. After overnight culture, type II cells were washed six times and incubated in 1 ml MEM containing various concentrations of inhibitors for 30 min. At the end of incubation, one set of dishes was removed for the zero time value. Other dishes were stimulated with secretagogues for 2 h. Lipids in the media and cells were extracted and counted. The secretion was expressed as (dpm in media/dpm in media plus dpm in cells). All data were subtracted from the zero time value. The viability of cells was monitored by lactic acid dehydrogenase (LDH) release [27].

2.4. Phalloidin staining of F-actin

Type II cells were cultured overnight before being stained with Oregon Green 488 Phalloidin. Cultured cells

were washed twice with MEM and incubated in MEM for 30 min in the presence or absence of 10 μM H89. Then, the cells were stimulated with 10 μM terbutaline or 1 mM 8-Br-cAMP for different time spans (0, 3, 5, and 10 min) at room temperature. At the end of stimulation, each dish was washed twice with phosphate-buffered saline (PBS). The cells were fixed for 30 min with 4% formaldehyde in PBS. After two washes with PBS, cells were permeabilized in 1% Triton X-100 for 10 min and incubated with 10% FBS and 1% BSA in PBS for 15 min. Cells were stained with 50 nM Oregon Green 488 Phalloidin for 30 min and washed three times with PBS. Fluorescence was observed with a fluorescent microscope.

2.5. Separation of G-actin and F-actin of type II cells

Overnight cultured type II cells were washed three times with 2 ml MEM and incubated in 1 ml MEM for 30 min. The cells were stimulated with 10 μM terbutaline for different time spans (1, 3, 5, 10, 15, and 30 min). At the end of stimulation, the dish was washed twice with 2 ml of cold PBS. In order to separate G-actin and F-actin fractions, 400 μl of extraction buffer (50 mM KCl, 0.3 M sucrose, 2 mM MgCl$_2$, 10 mM sodium phosphate, 0.5% Triton X-100, and 1 mM PMSF) was added to each dish. The dish was then incubated for 10 min on ice. The extract containing G-actin was analyzed for the content of actin and annexin II by Western blot.

2.6. Western blot

The Triton X-100 soluble fraction of type II cells (10–20 μl) was resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The gel was run for 1.5–2 h at 100 V. Thereafter, the gel was transferred to a nitrocellulose membrane at 100 mA for 2 h or 25 mA overnight. The blot was transiently stained with Ponceau S to monitor transfer efficiency of proteins and destained in water for 2–3 min. The lanes were marked with indelible ink. The blot was destained for 10 min with Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.5). Then, the blot was blocked with TBS containing 2% gelatin for 1 h. After blocking, the blot was placed in a plastic bag and incubated with the appropriate primary antibodies (anti-annexin II, 1:1000 dilution or anti-actin, 1:200 dilution) in TTBS (TBS plus 0.05% Tween 20) containing 1% gelatin for 2–3 h. The blots were removed from the bags and washed three times with TTBS for 5 min each. The washed blots were placed in a new bag and incubated with secondary antibody (HRP-conjugated anti-mouse or anti-rabbit IgG, 1:5000 dilution) for 1 h at room temperature. The blots were washed three times and visualized by enhanced chemiluminescence (ECL). The immunoblots were scanned using either the DeskScan or the HP Precision Scan Pro and quantitated using the ImageQuant.

2.7. Permeabilization of type II cells

Type II cells were permeabilized with 40 μM β-escin as previously described [26]. Overnight cultured cells on chamber slides were washed twice with permeabilization buffer (PB, 139 mM K-glutamate, 1 mM EGTA, 10 mM glucose, 20 mM HEPES, pH 7.0). Then the cells were equilibrated in PB buffer for 30 min at 37 °C. β-escin (40 μM) was added and incubated for 10 min at room temperature. At the end of incubation, fresh PB buffer containing 0.5 μM Ca^{2+} and 5 mM MgATP and/or 50 μg/ml AIIt were added and the incubation continued for another 10 min. The cells were washed twice with PBS and stained with Oregon Green 488 Phalloidin as described above.

2.8. F-actin binding and bundling

Annexin II tetramer (AIIt) was purified from bovine lung tissue by sequential column chromatography using DEAE-Sepharose CL 6B, Sephacryl S 300 and FPLC Mono S columns as previously described [26]. AIIt (20 μg) was incubated in 100 μl of bundling buffer (2 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM MgCl$_2$, 0.3 mM EGTA, 0.5 mM DTT, 0.1 mM CaCl$_2$, and 0.33 mM ATP) in the absence or presence of 1 mM NEM at room temperature for 10 min. Spin Column Chromatography was used to remove the excess NEM from the AIIt mixture after the incubation. In order to improve the recovery, the buffer in the spin column was changed first with 0.1% bovine serum albumin to reduce the non-specific binding and then three times with the bundling buffer. The sample (NEM-treated or untreated AIIt) was added in the spin column and spun at 1000 × g for 4 min.
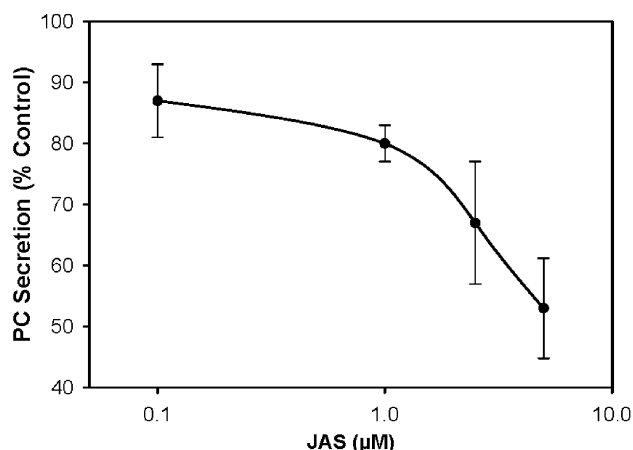

Fig. 1. A dose-dependent inhibition of JAS on terbutaline-stimulated surfactant secretion. [^{3}H] choline-labeled type II cells were treated with various concentrations of JAS for 30 min, and stimulated with 10 μM terbutaline for 2 h. Surfactant secretion was then assayed. The results were expressed as the percentage of the control (without JAS treatment). Data shown are mean ± S.E. (*n* = 3).

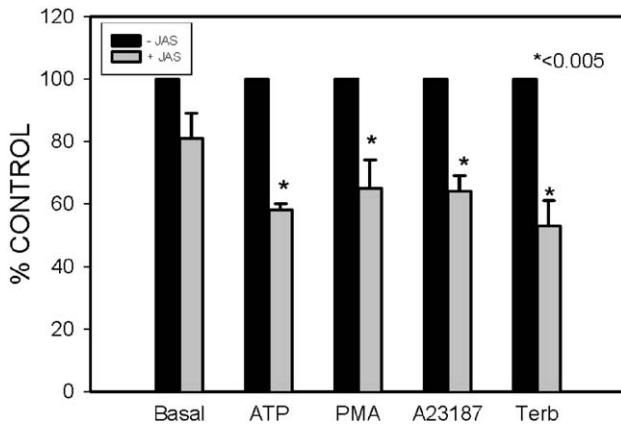Fig. 2. JAS inhibits surfactant secretion stimulated by various secretagogues. [³H] choline-labeled type II cells were treated with 5 μM JAS for 30 min, and then stimulated with no agents (basal), 1 mM ATP, 1 μM PMA or 0.1 μM Ca^{2+} ionophores A23187 or 10 μM terbutaline (Terb) for 2 h. Surfactant secretion was then assayed. The results were expressed as the percentage of the control (without JAS treatment). Data shown are mean ± S.E., $n=3$ for basal, ATP, and A23187, $n=4$ for PMA, $n=6$ for Terb. *$p<0.005$ vs. control.

The AIIt mixture was collected and used for the F-actin binding and bundling assay. G-actin (1 mg/ml) was converted to F-actin by incubating it in bundling buffer overnight. The AIIt mixture was combined with 12 μg F-actin in final volume of 150 μl bundling buffer plus 100 μM free Ca^{2+}. Aliquot (75 μl each) of the reaction mixture was analyzed at low speed (15,600 × g, 10 min) for F-actin bundling. The pellet was dissolved in 1× sample buffer at room temperature overnight and then analyzed on 10% SDS-PAGE.

2.9. Statistical analysis

Data are expressed as mean ± S.E. Statistical significance was evaluated by one-way analysis of variance (ANOVA). The level of significance was taken as $P<0.05$.

3. Results

3.1. Effect of Jasplakinolinde on surfactant secretion

Earlier studies using cytoskeletal disrupting agents such as cytochalasins showed a controversial results regarding a role of actin filaments in surfactant secretion [6,7]. We determined whether the stabilization of cytoskeleton affects surfactant secretion using Jasplakinolinde (JAS). JAS is a novel cell permeable cycolodepsipeptide, which binds to and stabilizes actin microfilaments in vitro [28] and in vivo [29]. As shown in Fig. 1, JAS caused a dose-dependent inhibition of the terbutaline-stimulated surfactant secretion. This inhibition is not due to a cytotoxic effect since the LDH release from the control cells and JAS-treated cells was the same (data not shown). While no significant inhibition was observed for the basal secretion, JAS inhibited all the secretagogue-stimulated surfactant secretion (Fig. 2).

3.2. Actin disassembly in type II cells upon stimulation

To test the hypothesis that cytoskeleton serves as a barrier in lung surfactant secretion, type II cells were stimulated for various times with terbutaline, and filamentous actin (F-actin) was stained with Oregon Green 488
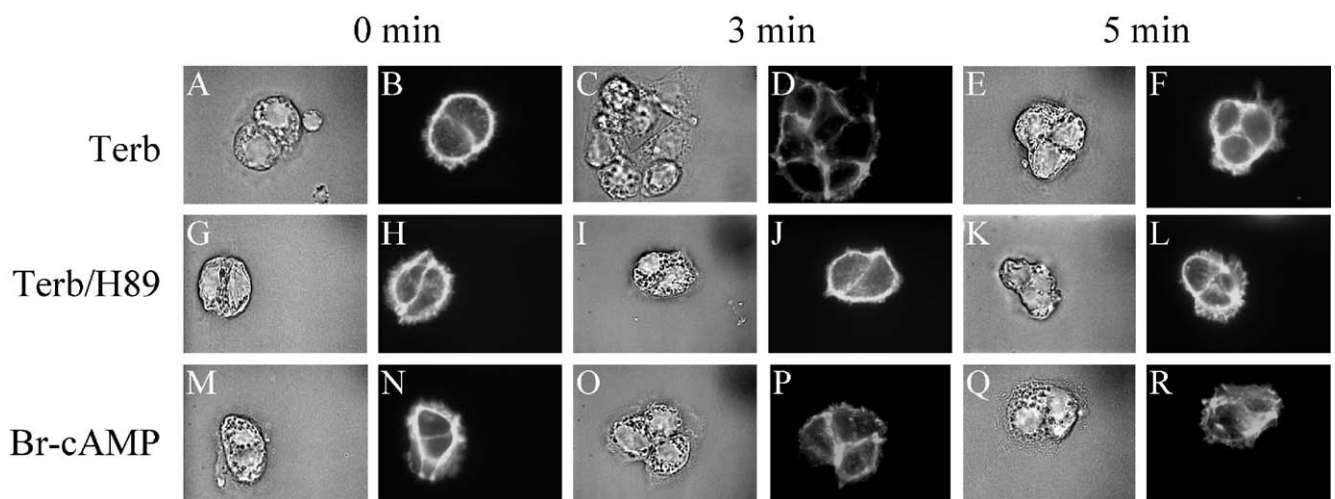

Fig. 3. Actin disassembly in lung type II cells upon stimulation. Overnight cultured alveolar type II cells were pre-incubated 30 min without (Panels A–F, M–R) or with 10 μM H89 (Panels G–M). Then, the cells were stimulated with 10 μM terbutaline (Terb, Panels A–L) or 1 mM 8-bromo-cAMP (Br-cAMP, Panels M–R) for 0 min (Panels A and B, G and H, M and N), 3 min (Panels C and D, I and J, O and P) and 5 min (Panels E and F, K and L, Q and R). At the end of incubation, the cells were fixed and stained with 50 nM Oregon Green 488 Phalloidin. Fluorescent staining is shown in Panels (B, D, F, H, J, L, N, P, and R). The Panels A, C, E, G, I, K, M, O, and Q provide a brightfield view of the corresponding cells.

Phalloidin. In resting cells (0 min), phalloidin fluorescence was most intense at the cell cortex (Fig. 3A and B), indicating that the F-actin was concentrated in that region. However, during a 3-min stimulation, there was a markedly decrease in the binding of phalloidin detected in the cell cortex region (Fig. 3C and D). Further stimulation (5–10 min) led to the reappearance of the cortical fluorescent ring (Fig. 3E and F, 10 min stimulation not shown). These results indicate that cortical actin filaments in type II cells are rapidly disassembled and reassembled following terbutaline stimulation. A similar result was observed in A549 cells, a lung epithelium-derived cell line (data not shown). Since terbutaline stimulation involves the activation of protein kinase A, we examined the effect of the protein kinase A inhibitor, H89 on the reorganization of cytoskeleton. Type II cells were pre-treated with 10 μM H89 and then stimulated with terbutaline. As shown in Fig. 3G–L, a transient disassembly and reassembly of F-actin caused by terbutaline was no longer observed. Furthermore, a cAMP analogue, 8-bromo-cAMP also caused the disassembly of F-actin during a short period of incubation (3 min). However, longer incubations (5 to 30 min) did not result in the recovery of the cortical fluorescent ring (Fig. 3M–R). This is probably because of continuous activation of protein kinase A by 8-bromo-cAMP.

3.3. Redistribution of G-actin and annexin II in stimulated type II cells

Disassembly of the cytoskeleton is necessary to allow lamellar bodies access to the plasma membranes following stimulation of type II cells. Annexin II has been shown to bundle actin filament in vitro [17,18]. Possible roles of annexin II in facilitating reorganization of cytoskeleton were investigated by determining the redistribution of annexin II and actin upon stimulation of type II cells. Overnight cultured type II cells were stimulated with 10 μM terbutaline for various times (0, 1, 3, 5, 10 and 30 min) and extracted with the extraction buffer containing 0.5% Triton X-100. The resulting soluble fraction was examined for the content of annexin II and actin by immunoblot using specific antibodies. In order to quantitate data from Western blot, samples of the control soluble fractions with various amount of total protein were analyzed using specific antibodies for annexin II and actin. A linear relationship between the amount of protein loaded and the immunolabeling was observed in the range of 0.5–7.5 μg total protein (data not shown). The amount of protein that fell in the linear range was chosen for the following experiments. As shown in Fig. 4A, there was an increase of G-actin in the soluble fraction during 1–3-min stimulation. However, G-actin contents returned to the control values after 5–30-min stimulation. Similarly, annexin II in the soluble pool was also increased at 1–3-min stimulation. A longer stimulation (5–30 min) resulted in a return of annexin II content to the resting levels (Fig. 4B).
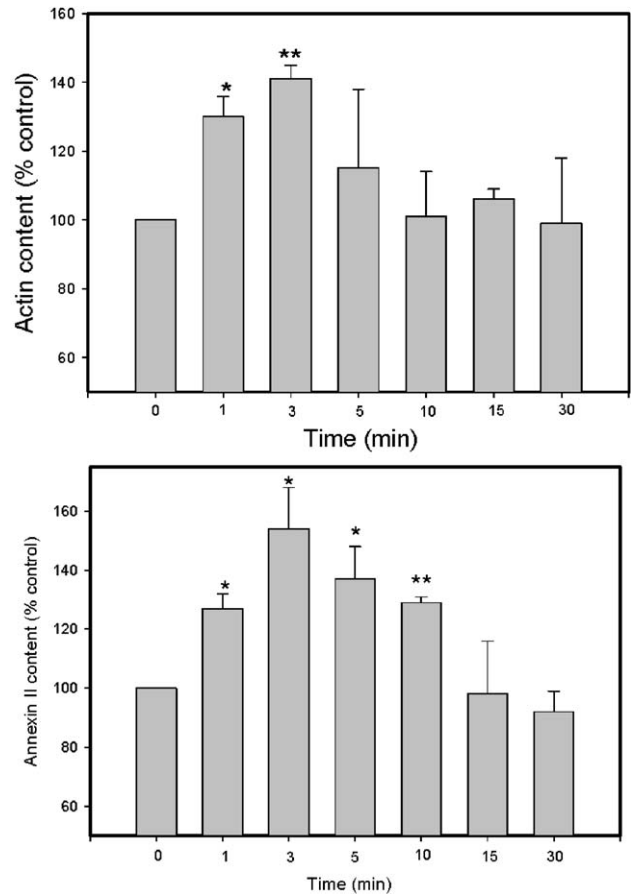

Fig. 4. Redistribution of G-actin and annexin II in stimulated type II cells. Alveolar type II cells were exposed to 10 μM terbutaline for different time spans. Triton X-100-soluble G-actin fractions were loaded onto SDS-PAGE and transferred to a nitrocellulose membrane. Blots were probed with anti-actin or anti-annexin II antibodies and visualized using ECL. The actin bands were quantitated by ImageQuant. The results were expressed as percentage of the control (0 min). Data shown are mean ± S.E. ($n = 3$).

3.4. Effect of exogenous AIIt on actin disassembly in permeabilized type II cells

To directly test the role of AIIt in reorganization of cytoskeleton, type II cells were permeabilized with β-escin and thus exogenous AIIt was able to be introduced into the cells. The cortical actin network in permeabilized type II cells was examined by Oregon Green 488 Phalloidin staining. The staining pattern in permeabilized cells was similar to that in intact cells, showing distinct cortical actin staining (Fig. 5A and B). No changes in phalloidin staining were observed when AIIt was added in the absence of Ca^{2+} and Mg^{2+} ATP (Fig. 5C and D). Addition of 0.5 μM Ca^{2+} and 5 mM Mg^{2+} ATP to permeabilized cells resulted in a minor disruption of the cortical actin staining (Fig. 5E and F). Furthermore, the addition of AIIt along with 0.5 μM Ca^{2+} and 5 mM Mg^{2+} ATP caused a significant disruption of the cortical fluorescent ring (Fig. 5G and H).

3.5. Distribution of F-actin in NEM-treated type II cells

N-ethylmaleimide (NEM) has been shown to stimulate secretion in chromaffin cells, possibly via disruption of cortical cytoskeleton network [30]. The effect of NEM on the distribution of F-actin in lung type II cells was therefore examined by labeling with Oregon Green 488 Phalloidin. In the control cells, there was a continuous fluorescent ring near the periphery of the cells (Fig. 6A and B). However, treatment of the cells with NEM resulted in a disruption of this fluorescent ring (Fig. 6C and D).

We have previously shown that NEM inhibited AIIt-mediated liposome aggregation [31]. The possibility that NEM-caused disassembly of cortical F-actin is due to the modification of annexin II was tested. AIIt was treated with 100 μM NEM and excess NEM in the reaction mixture by bio-spin columns. F-actin was incubated with the treated or untreated AIIt and examined by cosedimentation. F-actin bundling was analyzed by low-speed centrifugation (14,000 × g, 10 min). Under low-speed centrifugation, only F-actin bundles and AIIt associated with these F-actin bundles were sedimented. As shown in Fig. 6E, a greater amount of AIIt and F-actin in the untreated sample was
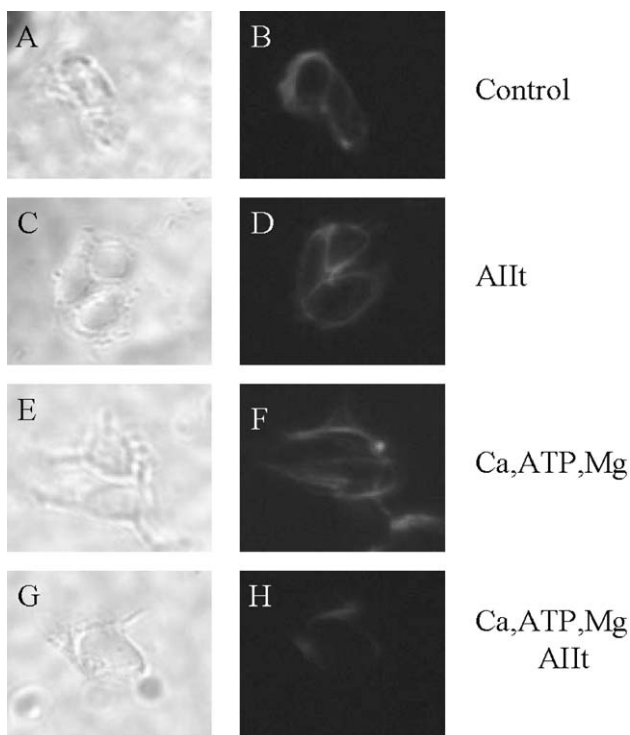

Fig. 6. Distribution of F-actin in NEM-treated type II cells. Panels A–D: Type II cells were incubated without (Panels A and B) or with 30 μM NEM (Panels C and D) for 20 min at room temperature. The cells were then fixed with formaldehyde, stained with 50 nM Oregon Green Phalloidin and observed using a fluorescence microscope. Panels on the right (B, D) show fluorescent staining. Panels on the left (A, C) provide a brightfield view of the corresponding cells. Panel E: AIIt was incubated in bundling buffer in the absence or presence of 1 mM NEM for 10 min. At the end of incubation, AIIt was passed through a spin column to remove excess NEM. The recovered AIIt was mixed with F-actin in bundling buffer plus 100 μM Ca^{2+}. Samples were analyzed for F-actin bundling by low-speed centrifugation (14,000 × g, 10 min). AIIt associated with the sedimented F-actin was analyzed on 10% SDS-PAGE.

recovered in the pellet from low-speed centrifugation compared to those in the NEM-treated sample. This suggests that NEM inhibits AIIt's ability to bundle F-actin.

4. Discussion

4.1. Cytoskeleton disassembly in type II cells

The stimulation of type II cells with terbutaline results in the reorganization of the cortical actin cytoskeleton that normally acts as a barrier to exocytosis of lamellar bodies. This cytoskeletal reorganization causes an increase in lung surfactant secretion. This conclusion is based on our present findings: (i) there is an abundance of F-actin in the cortical region of the type II cells (Fig. 3); (ii) there is a transient increase in G-actin in stimulated type II cells (Figs. 3 and 4); and (iii) the stabilization of cytoskeleton with JAS inhibited stimulated surfactant secretion, independent to the secretagogues used (Figs. 1 and 2).

Western blot analysis revealed an increase in the G-actin fraction in type II cells upon stimulation with terbutaline. The maximum increase was seen at 3 min. Then, G-actin

Fig. 5. Effect of exogenous AIIt on actin disassembly in permeabilized type II cells. Type II cells were permeabilized with 40 μM β-escin for 10 min at room temperature, followed by a 10-min incubation without any addition (Panels A and B) or with 50 μg/ml AIIt (Panels C and D), 0.5 μM Ca^{2+} and 5 mM MgATP (Panels E and F), or 50 μg/ml AIIt, 0.5 μM Ca^{2+} and 5 mM MgATP (Panels G and H). The cells were then fixed with formaldehyde and stained for F-actin using Oregon Green Phalloidin. Panels on the right (B, D, F and H) show the fluorescent staining. Corresponding panels (A, C, E and G) on the left provide the brightfield view of the cells.
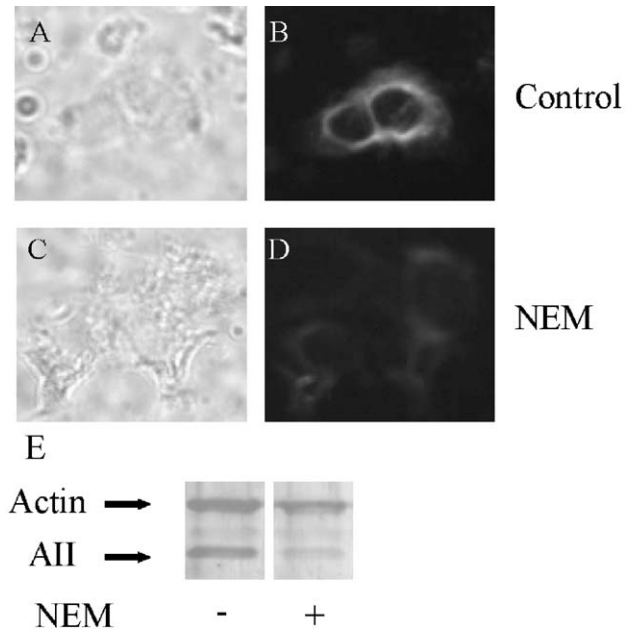
level returned to basal level. A similar study has shown an increase in the G-actin fraction concomitant with a decrease in the cytoskeletal F-actin fraction in adult rat lung type II cells stimulated with terbutaline for 1 min [32]. However, a detailed time course has not been done. Using phalloidin, it was observed that in resting cells the cortical region of the cell was particularly enriched in F-actin. This observation correlates well with previous studies showing the presence of numerous actin filaments in this region connecting the plasma membrane and secretory granules [33]. Upon stimulation with terbutaline, the phalloidin staining around the cortical region disappeared at 3 min and reappeared thereafter, suggesting a transient disassembly of F-actin.

Botulinum C_2 toxin is a binary toxin which ADP ribosylates nonmuscle G-actin. Rose et al. [34] found that the C_2 toxin caused a dose-dependent progressive depletion of the cellular F-actin content and a concomitant increase in the G-actin content. The F-actin decay induced by C_2 toxin was paralleled by a dose-dependent increase of basal surfactant secretion. At high toxin doses, there was more than two-fold increase in basal surfactant PC release. Furthermore, the study showed that pre-exposure of type II cells to C_2 toxin enhanced the surfactant secretory response to the secretagogues and mechanical stretch. Cytochalasins, which disrupt the microfilament organization, also enhance surfactant secretion [7]. In contrast, the stabilization of F-actin by JAS (this study) or phalloidin [34] causes a decrease in surfactant secretion. All these observations suggest that the altered state of actin assembly has a significant impact on the surfactant secretory process.

4.2. Annexin II and actin disassembly

The mechanisms of the cytoskeleton reorganization provoked by terbutaline in type II cells are unknown. One mechanism could be due to changes in actin-binding proteins. One of the candidates is annexin II, which has been shown to bind and bundle F-actin [35]. In the present study, it is found that annexin II undergoes a transient redistribution from the F-actin fraction to the G-actin fraction in type II cells stimulated with terbutaline, suggesting a role of annexin II in the reorganization of cytoskeleton. It is well known that terbutaline, a β-adrenergic agonist, raises intracellular cAMP level, activates cAMP-dependent protein kinase (PKA), and phosphorylates various proteins in the type II cells [2–5]. Several targets of PKA in type II cells have been identified [2,36]. The activated PKA might phosphorylate annexin II, cause its dissociation from F-actin, dismiss its F-actin bundling activity and therefore lead to the disassembly of cytoskeleton. Previous in vitro studies have shown that phosphorylated AIIt neither binds to nor bundles F-actin at physiological levels of Ca^{2+} [37]. Even high levels of Ca^{2+} up to 1 mM could not reverse the inhibitory effects of phosphorylation. Therefore, phosphorylation of AIIt is an inhibitory signal preventing the formation of F-actin bundles or network. Since a rise of cAMP

and the phosphorylation state of proteins in terbutaline-stimulated type II cells is transient [2,3], the speculation above correlates well with the present data that redistribution of both annexin II and actin occurs at the early stages of stimulation. This might be due to the presence of various phosphatases and other dephosphorylating mechanisms. A continuous activation of protein kinase A by 8-bromo-cAMP disassembled F-actin, but not reassembled it even after a prolonged incubation (Fig. 3). Furthermore, a protein kinase A inhibitor (H89) abolished terbutaline-induced F-actin disassembly/reassembly, suggesting an involvement of protein kinase A in this process.

The addition of AIIt to permeabilized type II cells in the presence of MgATP and Ca^{2+} resulted in a significant disruption of the cortical actin. It is possible that this is caused by AIIt's ability to sever F-actin filament [22]. This might seem controversial to the previous experiments in this study where annexin II is thought to bind and bundle F-actin. However, two forms of AIIt may exist in type II cells [25]. One is the AIIt bound to cytoskeleton in the resting conditions. Phosphorylation of this form of AIIt leads to the dissociation of AIIt from the cytoskeleton and thus disruption of cytoskeleton. Another is soluble AIIt that binds to cytoskeleton in response to a rise of Ca^{2+}. The binding sites of those two forms of AIIt may be different. While the former may directly bind to actin, the latter may bind to actin-binding proteins. Complex formation between AIIt, F-actin and fodrin has been shown to occur at high Ca^{2+} concentrations. The binding of AIIt to fodrin and F-actin is abolished by the addition of EGTA [23]. Other studies have also demonstrated a calcium-dependent interaction between AIIt and fodrin [24]. Therefore, it is possible that in the presence of high Ca^{2+} concentration, exogenous AIIt binds to the spectrin/fodrin and inhibits its F-actin cross-linking activity. This explains why the addition of exogenous AIIt in the presence of Ca^{2+} causes the disruption of cortical F-actin.

Previous studies have suggested the involvement of cytosolic Ca^{2+} in inhibition of actin cross-linking [38]. The addition of 10 μM free Ca^{2+} to digitonin-permeabilized cells results in a slower reduction in cytoskeletal actin than the actin changes in intact cells. However, the reduction of actin in permeabilized cells had a time course similar to that for catecholamine secretion. In chromaffin cells, it is believed that the effect of micromolar Ca^{2+} on actin might involve the Ca^{2+}-dependent actin severing protein gelsolin [38]. Severing is a Ca^{2+}-activated process in which the severing protein attaches to the actin subunit such as F-actin and induces its break with neighboring subunits [39,40]. The severing protein prevents the end of the filament form annealing with other actin filaments or binding actin monomers (G-actin). Villin, another actin-severing protein, severs actin filaments at high Ca^{2+} concentrations, but at low Ca^{2+} concentrations it cross-links actin filaments into bundles [41]. Therefore, an alternative explanation for effects of AIIt on cytoskeleton is that AIIt behaves in a similar manner.

AIIt binds and bundles F-actin at low physiological concentration of Ca^{2+}, but in the presence of high Ca^{2+} concentration, it disrupts the F-actin bundles.

4.3. NEM, cytoskeleton, and surfactant secretion

A study done by Wagner et al. showed that treatment of chromaffin cells with 30 μM NEM resulted in a decrease in the amount of cytoskeletal F-actin. They also observed an increase in the secretory activity in NEM-treated cells [30]. In the present study, when alveolar type II cells were treated with NEM, a disappearance of cortical F-actin was observed by staining the cells with Organ Green 488 phalloidin. How NEM depolymerizes the cortical F-actin is still unclear. The present study demonstrates that AIIt-mediated F-actin bundling is inhibited by NEM modification, suggesting that NEM may disrupt the F-actin network by inhibiting AIIt's ability to bundle F-actin. However, since NEM is a nonspecific sulfhydryl agent, we cannot exclude the possibility that NEM also modifies other cellular proteins such as N-ethylmaleimide sensitive factor.

Acknowledgements

References

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