Effect of surfactant protein A on granular pneumocyte surfactant secretion in vitro

Sandra R. Bates, Jian-Qin Tao, Kathleen Notarfrancesco, Kristine DeBolt, Henry Shuman, and Aron B. Fisher

The Institute for Environmental Medicine, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

Submitted 12 August 2002; accepted in final form 13 July 2003

Bates, Sandra R., Jian-Qin Tao, Kathleen Notarfrancesco, Kristine DeBolt, Henry Shuman, and Aron B. Fisher. Effect of surfactant protein A on granular pneumocyte surfactant secretion in vitro. Am J Physiol Lung Cell Mol Physiol 285: L1055–L1065, 2003.—Surfactant secretion by lung type II cells occurs when lamellar bodies (LBs) fuse with the plasma membrane and surfactant is released into the alveolar lumen. Surfactant protein A (SP-A) blocks secretagogue-stimulated phospholipid (PL) release, even in the presence of surfactant-like lipid. The mechanism of action is not clear. We have shown previously that an antibody to LB membranes (MAb 3C9) can be used to measure LB membrane trafficking. Although the ATP-stimulated secretion of PL was blocked by SP-A, the cell association of iodinated MAb 3C9 was not altered, indicating no effect on LB movement. FM1-43 is a hydrophobic dye used to monitor the formation of fusion pores. After secretagogue exposure, the threefold enhancement of the number of FM1-43 fluorescent LBs (per 100 cells) was not altered by the presence of SP-A. Finally, there was no evidence of a large PL pool retained on the cell surface through interaction with SP-A. Thus SP-A exposure does not affect these stages in the surfactant secretory pathway of type II cells.

PULMONARY SURFACTANT consists primarily of phospholipids (PL), especially dipalmitoyl phosphatidylcholine (DPPC), with a minor, albeit important, protein component. Surfactant functions to lower the surface tension at the air-liquid interface of the alveoli and to participate in host defense mechanisms (for review, see Ref. 35). Surfactant protein (SP)-A, the most abundant surfactant protein, is an octadecamer whose subunits (26- to 36-kDa molecular mass) are organized into a structure that resembles a “bunch of tulips” (39). Studies of the functions of SP-A examined under in vitro conditions concluded that SP-A enhances surfactant adsorption, participates in the formation of tubular myelin, regulates PL turnover, overcomes the surfactant protein inactivation of surfactant function, inhibits calcium-independent phospholipase A2, and aids in host defense (13, 14, 20, 40). In tissue culture studies with isolated type II cells, SP-A enhanced PL uptake and blocked both basal and stimulated PL secretion (13, 35). The role of SP-A in lung function in vivo is currently under intense scrutiny as mice with disruption of the SP-A gene survive in a normal environment (24). The importance of SP-A in innate immunity became clear when mice with SP-A gene disruption demonstrated a reduced ability to clear infectious agents from the lung on challenge (28). Lung lavage fluid from SP-A gene-targeted mice had significantly less tubular myelin and slight alterations in minimum surface tension parameters. PL metabolism under basal conditions was essentially normal in these animals, but the mice demonstrated an inability to respond adequately to stimuli that increased the rate of surfactant turnover (23).

Alveolar type II cells are the cuboidal epithelial cells that synthesize and secrete surfactant PLs and proteins. Before release, PLs are packaged as multilamellar whorls into the specialized type II cell secretory organelle, lamellar bodies. Secretion of surfactant PL from the type II cells occurs via a classic exocytotic mechanism where the lamellar bodies move to the cell surface; the limiting membrane of the lamellar body fuses with the plasma membrane, and the lamellar body contents are emptied into the alveolar lumen (36). Recently, Haller et al. (17) demonstrated that, on stimulation of secretion, the PL released from the lamellar body remains attached to the cell surface before its discharge into the media. PL secretion occurs at a basal rate that can be stimulated by a variety of agonists including exposure to secretagogues, increase in pH, or mechanical stretch (for review, see Refs. 8, 35). Inhibition of PL secretion results with use of pharmacological agents that block protein kinase C, affect calcium levels, or alter the cytoskeleton (for review, see Ref. 35). Both the lipid and SP-A moieties of surfactant can block PL release from rat type II cells, although SP-A is considerably more potent (12, 34). The inhibitory effect of SP-A on PL secretion is immediate and occurs in the presence of secretagogues that act through different pathways (35).

SP-A interacts with type II cells via specific cell surface receptors (31). The ability of SP-A to eliminate PL secretion requires the binding of SP-A to a specific
receptor on type II cells (25), through the carbohydrate recognition domain of the protein structure (30, 31, 34). However, the exact mechanism through which SP-A binding to a receptor downregulates type II cell secretion of PL is unknown. Because SP-A inhibits phosphatidylcholine (PC) release regardless of the secretagogue utilized, it has been postulated that the mode of action of the surfactant protein is late in the secretory pathway (8). The mechanism might involve either an indirect, or secondary, signaling pathway or a direct interaction between SP-A, lipid, and SP-A cell surface receptor.

The purpose of this study was to examine three of the possible mechanisms whereby SP-A could block surfactant PL secretion: 1) altering the trafficking of the lamellar bodies, 2) preventing the fusion of lamellar body membranes with the plasma membranes of the type II cells, or 3) binding the surfactant PL to the cell surface during the exocytotic process, thus preventing its release into the media.

MATERIALS AND METHODS

Cell preparation. Type II pneumocytes were isolated from the lungs of anesthetized male Sprague-Dawley rats essentially according to the method of Dobbs et al. (11), as previously described (2). Procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Rats were maintained under sterile conditions. Briefly, lungs were perfused through the pulmonary artery until free of blood, excised, and lavaged. The tissue was digested with elastase, and the lobes were separated and minced with a tissue chopper. The preparation was sequentially filtered through nylon screens (160-, 37-, and 10-μm mesh sizes) and enriched for type II cells by panning on rat immunoglobulin G (Sigma, St. Louis, MO)-coated petri dishes for 1 h at 37°C to remove contaminating macrophages. The pneumocytes were plated on 35-mm plastic tissue-culture dishes (Costar, Cambridge, MA) at 3 × 10⁶ cells/dish or on dishes with glass coverslips. Cells were cultured in 10% fetal calf serum (FCS) in Eagle’s minimal essential medium (MEM) at 37°C in a humidified incubator with 5% CO₂ in air. The purity of the type II cell preparation, as assessed by Papanicolaou stain, was >90% type II cells with macrophages as the principal contaminating cell type.

Purification of SP-A. Bronchoalveolar lavage fluid was obtained from lungs of alveolar proteinosis patients following therapeutic lavage at the Hospital of the University of Pennsylvania. Cellular material was removed by centrifugation, and surfactant was purified by density gradient centrifugation, followed by dialysis and lyophilization as described previously (2). We obtained SP-A according to the method of Hawgood et al. (19), using 1-butanol and β-n-glucopyranoside extraction, dialysis, and microconcentration, as described (2). The purity of the SP-A preparation was monitored by SDS-PAGE (27).

PC secretion. After isolation, type II cells were incubated overnight with 0.5 μCi/dish of [methyl-³H]choline (American, Arlington Heights, IL) in MEM containing 10% FCS to label cellular PLs. Cells were washed six times and incubated for 30 min in MEM. One set of cells was harvested at time 0 and served as control for PL secretion associated with the medium change, as described previously (3). The remaining cells were preincubated with or without SP-A, followed by additions of other agents (secretagogues, liposomes, or Sur- vanta) to the media in small volumes. The time of PC secretion in the table and figures is the time after the addition of secretagogues. SP-A preincubation varied between 15 min and 2 h. The time of preincubation with SP-A, within this time range, did not affect the results. Adenosine triphosphate (ATP), phorbol 12-myristate 13-acetate (PMA), and isoproterenol were obtained from Sigma. The medium was removed and centrifuged to remove detached cells. Methanol was added to the cell monolayer, and the cells were scraped from the dish. The cells and the media were extracted by the Bligh and Dyer method (5). The amount of PL secretion was calculated as the percentage of lipid degradations per minute (dpm) in the medium, relative to the total dpm in lipid present in the cells plus the medium. All experiments were performed in duplicate, and the values were averaged.

Monoclonal antibodies. Monoclonal antibody 3C9 (Mab 3C9) was obtained from BALB/c mice immunized with the lung membrane fraction of rat lung lamellar bodies, as previously described (42). After purification by precipitation from the hybridoma supernatant with (NH₄)₂SO₄, the MAb 3C9 was stored in aliquots.

Liposome preparation and Survanta. Lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Unilamellar liposomes were prepared from DPPC, egg PC, egg phosphatidylglycerol, and cholesterol (molar ratio, 10:5:2:3) using “The Extruder” (Lipex Biomembranes, Vancouver, BC, Canada) according to the directions of the manufacturer. Survanta, obtained from Abbott Laboratories (Columbus, OH), is a commercially available surfactant preparation isolated from bovine lung containing some SP-B and SP-C, but no SP-A. It has surface-active properties and is used for the treatment of neonates with respiratory distress.

Immunofluorescence of FM1-43. Type II cells on glass coverslips in tissue culture dishes were preincubated with SP-A (1 μg/ml) for 2 h, washed, and incubated with the indicated agents and FM1-43 (4 μM, Molecular Probes) for the specified time. At timed intervals, the cells were washed, fixed with 4% paraformaldehyde, and viewed on an inverted Nikon microscope. The number of type II cells (phase) and the number of FM1-43-positive LBs in each field were counted and expressed as number of labeled lamellar bodies/100 cells. At least five fields of 90 cells/field on average were counted under each condition for every experiment, and three to seven separate experiments were performed for each data point.

Iodination. Antibodies or SP-A were iodinated using Iodogen (Pierce, Rockford, IL) according to the directions provided by the manufacturer, and the iodinated protein was dialyzed extensively against Tris buffer. The specific activity for MAb 3C9 and SP-A was 90 ± 58 cpm/ng protein (n = 13) and 495 ± 110 cpm/ng protein (n = 9), respectively. The trichloroacetic acid (TCA) precipitability for MAb 3C9 and SP-A was 91.4 ± 1.1% (n = 13) and 92.8 ± 0.2% (n = 9), respectively. The iodinated proteins were stored at 4°C and used within 3 wk. Storage of MAb 3C9 or SP-A did not appreciably affect TCA precipitability.

Incubation of iodinated MAb 3C9 or SP-A with type II cells. Type II cells plated on plastic dishes were cultured for 20–24 h. Before the start of an experiment, nonadherent cells were removed by washing with MEM. For studies of the binding and uptake of MAb 3C9, type II cells were incubated with or without secretagogues and/or SP-A (1 μg/ml) for 30 min at 37°C, followed by the addition of iodinated MAb 3C9 for 2 h. To terminate the experiment, the nonbound 125I-MAb 3C9 was removed by three washes with MEM and two washes with phosphate-buffered saline (PBS) at 4°C. The cells were
harvested with trypsin and washed twice by centrifugation in PBS. Bound ligand was defined as trypsin-sensitive material, whereas trypsin-resistant material was assumed to represent internalized ligand.

For cell association of 125I-SP-A in the presence of liposomes or Survanta, 125I-SP-A was incubated with the type II cells for 15 min before the addition of liposomes. To terminate the experiment, we washed the cells as described for MAb 3C9 and harvested them with 0.2 N NaOH. Aliquots were taken for protein and radioactive determination. The amount of iodinated ligand associated with the cells was measured with a gamma counter. Background binding by dishes or wells without cells was determined in every experiment, and the amount of radioactivity in the absence of cells was subtracted from the samples. The results were normalized to cellular protein content as measured by the Lowry (29) or the Bradford (6) method.

Cell surface-associated PC. To test the hypothesis that, during the secretion of PC, there is one pool of PC that is released from the lamellar body directly into the media and a second pool that exits the lamellar body but remains attached to the cell surface; it was necessary to remove the latter from the plasma membrane with trypsin. However, after 20 min of trypsin treatment, the PC recovered from the trypsin solution consists of two pools of PC, a pool bound to the cell surface and released due to the action of trypsin (trypsin-sensitive PC), and a pool that left the lamellar body and entered the media immediately (medium PC). Thus, three sets of dishes of 3H-PC-labeled type II cells (sets A, B, and C) were incubated with ATP (1 mM) or ATP + SP-A (1 μg/ml) for 30 min to accumulate PC on the plasma membrane. One set (set A) was harvested to measure PC released into the media over the 30-min period. For the remaining dishes, the media containing the agents were removed, and either media alone (set B) or media containing 50 μg/ml trypsin (set C) were added. The incubation was continued for an additional 20 min at room temperature (total experimental time = 50 min), and the cells were harvested. In the samples with media alone (set B), the PC in the media represents the amount of PC released into the media from the cells during the 20-min period (medium PC). In the samples with trypsin (set C), the PC found in the trypsin solution after 20 min of trypsin treatment (total secreted PC) would represent the sum of the PC released into the medium (medium PC) plus the PC that was associated with the surface of the cell and released by trypsin (trypsin-sensitive PC). To determine the trypsin-sensitive pool of PC on the cell surface (trypsin-sensitive PC), set B (medium PC) was subtracted from set C (total secreted PC). The use of low levels of trypsin removes SP-A and SP-A receptors from the cell surface but does not remove the cells from the dish (9) and, thus, avoids centrifugation and damage to trypsin-treated cells with possible loss of PC.

Statistical analysis. Results are reported as means ± SE unless otherwise stated. Results were analyzed statistically by t-test or paired t-test using SigmaStat for Windows (Jandel, San Rafael, CA) where statistical significance is taken as P < 0.05. When SE bars are not visible in the figures, they are contained within the symbols.

RESULTS

We have previously shown that both bovine and human SP-A will suppress the release of PL from rat pneumocytes stimulated by PMA (3). Further experiments were performed to characterize the effects of the concentration of SP-A and the duration of treatment with SP-A necessary to eliminate secretagogue-induced PC secretion. The extent of SP-A inhibition of PC secretion into the media of type II cells stimulated by ATP was dependent on the concentration of SP-A (Fig. 1). Under these conditions, 1 μg SP-A/ml of media reduced the ATP-enhanced secretion of PC below basal values. The time course for the inhibition of PC release by SP-A is shown in Fig. 2. There was an initial release of PC in all samples after 15 min probably due to the media change. PC secretion in the control samples did not change substantially over the subsequent 2-h time period. Exposure to PMA resulted in a stimulation of PC secretion over control values that could be detected as early as 30 min into the incubation. The inhibitory effect of SP-A on secretion was immediate. In the presence of SP-A (1 μg/ml), secretion was below control values at the earliest time point (15 min) and remained low as the incubation proceeded. Coincubation of SP-A and PMA (10 nM) resulted in PC secretion values below those of the control cells although slightly higher than seen with SP-A alone. Because the level of secretion was variable between experiments, Table 1 (agonist, PMA) summarizes the data from several experiments where the results are expressed as a percentage of control values. After both 30 min and 2 h of incubation, the addition of 1 μg of SP-A/ml to the incubation media significantly reduced PC secretion under both control and secretagogue-stimulated (with 10 nM PMA) conditions.

The SP-A-mediated inhibition of the 3.4-fold stimulation of PC secretion by ATP (1 mM) was reversed by PC liposomes at a concentration of 45 μg PC/ml (Fig. 3A) in confirmation of earlier reports (for review, see Ref. 30). This effect of liposomes on the action of SP-A occurred whether SP-A was preincubated with the cells for 15 min before the addition of liposomes or whether SP-A was incubated with the liposomes for 30 min.

Fig. 1. Surfactant protein (SP)-A blocks phospholipid secretion stimulated by ATP. Type II cells were incubated with [3H]choline overnight, washed, and incubated without additions (Basal) or with 1 mM ATP and increasing concentrations of SP-A. SP-A was added 30 min before ATP. Phospholipid secretion was determined after a 2-h period as described in MATERIALS AND METHODS. Data are means ± SE as % phospholipid [phosphatidylcholine (PC)] secretion of n = 4–5 experiments. *Significantly different from basal; #significantly different from no SP-A (0 μg SP-A) with ATP; P < 0.05.
A set of cells was harvested at time 0 and served as control for phosphatidylcholine (PC) secretion associated with the medium change. The remaining cells were preincubated with or without surfactant protein A (SP-A) for up to 2 h did not affect the results. SP-A (1 μg/ml), PMA (10 nM), Control, 30 min ± 1.5 ± 0.4% PC secretion; Control, 2 h = 1.8 ± 0.1% PC secretion; ATP (10 μM), Control 2 h = 2.6 ± 0.5% PC secretion. *Significantly different from control; † significantly different from secretagogue; *P < 0.05.

**Table 1. Effect of SP-A on secretagogue-stimulated PC secretion by type II cells**

<table>
<thead>
<tr>
<th>Additions</th>
<th>30-min PMA</th>
<th>2-h PMA</th>
<th>2-h ATP + Isoproterenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100(3)</td>
<td>100(6)</td>
<td>100(3)</td>
</tr>
<tr>
<td>SP-A</td>
<td>55 ± 5%(3)</td>
<td>37 ± 14%(3)</td>
<td>20 ± 5%(3)</td>
</tr>
<tr>
<td>Agonist</td>
<td>196 ± 24%(3)</td>
<td>329 ± 44%(6)</td>
<td>322 ± 25%(3)</td>
</tr>
<tr>
<td>Agonist + SP-A</td>
<td>103 ± 4%(3)</td>
<td>87 ± 22%(6)</td>
<td>56 ± 20%(3)</td>
</tr>
</tbody>
</table>

Data are means ± SE of (n) number of experiments performed in triplicate and expressed as % of phosphatidylcholine (PC) secretion that occurred without additions (Control). Type II cells labeled overnight with [3H]choline were washed and incubated for 30 min. One set of cells was harvested at time 0 and served as control for phosphatidylcholine (PC) secretion associated with the medium change. The remaining cells were preincubated with or without surfactant protein A (SP-A) for up to 2 h did not affect the results. SP-A (1 μg/ml), PMA (10 nM), Control, 30 min ± 1.5 ± 0.4% PC secretion; Control, 2 h = 1.8 ± 0.1% PC secretion; ATP (10 μM), Control 2 h = 2.6 ± 0.5% PC secretion. *Significantly different from control; † significantly different from secretagogue; *P < 0.05.
special preparation of surfactant lacking SP-A, was less efficient at removing $^{125}\text{I}-\text{SP-A}$ from type II cells (Fig. 4) and thus did not affect PC secretion to the extent seen with PC liposomes at the same PL concentration. To reduce PC secretion to 40% of maximum stimulation measured in the presence of ATP and SP-A, 5 μg of liposome PC/ml (Fig. 3B) were required compared with 45 μg of Survanta PC/ml (Fig. 4). These data demonstrate that SP-A can modulate PC secretion in the presence of surfactant PL and that the form of PL was important in regulating SP-A-type II cell interactions.

Granular pneumocytes incubated with SP-A showed no changes in lamellar body trafficking, as measured by the binding and uptake of iodinated MAb 3C9, an antibody that recognizes ABCA3, an integral lamellar body membrane protein (32, 42). SP-A inhibited the PC secretion seen in both control cells and those stimulated by ATP (Fig. 5A). However, SP-A had no effect on the binding or uptake of iodinated MAb 3C9 whether SP-A was present alone or with ATP (Fig. 5, B and C). Similar results were seen with PMA under the conditions of these experiments. Binding or uptake of iodinated MAb 3C9 in the presence of PMA (10 nM) was $161 \pm 8$ or $170 \pm 9\%$ of control, respectively (mean ± SE, $n = 3$), and the addition of SP-A did not significantly affect the trafficking of the antibody [binding and uptake of MAB 3C9 with SP-A was $169 \pm 17$ and $158 \pm 5\%$ of control, respectively (means ± SE, $n = 3$)].

To determine whether SP-A influenced the fusion of the limiting membrane of the lamellar body with the plasma membrane and formation of a fusion pore, we used FM1-43, an amphipathic fluorescent dye. FM1-43 fluoresces on insertion into lipids but does not fluoresce in aqueous solutions, nor does it cross the plasma membrane readily. The dye gains access to the lamellar body lipids through the fusion pore, resulting in brightly fluorescent lamellar bodies (17). Type II cells were incubated without or with SP-A (1 μg/ml) for 2 h, followed by the addition of $^{125}$I-MAB 3C9 by type II cells. Type II cells were incubated for 15 min with the same additives described in A followed by the addition of iodinated MAB 3C9 (0.5 μg/ml) for an additional 2 h. The data are means ± SE for 5 experiments performed in duplicate. Control values for binding or uptake were 17.0 ± 5.2 and 33.6 ± 5.2 ng 3C9/mg cell protein, respectively. Significantly different ($P < 0.05$) from *control value, #ATP value.

**Fig. 5.** Effect of SP-A on phospholipid secretion and cell-association of MAB 3C9. A: PC secretion. Type II cells were incubated with $^{3}$H-choline overnight, washed, and incubated without additions (control) or with SP-A (1 μg/ml) for 30 min, followed by addition of ATP (1 mM) to selected dishes. The experiment was continued for 2 h. Data are means ± SE of phospholipid (PC) secretion as % of control, $n = 3$–4 experiments. Control value $= 1.9 \pm 0.2\%$ PC secretion. B and C: binding and uptake, respectively, of $^{125}$I-MAB 3C9 by type II cells. Type II cells were incubated for 15 min with the same additives described in A followed by the addition of iodinated MAB 3C9 (0.5 μg/ml) for an additional 2 h. The data are means ± SE for 5 experiments performed in duplicate. Control values for binding or uptake were 17.0 ± 3.5 or 33.6 ± 5.2 ng 3C9/mg cell protein, respectively. Significantly different ($P < 0.05$) from *control value, #ATP value.

**Fig. 4.** Survanta is less effective than liposomes at modulating ATP-stimulated surfactant secretion by SP-A. Type II cells were incubated with ATP (1 mM) and SP-A (1 μg/ml) for 15 min followed by no further additions or increasing concentrations of Survanta. In one series, the cells were incubated with $^{3}$H-choline overnight to label PC, and $^{3}$HPC secretion was measured for 2 h (○). In the other series, cell association of $^{125}$I-SP-A during a 2-h incubation was determined (●). For $^{3}$HPC secretion, data are means ± SE and are expressed as % of maximum secretion (1 mM ATP). ATP maximum = 10.2 ± 1.1% PC secretion, $n = 5$. For $^{125}$I-SP-A, data are means ± SE, $n = 3$, and are expressed as % of cell-association of $^{125}$I-SP-A in the absence of Survanta as a control (maximum). Maximum cell association of SP-A = 46 ± 7 ng $^{125}$I-SP-A/mg cell protein.

---

**AJP-Lung Cell Mol Physiol • VOL 285 • NOVEMBER 2003 • www.ajplung.org**
ture medium (Fig. 6A). Figure 6B quantitates the number of labeled lamellar bodies per 100 cells. The entrance of FM1-43 into control cells has reached a plateau by 30 min. After 60 min of incubation with secretagogues, there was significantly more labeled lamellar bodies in the secretagogue-treated cells. In the presence of secretagogues, SP-A did not reduce the number of labeled lamellar bodies, indicating that SP-A did not prevent the formation of fusion pores between lamellar bodies and the plasma membrane. However, as shown in Table 1, SP-A (1 μg/ml) blocked the secretagogue-stimulated release of PL into the medium under similar experimental conditions. The threefold increase in PC secretion stimulated by 10 μM ATP and 10 μM isoproterenol was completely blocked by SP-A.

Next, we measured whether there was a large membrane-bound pool of newly secreted PL in the presence of SP-A by exposing the type II cells to low levels of trypsin. PL associated with type II cells has been shown to be sensitive to trypsin treatment (7). To establish conditions where PC would accumulate on the plasma membrane, we incubated type II cells with SP-A (1 μg/ml) plus ATP (1 mM) for 30 min. ATP alone served as a control for the effects of ATP. One set of dishes was harvested, and PC release into the media was measured (Fig. 7A). At this time point, the inhibition of secretagogue-stimulated PC secretion by SP-A is already apparent as shown in Fig. 7A and Table 1. Next, the agents were removed, and two sets of dishes were incubated for an additional 20 min at room temperature without agents (Fig. 7B). One set was incubated in media alone and used to measure the PC directly released into the media (Fig. 7B, left), whereas the other set was incubated with trypsin (50 μg/ml) to measure the trypsin-sensitive PC pool (Fig. 7B, right)
(see MATERIALS AND METHODS). With cells that had been incubated previously for 30 min without additions (control), in the subsequent 20 min of incubation, 52% of the total released PC was associated with the surface of the cell in a trypsin-sensitive pool, whereas the remaining 48% was released into the media (Fig. 7B). Cells initially exposed to ATP for 30 min continued to demonstrate enhanced PC directly released into the media (Fig. 7B). Cells initially exposed to ATP for 30 min continued to demonstrate enhanced PC directly released into the media (Fig. 7B). In addition, there was a significant amount of PC in a trypsin-sensitive compartment on the plasma membrane of these type II cells, 60% more than seen in control cells (Fig. 7B, right). With cells exposed to media containing ATP + SP-A for 30 min, removal of the media resulted in an increase in both the directly released PC and the trypsin-sensitive PC over the next 20 min, presumably due to the ATP remaining in the cells and reversal of the SP-A-blocking mechanism (Fig. 7B). However, there was no evidence of a large pool of cell-associated PC that, theoretically, would have accumulated on the cell surface due to the prior 30-min exposure to ATP in the presence of SP-A. The medium PC and trypsin-sensitive PC pools were similar to or lower than those found from cells previously exposed to ATP alone.

It is possible that a membrane-associated pool of newly secreted PC might be internalized to a more significant extent with SP-A in the media, as we and others have shown that SP-A enhances the uptake of PL liposomes (2, 13). Type II cells, labeled overnight with [3H]choline, were incubated with ATP (1 mM) for 2 h. The media containing newly secreted [3H]-labeled PC was collected, SP-A (1 μg/ml) was added to some samples, and the PC-labeled media without or with SP-A was added to unlabeled type II cells. After 2 h, the cells were scraped from the dish, and the amount of [3H]PC incorporated into the cells was determined. Labeled PC associated with empty dishes treated identically was subtracted from the results. Of the total [3H]PC in the media, 8.4 ± 1.5% was associated with the type II cells in the absence of SP-A. The addition of SP-A to the media slightly, but significantly, increased the association of [3H]PC to the type II cells by 139% to 12 ± 2% (P = 0.005, means ± SE, n = 3). However, this relatively minor level of SP-A-stimulated cell association of PC does not occur at a sufficient extent to account for the complete blockage of PC secretion by SP-A, as similarly concluded by others (12, 34). Scraping the cells, as described here, or dissolving the monolayers in methanol, as described elsewhere (12, 34), will not determine whether the [3H]PC is internalized into the pneumocytes or bound to the cell surface. Thus the cells were harvested with 0.5% trypsin, and the [3H]PC in a trypsin-insensitive compartment was measured. A majority of the total cell-associated PC was trypsin insensitive without or with SP-A in the media (86 ± 3 or 80 ± 3%, respectively, means ± SE, n = 3, values are not significantly different). Thus under these conditions, SP-A slightly enhanced both binding and uptake of PC, ruling out a major role for SP-A-induced internalization of newly synthesized PC.

DISCUSSION

In this study of the potent inhibition of surfactant secretion from granular pneumocytes in culture by SP-A, we determined that incubation with SP-A did not affect three pathways critical for release of surfac-
tant PL. Trafficking of lamellar bodies between the interior of the cell and the plasma membrane, formation of fusion pores, and association of newly released PC with the plasma membrane before discharge into the media were not altered by exposure of the type II cells to SP-A. The results indicate that PLs were never exocytosed from type II cell lamellar bodies in the presence of SP-A. Although the mechanism for inhibition by SP-A remains unresolved, regulation of the expansion of the fusion pore remains an intriguing possibility.

The ability of SP-A to block surfactant PC secretion from alveolar pneumocytes in primary culture was reported several years ago (12, 34). SP-A isolated from all species tested to date, including human (3, 12, 31), canine (12, 34), bovine (3), and rat (12), have demonstrated the ability to block PC secretion. Binding of SP-A to its receptor on the surface of type II cells is critical for its action on surfactant secretion. Any perturbations to the receptor-SP-A interaction, such as the removal of calcium from the medium (12), release of SP-A by washing, the use of anti-SP-A antibodies (12, 34) or anti-SP-A receptor antibodies (10), or modifications to the SP-A protein structure (12, 31, 34), will inhibit its ability to block secretion. We demonstrate in this study that the addition of liposomes interfered with SP-A-cell contact, thereby preventing SP-A function. The mode of action may have been due to either liposome-mediated removal of SP-A from the cell surface or liposome interference with SP-A-receptor interactions. The high affinity of SP-A for DPPC is well documented (14, 26). For that reason, for some experiments, we preincubated the cells with SP-A before addition of liposomes to insure that the SP-A would have the opportunity to interact with SP-A receptors before exposure to lipids. Even with those experimental precautions, the liposomes were able to remove most of the SP-A from the type II cells. The results indicate that, during the experiment, the bulk of the SP-A either remained on the cell surface or was taken up by the cell and rereleased via a retroendocytic process (41). In either case, the SP-A was available for liposome interaction. When SP-A and liposomes were coincubated before exposure to the type II cells, the extent of SP-A-cell interactions was regulated by the concentration of liposomes, indicating an equilibrium between the affinity of SP-A for PC liposomes and the SP-A receptor. In addition, it is possible that binding of SP-A to PC liposomes alters the structure of the SP-A-receptor binding region of the SP-A molecule, affecting binding affinity.

The form of the PL made an important contribution to the results, as Survanta, a commercial preparation of PL that contains large aggregates, did not interfere with SP-A-receptor interactions or SP-A biological activity to the same extent as liposomes. At the physiological ratio of SP-A to PL in surfactant of 1:18, SP-A continues to inhibit PC secretion by ~80% in the presence of Survanta but only ~30% in the presence of liposomes. Such data provide support for the in vivo regulation of PC secretion by SP-A, as PC release is reduced by SP-A even in the presence of surfactant-like PLs at protein-lipid ratios found in natural surfactant. The experiments also provide further evidence that SP-A must remain associated with the cell to regulate PC secretion.

The mechanism through which SP-A works to inhibit PC extrusion remains unclear. Several possible direct effects of SP-A on PC metabolism had been excluded by others. SP-A did not affect PC synthesis (34), cause the deacylation of PC to lyso-PC (12), or significantly stimulate the reassociation of released PC to the cell (12, 34). On the basis of the observation that SP-A inhibits both basal and agonist-enhanced PC release regardless of stimulus, the action of SP-A was felt to be late in the secretory pathway. We assumed that, by whatever mechanism SP-A controls surfactant secretion, the result should be the cessation of movement of lamellar bodies to the periphery of the cell, inhibition of lamellar body docking to the plasma membrane, absence of fusion pores, decreased release of surfactant from the cell, or enhanced uptake of released PC. Thus we first followed the trafficking of lamellar bodies utilizing the MAb 3C9, which we had employed in previous studies (4, 37). MAb 3C9 recognizes the lamellar body membrane-associated protein ABCA3 (32, 42). Calphostin C, an agent that blocks PC secretion, also inhibited the binding and uptake of 3C9 antibody, in concert (4). However, the current studies demonstrate that SP-A, which also prevents PC secretion, did not interfere with the turnover of lamellar body membranes. Iodinated 3C9 antibody bound to the lamellar body membrane, presumably within a fusion pore, and became trypsin insensitive whether or not SP-A was present.

To provide additional confirmation that creation of fusion pores was not affected by SP-A, our next series of experiments followed the formation of fusion pores using FM1-43, a hydrophilic dye that fluoresces in a hydrophobic environment. This dye has been used to indicate the presence of fusion pores in several studies including the work of Haller et al. (17) examining the mechanisms involved in the exocytosis of surfactant from type II cells. We showed that there were more FM1-43-positive fluorescent lamellar bodies in secretagogue-exposed cells compared with controls and, thus, more fusion pores. However, SP-A did not affect the number of fusion pores formed, whereas secretagogue-stimulated PC secretion was markedly reduced in cultures with SP-A. Thus SP-A must act at a point more distal than lamellar body-plasma membrane fusion and formation of fusion pores.

Another possible mechanism for the action of SP-A would involve the surfactant protein binding to the SP-A receptor and blocking the release of PL either by preventing the opening of the fusion pore or by binding to PL as it is secreted through the fusion pore. The ability of SP-A to modulate fusion pore dynamics will be explored in future studies. In the current work, we examined the latter mechanism, which would involve either 1) sequestering the PL to the plasma membrane and preventing its release into the media or 2) promoting a rapid reuptake back into the type II cells. We
used two approaches: 1) determine whether there is a large membrane-bound pool of newly secreted PL in the presence of SP-A and 2) follow the reuptake of newly synthesized and released surfactant.

On stimulation of secretion, the formation of fusion pores is a fairly rapid event, whereas release of surfactant into the aqueous milieu is a slower process (17). Consequently, PC that exits the fusion pore of the pneumocyte would first enter a pool of PC that remains closely associated with the cell surface, followed, later, by release into the media (17). We explored the possibility that SP-A prevented PC release into the aqueous milieu by binding to both the SP-A receptors on the cell surface and the newly secreted surfactant PL and, thereby, increasing the pool of PC bound to the cell surface. We found evidence for a plasma membrane-associated PL pool that could be released from the cell surface by mild trypsin treatment. The trypsin-sensitive lipids may be similar to the extracellular PLs visualized by microscopic procedures (17). The size of the pool increased with secretagogue treatment. Thus previous quantitation of PC released into the media using conventional methods substantially underestimates the actual amount of surfactant secreted from lamellar bodies. However, there was no evidence for SP-A-mediated accumulation of a large trypsin-sensitive pool of newly secreted PC on the type II cell surface.

We next looked for an SP-A-mediated rapid reuptake of newly secreted PL. Previous reports as well as our current work have shown that SP-A causes a significant cell association of newly secreted PL (12, 34). However, the low levels of SP-A-mediated incorporation of newly secreted PL measured in these studies were not sufficient to support this pathway as a mechanism for the action of SP-A. The results were not unexpected as −10 μg SP-A/ml was necessary to cause a significant accumulation of intracellular PC from labeled liposomes (2), whereas only 1 μg of SP-A/ml was tested in the present experiments due to the fact that the latter concentration is sufficient to block PC secretion. In addition, we measured the internalization of newly secreted PC and determined that, in fact, the bulk of the PC reassociated with the cells was recovered in a trypsin-insensitive compartment without regard to the presence of added SP-A. However, the results of this type of experiment should be viewed with caution, as they may not represent physiological conditions. Once the tightly compacted surfactant lipid is released from the hydrophobic acidic environment of the lamellar body into a large volume of pH neutral aqueous media, structural changes may occur in the physical state of the surfactant. Thus assaying the interaction of SP-A with newly liberated surfactant lipids found in the media may not represent the situation that exists in the microenvironment of the cell’s surface where surfactant is in close approximation to the lipids of the plasma membrane.

There was no evidence that the lamellar body PL contents had ever emerged from the pneumocytes through the fusion pore in the presence of SP-A. The possibility remains that SP-A acts at the level of the fusion pore by either physically blocking fusion pore opening or preventing its further expansion. SP-A may form a physical barrier across the pore by associating with itself (15), by aggregation of lipids (19), or formation of tubular myelin figures (20, 40) that might plug the fusion pore entrance. However, preliminary electron microscopy data of type II cells incubated with ATP and SP-A did not show evidence of lamellar body fusion pores blocked by proteinaceous material. In any case, once the fusion pore widened sufficiently, surfactant PC should be able to overcome such a physical block by SP-A. Recently, Haller et al. (16, 18) raised the intriguing possibility that the size of the fusion pore itself may be one of the rate-limiting steps for the regulation of PC secretion by type II cells. Unlike in mast cells and neuronal cells where fusion pore formation is rapidly followed by enlargement of the pore and release of vesicle contents (1), in type II cells fusion pore formation is fairly slow (within 15 min of stimulation), and release of surfactant into the extracellular space can take hours (17). Haller et al. (16) observed that the fusion pores were much narrower than the attached lamellar bodies and that the surfactant seemed to squeeze out of these openings. The evidence presented in the current work would be consistent with SP-A mediation of the opening of the fusion pore. A possible means for SP-A control of fusion pore geometry includes regulation of intracellular calcium levels. This hypothesis is based on the observations that SP-A interaction with its receptor has been shown to modulate cytoplasmic calcium levels (38) and changes in intracellular Ca²⁺ levels were correlated with expansion of the diameter of the fusion pore in secretagogue-treated isolated type II cells in culture (16).

The ability of SP-A to regulate PL turnover of alveolar pneumocytes in culture raised the possibility that this surfactant protein would have a similar role in the intact lung. However, the absence of SP-A in SP-A gene-targeted (SP-A−/−) mice did not seem to have a detrimental effect on surfactant metabolism. Wild-type and SP-A−/− mice have shown similarities in surfactant pool sizes, PL synthesis, secretion, and rates of clearance, leaving the function of SP-A in surfactant turnover unclear (21, 22, 33). On the other hand, abnormalities in lipid clearance have emerged in SP-A−/− mice upon physiological stress such as in our studies of hyperventilation or secretagogue exposure (23) as well as those of others on LPS injury (33). It is quite possible that SP-A does not play a major role in the regulation of surfactant secretion in vivo, that measurements made at a single time point are not sufficient to show differences, as pointed out by the authors (33), or that surfactant metabolism is so crucial that redundant alternate mechanisms exist to compensate for the lack of SP-A. What is clear is that, for type II cells in culture, SP-A can completely block surfactant secretion. The present study demonstrates that this biological activity of SP-A occurred even in the presence of surfactant. Although the mechanism for the regulation of surfactant secretion from type II cells remains unclear,
the results of this study eliminate several possibilities and are consistent with an important regulatory process involving the control of fusion pore expansion.

In conclusion, we have shown that several of the critical stages involved in type II cell surfactant secretion are not affected by SP-A. Neither trafficking of lamellar bodies nor formation of fusion pores was altered by exposure to SP-A. In addition, there was no evidence that SP-A promoted the enlargement of the cell-surface adherent pool of newly released PC. In fact, SP-A completely prevented the release of PL from the lamellar body fusion pore. Therefore, these results suggest that SP-A acts at a very late or final stage of secretion. The fact that SP-A blocks secretion in the presence of surfactant lends support to a regulatory role for SP-A in surfactant release in the intact lung.

The authors thank Dr. Susan Gutten tag for the gift of Survanta.

DISCLOSURES

This research was supported by National Heart, Lung, and Blood Institute Grant HL-19737. Preliminary results of portions of this work were presented at the 2001 American Thoracic Society International Conference in San Francisco, CA.

REFERENCES


