Secretagogues Increase the Expression of Surfactant Protein A Receptors on Lung Type II Cells*

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Since secretagogues have been shown to increase the internalization of surfactant phospholipid and protein by lung cells, we postulated that their action occurred through a mechanism involving increased surfactant protein A (SP-A) receptor density. Therefore, we evaluated the influence of secretagogues on the binding of iodinated SP-A to alveolar type II cells. Type II cells were isolated from rat lung and maintained in primary culture for 18 h on Transwell membranes. Upon exposure to 8-bromo-cyclic AMP (cAMP, 0.1 mM), phorbol 12-myristate 13-acetate (PMA, 0.1 mM), terbutaline (0.1 mM), or ATP (1 mM), the binding of SP-A increased 1.5–2-fold. This stimulation was cell substrate-dependent since type II cells plated on plastic dishes did not show this effect. A time course of the stimulation of SP-A binding due to secretagogues showed that both cAMP and PMA increased SP-A binding by 2-fold after 20 min. With cAMP, binding remained elevated for 2 h, while binding in the presence of PMA had returned to control values. The effects of submaximal concentrations of cAMP and PMA on binding were additive. Inhibition of cellular protein synthesis with cycloheximide did not alter the increase of SP-A binding stimulated by the secretagogues. Type II cells pretreated with PMA responded to subsequent treatment with cAMP by increasing SP-A binding, while these cells were refractory to subsequent treatment with PMA. Both constitutive and regulated binding of SP-A to type II cells were sensitive to trypsin. The binding of SP-A to type II cells showed saturation at a concentration of 1 μg/ml SP-A under control and secretagogue-stimulated conditions, with both total and calcium-dependent binding showing a 2-fold increase upon secretagogue exposure. The data are consistent with the hypothesis that secretagogues stimulate surfactant uptake, at least in part, through recruitment of SP-A receptors to the type II cell surface, resulting in an increase in the number of SP-A binding sites.

Pulmonary surfactant is a complex mixture of phospholipids and proteins secreted by the type II alveolar epithelial cells. Surfactant functions as the surface tension-reducing material at the air–liquid interface of the alveoli. In order to maintain an appropriate amount of functional surfactant in the alveolar space, surfactant turnover should be tightly regulated. The rate of surfactant lipid uptake by intact lungs and isolated type II cells has been shown to be stimulated by agents that promote surfactant secretion. Secretagogues have augmented the clearance of lipid from rabbit lungs (Pettenazzo et al., 1989) and the uptake of surfactant protein and dipalmitylophosphatidylcholine into both isolated, perfused rat lungs (Fisher et al., 1985, 1989, 1991) and type II cells cultured on microporous membranes (Chinoy et al., 1993). Endocytosis of phospholipids is enhanced also by surfactant protein A (SP-A),† a major protein constituent of lung surfactant. SP-A (26–38 kDa, reduced) is a glycoprotein that shows calcium-dependent binding to phospholipid vesicles through an internal hydrophobic domain (Ross et al., 1986). Several studies have shown that SP-A stimulates the incorporation of phospholipid by primary cultures of type II cells (Wright et al., 1987; Tsuzuki et al., 1993; Bates et al., 1994). SP-A also functions to inhibit surfactant phospholipid secretion by type II cells (Dobbs et al., 1987; Kuroki et al., 1988). Thus, SP-A may play a pivotal role in the regulation of surfactant turnover. Type II cells express a high affinity receptor for SP-A (Kuroki et al., 1988, Wright et al., 1989) that recognizes the carboxyl-terminal portion of the SP-A molecule (Wright et al., 1989; Murata et al., 1993). Using an anti-idiotypic antibody approach, two groups have identified type II cell membrane proteins specific for SP-A with molecular masses of either 30-kDa (Strayer et al., 1993) or 55 kDa (Stevens et al., 1995). The cDNA for the 30-kDa receptor protein has been cloned and sequenced and the structure of the encoded protein deduced. Strayer et al. (1996) have shown that antibodies to the 30-kDa protein inhibit the binding of SP-A and mimic the functional ability of SP-A to down-regulate phospholipid secretion stimulated by secretagogues in isolated type II cells. Evidence has been presented that the receptors function physiologically since binding to type II cells isolated from rats after silica treatment showed a 2-fold increase in saturable SP-A binding over controls (Suwabe et al., 1991). In addition, SP-A metabolism was cell substrate-dependent. Type II cells plated on microporous membranes maintained their cuboidal shape and other differentiated characteristics and also demonstrated elevated binding and incorporation of SP-A as compared with cells plated on plastic tissue culture dishes (Bates et al., 1994; Chinoy et al., 1993). Since secretagogues stimulated the uptake of biosynthesized SP-A and phosphatidylcholine (Chinoy et al., 1993), in this report we have examined the possibility that secretagogues induce SP-A receptor activity and have found that secretagogues enhance SP-A binding to type II cells.

MATERIALS AND METHODS

Isolation and Culture of Type II Cells—Type II cells were isolated from anesthetized pathogen-free male Sprague-Dawley rats, weighing 200–250 g by the method of Dobbs et al. (1986), as described previously.

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‡ The abbreviations used are: SP-A, surfactant protein A; PMA, phorbol 12-myristate 13-acetate; MEM, Eagle’s minimal essential medium; PKC, protein kinase C; PKA, protein kinase A.
in detail (Chinoy et al., 1993). Briefly, after perfusion via the pulmonary artery with a low concentration of trypsin, lungs were minced with elastic and minced in the presence of deoxyribonuclease (DNase, Sigma) and fetal bovine serum (fetal calf serum, ICN/Flow Laboratories, ICN Biochemicals, Costa Mesa, CA). Cells were separated by filtration and enriched by blotting on rat immunoglobulin G (IgG, Sigma)-coated Petri dishes. The purity of the freshly isolated type II cell preparation was routinely >98% by modified Papanicolaou stain, and the viability was >98% by vital dye exclusion.

Cells were plated at 5 \times 10^5 or 1.5 \times 10^5 type II cells in 24- or 12-mm inserts of Transwell microporous membranes (Costar, Cambridge, MA, 3-μm pore size), respectively, or 35-mm plastic tissue culture dishes (Costar) at 3 \times 10^5 cells/dish. Cells were cultured in 10% fetal calf serum in Eagle's minimal essential medium (MEM) at 37°C in a humid incubator with 5% CO₂ in air. After overnight culture and removal of nonadhered cells, the purity of the type II cells was >90%.

Isolation and Labeling of SP-A—Alveolar lavage fluid was obtained from the lungs of bovine (slaughterhouse) or alveolar proteinosis patients. The surfactant was purified using density gradient centrifugation followed by dialysis and lyophilization as described previously (Fisher et al., 1991). SP-A was isolated from surfactant using 1-butanol and (E)-4-phenyl-2-pentenoic acid extraction, deproteinization, and incubated according to the method of Hawgood et al. (1985). SP-A was iodinated using IODO-GEN (Pierce) according to the directions provided by the manufacturer, and the iodinated protein was dialyzed extensively against Tris buffer. An aliquot of 125I-SP-A was analyzed for protein concentration by the Lowry method (Lowry et al., 1951). An aliquot of the samples was analyzed for protein concentration by the Lowry method (Lowry et al., 1951). Dishes or wells without cells were carried through the experiment concurrently, and the amount of background radioactivity was subtracted from the samples with cells. This correction varied between experiments and ranged from 10 to 30% of total counts. Each experiment was carried out with duplicate or triplicate samples.

Statistical Analysis—Results are expressed as mean ± S.E. for the number of experiments indicated (n). Statistical analysis was performed using one-way analysis of variance followed by Dunnett’s test and Student’s t test. Significance was set at p < 0.05.

RESULTS

The Effect of Secretagogues on the Binding of SP-A—Our previous reports had shown an increase in the binding of SP-A and in the uptake of surfactant phospholipid, liposomal phospholipid, and bovine SP-A by type II cells on Transwell membrane as compared with cells on plastic dishes (Bates et al., 1994; Chinoy et al., 1993). In the present study, the binding at 4°C of human 125I-SP-A to type II cells was examined. Type II cells plated on plastic dishes bound 5.7 ± 0.7 ng of SP-A/mg of cell protein (n = 8), whereas the binding of SP-A to cells plated on Transwell membranes was 27.7 ± 2.7 ng of SP-A/mg of protein (n = 13), approximately five times higher than that for cells on plastic (p < 0.01). Upon exposure of cells on Transwell membranes to 8-bromo-cAMP (0.1 mM), terbutaline (0.1 mM), or ATP (1 mM) for 2 h, the binding of SP-A increased 1.4–2-fold, over controls, as shown in Fig. 1A. There were no significant changes in SP-A binding in the presence of PMA (10 nM). In contrast, type II cells plated on plastic did not show significant changes in SP-A binding upon exposure to any of the secretagogues (Fig. 1B).

Time Course of the Effect of cAMP and PMA—To determine the time of incubation necessary for the augmentation of SP-A binding, a time course of the effect of cAMP and PMA on the stimulation of SP-A binding was performed. Cells cultured on Transwell membranes were incubated with cAMP or PMA for the indicated time at 37°C and washed, and the binding assay was performed at 4°C. After 20 min, cAMP exposure increased SP-A binding by 2-fold over control values, and SP-A binding remained elevated at 2 h (Fig. 2A). Type II cells incubated with PMA also showed a 2-fold elevation in SP-A binding at 20 min (p < 0.01). The increased level with PMA was not maintained, and after 60 min of exposure to PMA, SP-A binding had returned essentially to control level (125.7 ± 4.2% of control, n = 3). As shown in Fig. 2B, type II cells treated with PMA for 2 h, washed, and reincubated with PMA did not show increased SP-A binding in response to the second PMA treatment. However, cAMP was able to induce a 2-fold increase in binding of SP-A in the PMA-pretreated type II cells.

The increase in SP-A binding to type II cells on Transwell membranes was dependent on the concentration of cAMP or PMA, as shown in Fig. 3. Type II cells were exposed to 0.1–150 μM cAMP or 0.1–50 nM PMA for 20 min, cooled to 4°C, and the SP-A binding assay performed. The secretagogue concentrations that resulted in maximal binding of SP-A to type II cells were 100 μM cAMP or 10 nM PMA.

Short-term inhibition of protein synthesis with cycloheximide did not affect the cellular binding of SP-A. Type II cells were incubated without or with 50 μM cycloheximide for 30 min at 37°C. The cells were cooled and the SP-A binding assay was performed. Cycloheximide treatment did not significantly alter the binding of SP-A (96.3 ± 1.4%, n = 3 of control values). In the presence of cycloheximide, the cells responded to a 20-min exposure to cAMP (193.7 ± 8.1%, n = 3) or PMA (198.5 ± 4.4%, n = 3) in a similar manner as type II cells incubated with...
Secretagogues when protein synthesis was not inhibited (Table I, Expt. 1).

The Additive Effect of Secretagogues—The data indicated that both cAMP and PMA increase SP-A binding but probably function via two different pathways. The effect of cAMP on surfactant secretion by type II cells has been shown to act via protein kinase A (PKA) while PMA activates PKC (Griese et al., 1993). Thus, the two secretagogues were tested for additive effects on SP-A binding. Fig. 3 indicates that a concentration of 0.1 mM cAMP and 10 nM PMA stimulated SP-A binding to the maximal extent. As shown in Table I (Expt. 1), the exposure of type II cells on Transwell membranes to a combination of 0.1 mM cAMP and 10 nM PMA did not enhance SP-A binding above the 2-fold stimulation produced by the secretagogues alone. Due to the possibility that the number of SP-A receptors was limited and that a 2-fold stimulation was maximal, experiments were performed using concentrations of secretagogues that produced a half-maximal response. Either 0.1 mM cAMP or 1 nM PMA stimulated SP-A binding by approximately 140% over controls, after 20 min of incubation (Table I, Expt. 2). Exposure of type II cells to both 0.1 mM cAMP and 1 nM PMA for 20 min increased SP-A binding to 202%, consistent with the possibility that the effects of the secretagogues were additive under these conditions.

The Effect of Protease Treatment on SP-A Binding—To determine whether SP-A was binding to a trypsin-sensitive site on the cell membrane, type II cells plated on plastic dishes were incubated at room temperature for 20 min with increasing concentrations of trypsin from 12 to 250 μg/ml, washed with ice-cold media, and assayed for 125I-SP-A binding at 4°C (see “Materials and Methods”). Trypsin at 12 μg/ml had no effect on SP-A binding. However, trypsin at 50 μg/ml reduced SP-A binding to 42 ± 5% (n = 6) of control values. Increasing the trypsin concentration up to 250 μg/ml did not reduce binding further (43 ± 5%, n = 2).

The reduction of type II cell surface SP-A binding sites by trypsin was reversible as shown in Fig. 4A. After trypsin treatment (50 μg/ml), the cells were incubated in trypsin-free media with or without secretagogues for an additional 20 min. Trypsin-treated type II cells (no additions) showed a return of SP-A binding to resting values (106.3 ± 7.7%, n = 3). Type II cells that were exposed to cAMP or PMA following trypsin treatment were able to enhance SP-A binding to levels that were signifi-
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**Fig. 3. Effect of concentration of secretagogues on enhancement of SP-A binding to type II cells.** Type II cells on Transwell membranes were incubated with increasing concentrations of cAMP (C) or PMA (○) at 37 °C for 20 min. The cells were cooled to 4 °C, and the binding of 125I-SP-A to type II cells was performed. Control values from untreated cells in the cAMP or PMA experiments were 31.8 ± 4.1 and 30.3 ± 3.9 ng of SP-A/mg of cell protein, respectively. The values are mean ± S.E. of three to seven experiments.

![Diagram](image)

The Characterization of SP-A Binding to Type II Cells in the Presence of Secretagogues—To determine whether the increase in SP-A binding to type II cells in the presence of secretagogues might be due to a change in receptor affinity or in nonspecific binding, the dose-response for SP-A binding to type II cells was characterized after secretagogue treatment. As shown in Fig. 5, the binding of SP-A varied with the exogenous SP-A levels and showed saturation at an SP-A concentration of 1 μg/ml. After exposure to either cAMP or PMA, the binding was significantly greater than untreated controls at each concentration of SP-A tested, but the concentration required for saturation was similar. The extent of SP-A binding was comparable for both secretagogues. To evaluate the amount of calcium-dependent specific binding, the binding assay was performed in the presence or absence of 10 mM EGTA. Specific binding was determined by subtracting the calcium-independent binding (with 10 mM EGTA) from the total binding value (no EGTA). Of the total binding of SP-A to type II cells, the calcium-dependent binding of SP-A was 68.2 ± 4.4% for control cells, 69.6 ± 4.2% for PMA, and 68.1 ± 1.5% for cAMP-treated cells (mean ± S.E., n = 3–4) as shown in Fig. 6. Both PMA and cAMP stimulated specific SP-A binding 2-fold while also increasing calcium-independent binding. With increasing levels of SP-A in the media (Fig. 7), both the calcium-dependent (Fig. 7A) and the calcium-independent (Fig. 7B) binding were saturable and showed a similar binding pattern as the total binding values presented in Fig. 5.

**Table I**

| Additions | Concentrations | Binding | Control |
|-----------|----------------|---------|---------|
| Expt. 1   |                | %       | n       |
| Control   |                | 100.0   | 3       |
| cAMP 0.1 mM | 54.3 ± 4.3% | 200.2  | 3       |
| PMA 10 nM | 55.7 ± 3.3% | 205.4  | 3       |
| cAMP 0.1 mM + 10 nM | 55.9 ± 3.9% | 206.3  | 3       |
| + PMA     |                |         |         |
| Expt. 2   |                |         |         |
| Control   |                | 100.0   | 8       |
| cAMP 0.1 μM | 50.5 ± 3.9% | 145.2  | 8       |
| PMA 1 nM | 48.5 ± 4.3% | 139.4  | 6       |
| cAMP 0.1 μM + 1 nM | 70.2 ± 7.6% | 201.6  | 5       |

*Significantly different from control values at p < 0.05.

Although the calcium-independent binding of SP-A was enhanced with secretagogue treatment, the bulk of the observed increases in total binding were due to a rise in the specific calcium-dependent binding.

**DISCUSSION**

As a major surfactant protein constituent, SP-A has been proposed to have several functions in the alveoli of the lung. The capacity of SP-A to function as the modulator of surfactant secretion and turnover appears to involve the binding of SP-A to type II cells, probably via specific cell surface receptors (Murata et al., 1993; Wright et al., 1989; Kuroki et al., 1988). Previously we have reported that secretagogues enhanced the uptake of lipid and protein components of lung surfactant by isolated perfused lungs (Fisher et al., 1991) and by type II cells (Chinoy et al., 1993). This effect also may be associated with an increase in binding sites for SP-A. Therefore, our present effort characterized the cell surface receptor for SP-A and evaluated the effect of secretagogues on the activity of the receptor.

Previously, we reported that the secretagogues, cAMP and terbutaline, significantly stimulated the uptake of surfactant 35S-SP-A protein and 32P-phosphatidylcholine by type II cells, whereas PMA did not. This stimulation was cell substrate-dependent, as type II cells plated on plastic were not affected by any secretagogues. In the present study, we found that a 2-h exposure of type II cells on Transwell membranes to cAMP or terbutaline increased the binding of SP-A, whereas PMA did not show significant stimulation, which paralleled the results seen for surfactant uptake (Chinoy et al., 1993). Since the amount of surfactant uptake is likely to be proportional to its binding, the data support the hypothesis that changes in SP-A binding due to secretagogues account for the observed enhancement of surfactant incorporation.

The effect of secretagogues on SP-A binding showed that cAMP and PMA followed a different time course. 20 min of exposure to both secretagogues was sufficient to promote SP-A binding. However, the PMA-induced elevation was not maintained as SP-A binding returned to control levels within 2 h. This finding is consistent with a rapid desensitization of PKC by PMA as was reported in studies with a variety of cell types (Philips and Jaken, 1983; Solanki et al., 1981). In response to...
PMA treatment, type II cell PKC activity increased rapidly in the membranes and decreased in the cytosol fraction. The membrane-associated PKC activity reached a maximum by 30 min and declined to control levels after 2 h (Chander et al., 1995). Such results would suggest that the reason for our failure to detect stimulation of natural surfactant uptake by PMA after a 2-h exposure seen in our previous report (Chinoy...
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FIG. 7. SP-A concentration dependence of calcium-dependent and calcium-independent binding of SP-A to type II cells. Control (nontreated, ○), PMA (10 nM PMA, ●), and cAMP (0.1 mM cAMP, □)-treated type II cells were incubated at 4°C with increasing concentrations of 125I-SP-A in the absence or presence 10 mM EGTA. Total (no EGTA) and calcium-independent binding (10 mM EGTA) were measured. Calcium-dependent, specific binding was determined by subtracting the calcium-independent binding from the total binding. A, calcium-dependent binding; B, calcium-independent binding. The values are the mean ± S.E. of duplicates from three to six experiments. The data were calculated as a percentage of the total binding values of the untreated control cells = 100%. Mean (± S.E.) binding of SP-A to control cells was 114.2 ± 8.7 ng of SP-A/mg of cell protein. Where not visible, the error bars are within the symbols.

may have been that the SP-A binding sites were not elevated for a sufficient period. The cAMP-induced increase in SP-A binding to type II cells after 20 min exposure was sustained for up to 2 h and, thus, could have been present long enough to produce a detectable increase in surfactant uptake by cAMP.

The regulation of cell surface receptors can be controlled through four general mechanisms, changes in receptor protein synthesis, affinity, internalization, and externalization. Synthesis of the receptor protein molecule seems unlikely to be involved in the regulation of SP-A binding to type II cells, since acceleration in SP-A binding occurred too rapidly to be regulated by new protein production and cycloheximide did not prevent the increase of SP-A binding by secretagogues. As the targeting material of two major signal transduction pathways, cAMP and PMA have been reported to regulate the cycling of a variety of cell surface receptors through serine/threonine phosphorylation mediated by an activation of PKA or PKC, respectively (Hu et al., 1990; Larose et al., 1992; Zacharias et al., 1995). The effects of phosphorylation have been shown to be either stimulatory or inhibitory. Insulin growth factor II/mannose 6-phosphate receptor in microvascular endothelial cells moved from an intracellular pool to the cell surface when treated with PMA, causing an elevation of ligand binding accompanied by a reduction of intracellular receptor stores (Hu et al., 1990). On the other hand, exposure to PMA led to an absolute decrease of surface asialoglycoprotein receptor number in a hepatoma cell line (Hep G2 cells) due to internalization of receptors. Finally, transferrin binding on these cells was down-regulated by a reduction in the binding affinity of the transferrin receptor for the ligand after incubation with PMA (Fallon and Schwartz, 1986). By measuring the characteristics of binding in the presence of secretagogues, we determined that the affinity of the receptors was not changed, whereas the amount of specific, calcium-dependent binding was significantly elevated. Thus, the most likely mechanism whereby cAMP and PMA enhanced the binding of SP-A to type II cells was that these two secretagogues phosphorylated the SP-A receptors, stimulating their movement from the cytosol to the plasma membrane, resulting in an increase in SP-A binding sites on the type II cell surface.

The SP-A binding site on the type II cells shows the characteristics of a protein in that it was sensitive to trypsin treatment. Previous studies on the effects of trypsin on the binding of SP-A to type II cells have been inconclusive, probably due to the various concentrations of trypsin used. The present study confirmed the observations of Kuroki et al. (1988) that treatment of type II cells with 12 μg/ml trypsin does not reduce the SP-A binding, and also confirmed the observations of Wright et al. (1989) that 250 μg/ml trypsin reduced binding by approximately 50%. Data presented here determined that 50 μg/ml trypsin was sufficient to produce the maximum removal of binding sites, since higher concentrations of trypsin did not reduce binding further. The data indicated that trypsin treatment did not damage cells because the cells were able to respond normally to subsequent treatment with secretagogues by increasing SP-A binding sites. Our results also suggest that approximately half of the SP-A binding sites were trypsin-sensitive, in that increasing trypsin to concentrations where cell detachment occurred did not reduce the binding further. In addition, all of the SP-A binding sites stimulated by cAMP or PMA were trypsin-sensitive providing evidence that these binding sites were proteins. Our results correspond with the observations of Kuroki et al. (1988) and Wright et al. (1989)
that SP-A binding to type II cells was dependent on calcium and that calcium-independent binding accounted for approxi-
mately one-third of the total binding.

In summary, the present work suggests that the secreta-
gogues increased the binding of SP-A to type II cells by increas-
ing SP-A receptor activity on the plasma membrane of type II
cells. The enhancement of surfactant uptake due to either
secretagogues or to the use of microporous membranes as a cell
substrate has been shown to occur in conjunction with an
increase in the number of SP-A binding sites. Regulation of
these binding sites may well be the mechanism whereby the
turnover of SP-A, and perhaps surfactant, occurs in the intact
lung.

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