Adrenergic and Cholinergic Regulation of Lung Surfactant Secretion in the Isolated Perfused Rat Lung and in the Alveolar Type II Cell in Culture*

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The purpose of this study is to define the hormonal regulation of pulmonary surfactant secretion in two models, the isolated perfused rat lung and the isolated alveolar type II cell in culture. In the perfused lung, both cholinergic and adrenergic stimulation independently increased labeled disaturated phosphatidylcholine secretion, the major phospholipid component of surfactant, each by 2.3-fold. A concomitant increase in lung cGMP and cAMP concentration of 275- and 25-fold, respectively, was observed. The effect of each agonist was inhibited only by its appropriate antagonist.

In alveolar type II cells in culture, both adenylyl and guanylyl cyclase responded to their appropriate agonists and antagonists. The release of $^3$H-labeled disaturated phosphatidylcholine was enhanced by $\beta$-adrenergic but not $\alpha$-adrenergic or cholinergic effectors. The effect of isoproterenol (10 $\mu$M) on surfactant release seen was by 2.5 min, and secretion was stimulated 2.9-fold at a half-maximal concentration of 1 nM. Cyclic AMP levels were increased by 4.9-fold by isoproterenol at a half-maximal concentration of 40 nM. These results indicate that while in the perfused lung, secretion is stimulated by both adrenergic and cholinergic effectors, in the type II cell model, surfactant secretion is under only $\beta$-adrenergic control.

Lining the alveolar surface of the lung is a lipid-protein complex, termed pulmonary surfactant, which prevents alveolar collapse at end-expiratory volume. The major lipid component of this surfactant is phosphatidylcholine containing two saturated fatty acids, most often palmitate (3, 4). While the lung is said to contain approximately 40 cell types, it is now well documented that the type II alveolar epithelial cell is responsible for surfactant synthesis and secretion, and that the surfactant is stored in lamellar bodies of the type II cell prior to secretion (5, 6). The secretion of surfactant in vivo has been reported to be affected by hyperventilation (7-10), cholinergic mechanisms (9-16), $\alpha$- and $\beta$-adrenergic mechanisms (13, 16-22), prostaglandins (22, 23), and thyroxine (24). In $\textit{in vivo}$ experiments, it is difficult to discern the direct effects of such effectors from effects mediated through other organ systems on the lung. As well, no data concerning the responsiveness of adenylyl or guanylyl cyclase in lung to these effectors have been reported.

While it is important to consider the regulators of surfactant secretion in the whole lung, which is reported as part of the present study using the isolated, perfused lung model, a more direct approach would be to study the action of mediators directly on the type II alveolar epithelial cell. Limited studies have been carried out previously using both the A-549 tumor cell line, derived from a human lung carcinoma, and in isolated adult rat lung type II cells in primary culture. In the A-549 cells, the release of disaturated phosphatidylcholine was reported to be stimulated by both cholinergic and adrenergic agents (25). In another study using the A-549 cells (26), it was found that the addition of cyclic AMP analogs increased secretion of disaturated phosphatidylcholine as well as the incorporation of $[^3]$H cholinoine into this surfactant phospholipid. Cyclic GMP analogs had no effect. The use of A-549 cells as a model for the type II epithelial cell may be questioned, as it is known to contain a much lower content of disaturated phosphatidylcholine (27, 28) and may be morphologically different from the type II cells (29). Secretion of disaturated phosphatidylcholine by isolated adult rat type II epithelial cells placed in primary culture has been reported to be stimulated by adrenergic but not cholinergic agonists (30).

In this present study, the isolated, perfused rat lung and type II cells in monolayer culture from normal adult rat lung were used to determine the effects of adrenergic and cholinergic mediators on the secretion of disaturated phosphatidylcholine. In addition, the levels of tissue and cellular cAMP and cGMP were determined in order to correlate their change in concentration with the secretion of surfactant.

**EXPERIMENTAL PROCEDURES**

$[^1]$The "Experimental Procedures" are presented in miniprint as prepared by the authors. The abbreviations used in the miniprint are: TCA, trichloroacetic acid; DSPC, disaturated phosphatidylcholine; BSA, bovine serum albumin. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80-M-1805, cite authors, and include a check or money order for $1.00 per set of photocopies. Full size photocopies are also included with the microfilm edition of the Journal that is available from Waverly Press.

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circulating perfusate 5 minutes prior to the addition of the agonist.

Lung lavage was performed for 10 minutes at 1,000 g to remove alveolar macrophages. The samples were dried, resuspended with sodium bicarbonate and chromatographed on an ion-exchange column to isolate DSGC (34). An aliquot of each DSGC sample was used for radioactivity determination by liquid scintillation technique, using the channel ratio method to determine the efficiency of counting. A second aliquot of DSGC sample was used for phosphorus determination (35).

RESULTS

Perfused Lung

The effect of 15 µM l-isoproterenol on the secretion of disaturated phosphatidylcholine in the isolated perfused rat lung is shown in Table I. Isoproterenol caused a 2.3-fold increase in surfactant secretion over the control. The stimulation effected by isoproterenol was inhibited by the adrenergic antagonist alprenolol, but was not inhibited by the cholinergic antagonist atropine. Alprenolol alone caused no effect. Isoproterenol also caused a 25-fold increase in CAMP concentration over the control. The effect was completely inhibited by alprenolol. Atropine partially inhibited the stimulation of CAMP, but did not inhibit the stimulation of secretion. Isoproterenol did not increase the concentration of cGMP.

Isolated Alveolar Type II Cells

(a) P-Adrenergic Response—The results of studies of the effects of p-adrenergic mediators on secretion of disaturated phosphatidylcholine and cyclic nucleotide levels in isolated type II cells in culture are presented in Table III. As in the perfused lung, the addition of isoproterenol caused a 2.3-fold increase in surfactant secretion over the control. The stimulation effected by isoproterenol was inhibited by the adrenergic antagonist alprenolol, but was not inhibited by the cholinergic antagonist atropine. Alprenolol alone caused no effect. Isoproterenol also caused a 25-fold increase in cAMP concentration over the control. The effect was completely inhibited by alprenolol. Atropine partially inhibited the stimulation of cAMP, but did not inhibit the stimulation of secretion. Isoproterenol did not increase the concentration of cGMP.

Effect of a p-adrenergic mediator on surfactant disaturated phosphatidylcholine secretion and cyclic nucleotide concentration in the isolated perfused lung

<table>
<thead>
<tr>
<th>Effector(s) added</th>
<th>Lavage disaturated phosphatidylcholine pmol/mg DNA</th>
<th>Lung cAMP pmol/mg DNA</th>
<th>Lung cGMP pmol/mg DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>101 ± 11</td>
<td>53.0 ± 4.1</td>
<td>0.15 ± 0.15</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>235 ± 11</td>
<td>1847 ± 194</td>
<td>1.82 ± 0.17</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>153 ± 13</td>
<td>114.6 ± 4.1</td>
<td>1.49 ± 0.31</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>274 ± 14</td>
<td>1159 ± 48</td>
<td>1.55 ± 0.12</td>
</tr>
<tr>
<td>Alprenolol</td>
<td>116 ± 10</td>
<td>46.2 ± 5.2</td>
<td>1.62 ± 0.3</td>
</tr>
</tbody>
</table>

Lung lavage, as described under "Experimental Procedures," was carried out at the end of 3 h of perfusion with effectors.

Cyclic nucleotide concentration was determined in lung tissue as described under "Experimental Procedures" after 15 min of perfusion with effectors. Values represent the mean ± S.E. values, determined by the Student's t test less than 0.02 compared to the control values, are given in parentheses.

Experimental Procedures—The isolation of surfactant was performed as described under "Experimental Procedures." All values represent the mean ± S.E. values, determined by the Student's t test less than 0.02 compared to the control values.
The secretion of 3H-labeled disaturated phosphatidylcholine is expressed as the percentage of cellular 3H-labeled disaturated phosphatidylcholine at zero time released into the media at the end of the 1.5-h incubation period.

Values represent the mean ± S.E. p values, determined by Student's t test, less than 0.02 compared to the control values, are given in parentheses.

Values represent percentage increase of [3H]adenosine incorporation into cAMP over basal incorporation.

The time course for the release of disaturated phosphatidylcholine and the effect of 10^{-5} M isoproterenol on its release is demonstrated in Fig. 1. At 3 h, the basal release was 4.1 ± 0.5% (mean ± S.E. n = 8) of total cellular disaturated phosphatidylcholine. Isoproterenol stimulated disaturated phosphatidylcholine release by 2.9 ± 0.6-fold (n = 8) over the basal level. This correlates well with the increase of 2.30 ± 0.5-fold caused by 15 μM isoproterenol in the isolated perfused lung (Table I). From the data presented in Fig. 1, isoproterenol is seen to stimulate the release of disaturated phosphatidylcholine by 2.8 ± 0.3 times over the basal as early as 2.5 min after its addition. This would imply that isoproterenol is affecting secretion rather than synthesis or degradation. The effective

![Fig. 1. Effect of time on the release of disaturated phosphatidylcholine (DSPC) from alveolar type II cells in culture, in the presence and absence of l-isoproterenol. After a 30-min equilibration period, 10 μM l-isoproterenol was added (zero time). Each point represents the mean of six experiments, and the bars represent standard errors.](image1)

![Fig. 2. Effect of l-isoproterenol concentration on disaturated phosphatidylcholine (DSPC) release from alveolar type II cells in culture. After a 30-min equilibration period (zero time), l-isoproterenol was added and the incubation was continued for 90 additional min. Each point represents the mean of experiments, and the bars represent standard errors.](image2)
concentration of l-isoproterenol at half-maximal stimulation is 1 nM, as shown in Fig. 2. This correlates well with the value of 4 nM given as the effective concentration of l-isoproterenol for half-maximal stimulation given by Dobbs and Mason (30) using isolated type II cells obtained by elastase digestion. Isoproterenol caused a 4.8 ± 0.3-fold increase in cAMP over the basal level of 0.90 ± 0.05 pmol/10^5 cells at 1 min 45 s (Fig. 3). Throughout the time course study, the basal level of cAMP remained constant within experimental error. The stimulation induced by isoproterenol falls within the range of results using lung fragments (45) and lung tissue slices (46) in which 15 μM isoproterenol increased cAMP to 3.0 and 4.8 times the basal level, respectively. The effective concentration at half-maximal stimulation was 40 nM (Fig. 4).

(b) α-Adrenergic Response—The concentration of phenylephrine, an α-adrenergic agonist, needed to produce half-maximal stimulation of surfactant release was 8.0 μM (Fig. 5). Dobbs and Mason (30) obtained a value of 13 μM with phenylephrine using type II cells isolated by elastase digestion. Half-maximum stimulation of cAMP level by phenylephrine was 7.5 μM (Fig. 6). Therefore, phenylephrine is a less potent stimulator of both disaturated phosphatidylcholine secretion and cAMP synthesis than is l-isoproterenol. Importantly,
TABLE IV
Effect of phenolamine in blocking the secretion of disaturated phosphatidylcholine and increased cAMP concentration induced by phenylephrine (an &-adrenergic mediator) in alveolar type II cells in culture

<table>
<thead>
<tr>
<th>Effector(s) added</th>
<th>Cellular disaturated phosphatidylcholine secretion induced%</th>
<th>Cellular cAMP concentration induced%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylephrine (10 μM)</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Phenolamine (25 μM) + phenylephrine (10 μM) (n = 8)</td>
<td>99.1 ± 1.1*</td>
<td>97.4 ± 1.9</td>
</tr>
<tr>
<td>Phenolamine (50 μM) + phenylephrine (10 μM) (n = 8)</td>
<td>94.7 ± 2.2</td>
<td>94.5 ± 1.6</td>
</tr>
<tr>
<td>Phenolamine (100 μM) + phenylephrine (10 μM) (n = 8)</td>
<td>96.1 ± 1.9</td>
<td>96.1 ± 2.1</td>
</tr>
<tr>
<td>Alpenrol (50 μM) + phenylephrine (10 μM) (n = 8)</td>
<td>29.4 ± 2.0</td>
<td>14.5 ± 1.2</td>
</tr>
</tbody>
</table>

* Cells were incubated for 22 h with 0.05 μM [9,10-3H]-palmitic acid. The secretion of 3H-labeled disaturated phosphatidylcholine is expressed as the percentage of cellular 3H-labeled disaturated phosphatidylcholine at zero time released into the media at the termination of the 1.5-h incubation period.

Fig. 7. Effect of acetylcholine concentration on disaturated phosphatidylcholine (DSPC) release from alveolar type II cells in culture. After a 30-min equilibration period (zero time), acetylcholine was added and the incubation was continued for 90 additional min. Each point represents the mean of six experiments, and the bars represent standard errors.

DISCUSSION
Using intact animals, hyperventilation (7-10), cholinergic mediators (9-15), adrenergic mediators (13, 16-22), prostaglandins (22, 23), and thyroxine (24) have been shown to facilitate the secretion of surfactant. Since whole animals were used, it is difficult to ascertain the contribution of other organ systems in the stimulation of pulmonary surfactant secretion. The secondary effects of equilibrating adrenergic mediators on cyclic nucleotides cannot be judged.

Using the isolated perfused rat lung, it is shown here that both cholinergic and adrenergic mediators stimulated independently an increased appearance of 14C-labeled disaturated phosphatidylcholine in the lavage fluid. With cholinergic or adrenergic stimulation, there was a concomitant increase in cGMP or cAMP, respectively. Each agonist was sensitive to inhibition by its appropriate antagonist but not by the opposing antagonist. Neither pilocarpine nor isoproterenol caused cross-stimulation. The increase in both cAMP and cGMP upon stimulation was large. It is possible that the sharp rise in cyclic nucleotide concentration is a result of the stimulation.
of more than the type II cell among the 40 cell types in the lung.

It is concluded that in the isolated perfused rat lung, the increase in disaturated phosphatidylcholine is attributable to both cholinergic and adrenergic action, although each is independent. This conclusion is supported by the studies of Corbet et al. (21) and Olsen (13). Corbet et al. (21), using fetal rabbits, found improved static pressure volume after injections of pilocarpine and isoxsuprine. When Olsen (13) administered single injections of pilocarpine and isoproterenol to rats, an increase in phospholipids was found after 1 h in the lavage as well as a decrease in the number of lamellar bodies in the alveolar type II cells. Although the effects of other organ systems may be ruled out, the effects of other cells on the type II cell cannot be excluded.

Because of the heterogeneity of the lung, biochemical experiments with perfused lung do not yield data pertinent to any specific cell type. Through the utilization of isolated type II cells, a more precise understanding of the factors affecting secretion may be obtained.

Dobbs and Mason (30), using type II cells isolated by means of elastase digestion, reported that the secretion was stimulated by adrenergic agonists only. In earlier studies, they found that secretion was stimulated by 12-o-tetradecanoyl phorbol-13-acetate and by the divalent cation ionophore A23187 (47). The effect of isolation of cells by proteolytic dissociation may alter specialized cell function (48). Depending on the enzyme and cell system studied, stimulatory or inhibitory responses may result from the severe treatment necessary to separate lung cells. In the studies of Dobbs and Mason (30) it was not established that guanylate cyclase or adenylate cyclase had maintained their function throughout the isolation process. Some or all of the receptors may have lost their responsiveness. It is possible that the adrenergic agonists were stimulating through another means such as Ca++ mediation rather than through adenylate cyclase. Although 12-o-tetradecanoyl phorbol-13-acetate was shown to enhance secretion of disaturated phosphatidylcholine, the mechanism is unknown. It has been postulated that this substance induces a stimulation in cellular cGMP correlated with a stimulation of secretion in the polymorphonuclear leukocyte (49). This hypothesis was not supported in the secretion of the pancreas (50). The cyclic nucleotide content of type II cells upon treatment with 12-o-tetradecanoyl phosphol-13-acetate was not determined. The results reported here in the isolated alveolar type II cell in culture indicate that both the guanylate cyclase and adenylate cyclase receptors are present and responsive to stimulation. The possibility cannot be excluded that the isolation procedure increased the number of receptors by unmasking receptors or altering their affinity or responsiveness.

While the response to β-adrenergic effectors is clearly supported by the present data, the response to α-adrenergic and cholinergic effectors must also be considered. It has been demonstrated in vivo that a decrease in lung compliance and an augmented minimum surface tension of isolated lung surfactant after electrical stimulation of the stellate ganglion can be blocked by α-adrenergic antagonists (51). Those results suggested an α-adrenergic involvement in surfactant release. In the present studies using the alveolar type II cells in culture, stimulation of disaturated phosphatidylcholine secretion and an increase in cAMP levels by an α-adrenergic agonist was inhibited only by a β-adrenergic antagonist. Dobbs and Mason (30) observed that phenylephrine stimulation of surfactant release was inhibited by propranolol, a β-adrenergic antagonist. Both observations support the suggestion by Dobbs and Mason (30) that the α-adrenergic mediator was acting through the β-adrenergic receptor, not unexpected at the high concentrations used to obtain the observed effects (52).

The secretion of disaturated phosphatidylcholine was stimulated in response to 100 but not 10 μM acetylcholine. Although lactate dehydrogenase leakage and leucine incorporation into protein did not indicate cellular damage, the secretion in response to 100 μM acetylcholine is not felt to be significant. The stimulation of secretion was not inhibited by atropine, nor did it demonstrate the usual dose-response curve, while cGMP was responsive at 10 μM acetylcholine. In view of these findings, it is concluded that isolated type II cells do maintain functional adenylate and guanylate cyclase receptors, but disaturated phosphatidylcholine secretion is subject only to β-adrenergic stimulation. This response occurs as early as 2.5 min, indicative of an effect on secretion and not synthesis. This is contrary to findings in the isolated perfused lung, where the release of disaturated phosphatidylcholine was responsive to both cholinergic and adrenergic stimulators. Perhaps the isolated cells have lost important cell polarity, cell-cell contacts, or feedback from ventilation. One or all of these factors may involve cGMP or α-adrenergic stimulation, but in isolated alveolar type II cells, secretion of surfactant is affected by the β-adrenergic system alone.

REFERENCES

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