Role of clathrin- and actin-dependent endocytotic pathways in lung phospholipid uptake

Peter Rückert, Sandra R. Bates, and Aron B. Fisher
Institute for Environmental Medicine, University of Pennsylvania
School of Medicine, Philadelphia, Pennsylvania 19104

Submitted 18 November 2002; accepted in final form 5 February 2003

Rückert, Peter, Sandra R. Bates, and Aron B. Fisher. Role of clathrin- and actin-dependent endocytotic pathways in lung phospholipid uptake. Am J Physiol Lung Cell Mol Physiol 284: L981–L989, 2003. First published February 28, 2003; 10.1152/ajplung.00392.2002.—We evaluated the contribution of endocytotic pathways to pulmonary uptake of surfactant lipids from the alveolar space. Resting and stimulated 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) uptake of unilamellar liposomes labeled with either [3H]dipalmitoylphosphatidylcholine ([3H]DPPC) or 1-palmitoyl-2-[12-(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] dodecanoyl-phosphatidylcholine (NB-DPC) was studied in isolated perfused rat lungs and isolated type II cells. Amantadine and phenylarsine oxide, inhibitors of clathrin-mediated endocytosis, each decreased [3H]DPPC uptake under resting conditions by ~40%; their combination had no additional effect. Cytochalasin D, an inhibitor of actin-dependent processes, reduced lipidosome uptake by 55% and potentiated the effect of either clathrin inhibitor alone. Relative inhibition for all agents was higher in the presence of 8-Br-cAMP. The effect of inhibitors was similar for liposomes labeled with [3H]DPPC or NB-DPC. By fluorescence microscopy, NB-DPC taken up by lungs was localized primarily to alveolar type II cells and was localized to lamellar bodies in both lungs and isolated cells. These studies indicate that both clathrin-mediated and actin-mediated pathways are responsible for endocytosis of DPPC-labeled liposomes by alveolar type II cells in the intact lung.

PULMONARY SURFACTANT, a complex mixture of lipids and specific proteins lining the alveolar surface, promotes alveolar stability by lowering the surface tension, thus enabling the even ventilation of alveoli. The composition and amount of surfactant in the alveolar lumen appear to be tightly regulated, and imbalances are associated with pathological states such as acute respiratory distress syndrome (deficiency) and alveolar proteinosis (excess). The lung epithelium is responsible for production of lung surfactant and also primarily responsible for its clearance. Radiolabeled dipalmitoylphosphatidylcholine (DPPC) liposomes or biosynthesized natural surfactant instilled into rat lungs is cleared from the alveoli, and a significant fraction becomes associated with the lamellar body fraction (12, 13, 15, 19, 28). In lungs from adult rabbits, ~20–50% of intratracheally instilled DPPC is recycled, that is, resecreted after internalization (20, 31).

Removal of surfactant lipids from the alveolar space occurs predominantly by type II pneumocytes and to a lesser degree by alveolar macrophages (11). Studies with isolated type II cells in primary culture have shown net uptake of phospholipid vesicles (6, 38) and natural surfactant (13). However, the pathways for surfactant trafficking through the type II cells and the mechanisms involved in the regulation of surfactant turnover remain poorly defined.

Eukaryotic cells possess several routes for the uptake of extracellular material, including phagocytosis and pinocytosis, both commonly referred to as endocytosis. Phagocytosis is strictly an actin-dependent uptake pathway for larger particles. Pinocytosis, on the other hand, is a pathway for uptake of smaller (~0.2 μm diameter) vesicles by either clathrin-dependent or clathrin-independent processes. The latter includes both caveolar-mediated uptake and macropinocytosis (24). Uptake of lipids could additionally occur through monomer diffusion as well as fusion of liposomes with the plasma membrane (27, 29).

Previous studies with isolated rat granular pneumocytes have suggested the presence of both clathrin-dependent as well as actin-dependent pathways for uptake of surfactant lipids (2, 25, 36). Clathrin-mediated endocytosis by type II cells also has been demonstrated for surfactant protein A (SP-A), the major surfactant protein, as phenylarsine oxide (PAO) (3) and potassium depletion (36), both inhibitors of clathrin-mediated endocytosis, blocked the uptake of SP-A. In addition, immunogold-labeled SP-A has been found in coated pits and coated vesicles of type II cells compatible with a role for receptor-mediated endocytosis (32). SP-A enhances the uptake of phospholipid liposomes by pneumocytes (2, 38), possibly by interaction of a SP-A/phospholipid complex with specific clathrin-associated receptors for SP-A present on the surface of these cells (9, 22, 35, 37). The actin-dependent pathway appears to be largely clathrin independent. As one
possibility for the mechanism, the actin-dependent pathway of type II cells may represent retrieval of specialized membrane patches containing lamellar body membrane proteins. We have used 3C9, a monoclonal antibody that recognizes a 180-kDa protein recently identified as ABCA3 in the limiting membrane of lamellar bodies (26, 39), to follow trafficking of lamellar body membranes (3, 33). We determined that treatment with secretagogues enhanced the turnover of this protein at the surface of type II cells. This pathway was defined as actin dependent since it was inhibited by cytochalasin D but was insensitive to inhibitors of clathrin-mediated internalization (3).

The purpose of the current study was to evaluate the role of clathrin- and actin-dependent pathways for the uptake of liposomes by the intact lung under resting and secretagogue-stimulated conditions. The isolated perfused lung was utilized as it provides a model in which type II cell function can be studied in a physiological environment, while agonists/antagonists can be delivered intratracheally and/or through the pulmonary circulation.

**MATERIALS AND METHODS**

**Materials.** Sprague-Dawley pathogen-free male rats weighing ~200–250 g were obtained from Charles River Breeding Laboratories (Kingston, NY). Authentic lipids and 1-palmitoyl-2-[12-(7-nitro-2,3-benzoxadiazol-4-yl) amino] dodecanoyl (NBD)-labeled phosphatidylcholine were purchased from Avanti (Birmingham, AL). [3H-methyl] dipalmitoylphosphatidylcholine ([3H]DPPC) was purchased from New England Nuclear (Boston, MA). 8-Bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP), PAO, cytochalasin D, and amantadine HCl were purchased from Sigma (St. Louis, MO). Bovine serum albumin (BSA) and fatty acid-free BSA (faff BSA) were obtained from Boehringer-Mannheim (Indianapolis, IN). LysoTracker Red and the Live/Dead Cell Assay kit were obtained from Molecular Probes (Eugene, OR).

**Liposome preparation.** We prepared liposomes from 1-α-DPPC, egg PC, egg phosphatidylglycerol, and cholesterol in molar ratio 10:5:2:3 with or without trace amounts of [3H]DPPC by evaporating the lipids to dryness under nitrogen. The dried lipids were resuspended in phosphate-buffered saline (PBS) without calcium-magnesium. The mixture was frozen and thawed three times by alternating liquid ered saline (PBS) without calcium-magnesium. The mixture were stored at 4°C and used within 24 h. This method resulted in unilamellar liposomes of 103 ± 7.7 nm (means ± SE, n = 4) as assessed by light scattering using a 90 Plus particle size analyzer (Brookhaven Instrument, Austin, TX). For fluoroscentially labeled liposomes, 50% of the usual DPPC was replaced by NBD-PC. NBD-PC-labeled liposomes had a diameter of 132 ± 11 nm (means ± SE, n = 3).

**DPPC uptake by the isolated perfused lung.** To measure DPPC uptake, we anesthetized rats with intraperitoneal pentobarbital sodium (50 mg/kg body wt) and cannulated their tracheas. Liposomes (0.1 μmol of total PC in 0.1 ml of PBS representing ~10% of the endogenous PC pool) were instilled into the airways with a Hamilton syringe inserted into the trachea through a cannula at the level of carina. The rats were then placed on a ventilator, and lungs were ventilated with 5% CO2 in air at 60 cycles/min, 2–2.5 ml of tidal volume, and 2 cmH2O end expiratory pressure. The chest wall was incised, and the pulmonary artery was cannulated. Lungs were cleared of blood by perfusion with Krebs-Ringer bicarbonate buffer (KRB) containing 3% BSA ± inhibitors before being removed from the thorax for isolated organ perfusion. Lungs were perfused at 10–12 ml/min in a recirculating system with 40 ml of KRB (pH 7.4) containing 10 mM glucose and 3% faf BSA. Time between instillation of the liposomes and start of perfusion was ~5 min. In some experiments, amantadine was added either to the perfusate or to the suspended liposomes before intratracheal installation; the effects were similar for the two routes of administration, and the results were combined. In other experiments, PAO or cytochalasin D was added to the perfusate. Where noted, 0.1 mM 8-Br-cAMP was added to the perfusate to stimulate DPPC uptake pathways (15). Tracheal and pulmonary artery pressures were continuously monitored and recorded on a data acquisition system. Lungs were weighed at the end of the experiment. Those lungs that demonstrated a significant increase in ventilation or perfusion pressure or lung wet weight, indicative of pulmonary edema, were not further analyzed. To evaluate potential toxic effects of the inhibitors, we compared lactate dehydrogenase (LDH) release into the perfusate in lungs perfused with or without the inhibitors.

Lungs were evaluated for [3H]DPPC uptake at 5 min after liposome instillation or after perfusion for 2 h. To measure uptake, we lavaged lungs five times with ice-cold 0.9% saline through the tracheal cannula. For each lavage, we slowly instilled and aspirated 7 ml of fresh saline while gently shaking the lung. The postlavage lung tissue was homogenized in saline using a Polytron (Brinkmann, Westbury, NY) followed by homogenization with a motor-driven Teflon pestle in a Potter-Elvehjem vessel (Thomas Scientific, Philadelphia, PA). Aliquots of the homogenate were analyzed for radioactivity by scintillation counting with quench corrections based on internal standards. Uptake of [3H]DPPC by the lung was expressed as the percentage of instilled dpm remaining in the lung after lavage. Net uptake was calculated as the difference between the 5-min and 2-h values.

Experiments with NBD-PC-labeled liposomes were performed similarly except that liposomes with 0.5 μmol of total PC were instilled in 100 μl of PBS. After lavage and homogenization, lipids were extracted from the lung tissue as previously described (14, 15). We dried and redissolved the lipids in equal amounts of chloroform before quantitating the fluorescent intensity with a PTI spectrofluorometer (Photon Technology International, Bricktown, NJ) with excitation/emission set at 460/534 nm and the slits at 5 nm. Net uptake was calculated as the difference in lung-associated fluorescence between the 5-min and 2-h uptake values.

**Microscopy.** Some lungs were utilized for fluorescence microscopy after instillation of NBD-PC-labeled liposomes as described above. For some of these experiments, LysoTracker Red was added to the liposome suspension before instillation at a final concentration of 0.01 μM. The animals were maintained on the ventilator for 30 min after liposome instillation, and then the lungs were cleared of blood by perfusion with KRB/BSA through the pulmonary artery and placed in a custom-made glass-bottomed box. Lungs were inflated with 5 ml of air, and subpleural alveoli were visualized with a Nikon Diaphot inverted microscope using a ×60 oil-immersion lens and commercial FITC/rhodamine filter cubes. LysoTracker Red fluorescence was visualized at a wavelength of 488 nm and NBD-PC at 568 nm. Images were acquired with a Hamamatsu camera and processed using Metamorph Imaging software (Universal Imaging, West Chester, PA).
Isolation of type II cells and measurement of DPPC uptake.

Alveolar type II cells were isolated by elastase digestion (10). The cells were cultured for 24 h on 35-mm glass-bottomed dishes (MatTek, Ashland, MA) in minimal essential medium containing 10% fetal calf serum in 5% CO₂ in air at 37°C. To begin an experiment, the pneumocytes were washed three times with phenol red-free Dulbecco's modified Eagle's medium (DMEM). Cells then were incubated for 20 min in DMEM ± inhibitors. Liposomes were added to the incubation media at a final concentration of 150 μg/ml and incubated for 30 min. In some experiments, LysoTracker Red was added at 0.01 μM during the last 10 min of incubation. After the uptake phase, we performed a lipid back exchange step by washing the dishes once with ice-cold DMEM containing 3% faf BSA, twice with ice-cold medium with 0.1% faf BSA, and three times with ice-cold Ca/Mg-free PBS. In between each wash, the cells were kept for 5 min on ice to remove surface-bound liposomes. Uptake of fluorescent liposomes was visualized under a Nikon Eclipse TE 300 microscope with a 60 x 1.4 oil immersion lens connected to a Radiance 2000 confocal system equipped with an argon-krypton laser (Bio-Rad, Richmond, CA). In double-labeling experiments with LysoTracker Red and NBD-PC liposomes, the 488-nm and 568-nm excitation channels were acquired separately. Cross talk was excluded by single-label controls. Acquisition settings were kept identical for each sample, and postprocessing modifications were done in parallel for all images.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was done by ANOVA and t-test using SigmaStat software (Jandel Scientific, San Rafael, CA). The level of statistical significance was taken as P < 0.05.

RESULTS

Effect of inhibitors on the uptake of radiolabeled liposomes by isolated perfused lung. We used amantadine, PAO, and cytochalasin D as inhibitors with which to evaluate the pathways responsible for the uptake of lipids in the isolated perfused lung. We examined the optimal dose for inhibitors by establishing dose response curves for the uptake of intratracheally instilled [³H]DPPC-labeled liposomes over a 2-h perfusion period in the presence of 0.1 mM 8-Br-cAMP. Amantadine was added to the liposome preparation at concentrations ranging from 0.25 to 5 mg/ml. PAO and cytochalasin D were added to the perfusate at concentrations ranging from 0.5 to 5 μg/ml. Each of the inhibitors resulted in a dose-dependent reduction in liposomal PC incorporation into lung tissue (Fig. 1). The lowest concentration of inhibitor that resulted in maximum effect (1 mg amantadine/ml of liposome suspension, 2 μg PAO/ml of perfusate or 2 μg of cytocha-

![Fig. 1. Concentration-dependent effects of inhibitors on the uptake of dipalmitoylphosphatidylcholine (DPPC) by the isolated, perfused lung. The effect of inhibitor concentration on the net uptake of [³H]DPPC-labeled liposomes by the isolated perfused lung in the presence of 0.1 mM 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) added to the perfusate. Unilamellar liposomes (0.1 μmol of total PC in 100 μl of PBS) labeled with [³H]DPPC were instilled intratracheally at zero time. After 2-h perfusion, the lungs were lavaged five times with saline and homogenized, and aliquots of the homogenate were counted in a scintillation counter to measure the tissue-associated radioactivity. To calculate net uptake, lung-associated dpm at 5 min (basal) was subtracted. Uptake represents tissue-associated radioactivity as a percentage of total radioactivity instilled. Amantadine (A) was instilled intratracheally with liposomes; phenylarsine oxide (PAO, B) and cytochalasin D (C) were added to the perfusate. Values with error bars are means ± SE for n = 4; other points are for n = 1.](http://ajplung.physiology.org/)

AJP-Lung Cell Mol Physiol • VOL 284 • JUNE 2003 • www.ajplung.org
Lasin D/ml of perfusate) was chosen for further study. None of the chosen inhibitor concentrations led to an increased LDH release into the perfusate measured at the end of the experiment or resulted in increased incidence of pulmonary edema (data not shown).

Uptake of [3H]DPPC-labeled liposomes was studied under resting as well as cAMP-stimulated conditions. Net uptake of [3H]DPPC increased from 8% of the instilled counts under resting conditions to 26% in the presence of 8-Br-cAMP (Fig. 2A). Inhibitors of clathrin-mediated endocytosis (amantadine and PAO) led to a significant reduction of lipid uptake (calculated as percentage of instilled PC) under both resting and stimulated conditions (Fig. 2A). These results also were expressed as percent inhibition of [3H]DPPC uptake compared with control (no inhibitors) (Table 1). The

![Fig. 2. Effect of inhibitors on the resting and stimulated 8-Br-cAMP uptake of [3H]DPPC-labeled liposomes by isolated perfused lung (A) and uptake of NBD-PC-labeled liposomes (B). A: [3H]DPPC-labeled liposomes were instilled intratracheally, and the lungs were excised and perfused for 2 h with 0.1 mM 8-Br-cAMP and with or without one or more inhibitors. Amantadine (Amant, 1 mg/ml) was added to liposomes; cytochalasin D (Cyto D, 2 μg/ml) and PAO (2 μg/ml) were added to the perfusate. Lung tissue-associated radioactivity was quantitated after removal of extracellular [3H]DPPC by lung lavage. Dpm from identically treated 5-min lungs (basal uptake) were subtracted to obtain net uptake, which was expressed as percentage of instilled dpm. The effect of each inhibitor on net uptake was statistically significant under resting (solid bars) as well as stimulated (open bars) conditions. Values are means ± SE for n = 3–5 for each condition. B: liposomes were fluorescently labeled by replacing 50% of the DPPC with 1-palmitoyl-2-[12-(7-nitro-2,1,3-benzoxadiazol-4-yl) amino] dodecanoyl (NBD)-PC. The experiment was performed as described in A in the absence of 8-Br-cAMP. The results were calculated as relative fluorescence units. The values representing net uptake in percentage of control (no inhibitors) are the means ± SE (n = 3). *Significantly higher (P < 0.05) than uptake in the presence of inhibitors; #significantly lower (P < 0.05) than any single inhibitor under resting or stimulated conditions.

Table 1. Relative effect of inhibitors of endocytosis on phospholipid uptake by the isolated perfused rat lung

<table>
<thead>
<tr>
<th></th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amant</td>
</tr>
<tr>
<td>[3H]DPPC resting</td>
<td>39 ± 1.4</td>
</tr>
<tr>
<td>[3H]DPPC stimulated</td>
<td>50 ± 0.7</td>
</tr>
<tr>
<td>NBD-PC resting</td>
<td>61 ± 4.5*</td>
</tr>
</tbody>
</table>

Effect of inhibitors on the uptake of [3H]dipalmitoylphosphatidylcholine ([3H]DPPC) and 1-palmitoyl-2-[12-(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] dodecanoyl-phosphatidylcholine (NBD-PC)-labeled liposomes by the isolated perfused rat lung in the absence (resting) or presence (stimulated) of 8-Br-cAMP. Experiments were performed as described in Figs. 2 and 3. Results are expressed as % inhibition compared to control values for each experimental condition. Each value represents the mean ± SE for n = 3–5. All values show significant inhibition by comparison to control. *P < 0.05 for comparison of resting [3H]DPPC and NBD-PC uptake. ND, not done; Amant, amantadine; PAO, phenylarsine oxide; Cyto D, cytochalasin D.
degree of inhibition achieved with amantadine and PAO was comparable. Under resting conditions, both clathrin inhibitors reduced the net uptake of [3H]DPPC in 2 h from ~8 to ~5% of instilled DPPC. With 8-Br-cAMP stimulation, uptake in the presence of inhibitors was decreased by 50% from ~26 to ~13% of instilled counts. The combination of amantadine and PAO did not significantly decrease the PC uptake by the lung beyond that for each drug alone. Cytochalasin D also caused a significant reduction of net [3H]DPPC uptake. The resting and stimulated uptake decreased to 4 and 9%, respectively, of the instilled [3H]DPPC, a reduction of 55–65% compared with control values. The combination of cytochalasin D with either amantadine or PAO further inhibited [3H]DPPC uptake to ~2.5% of instilled dpm under resting and ~5% of instilled dpm in the presence of 8-Br-cAMP.

Uptake of NBD-PC-labeled liposomes by intact lung. Uptake of fluorescent NBD-PC-labeled liposomes by the isolated perfused lung was analyzed under resting conditions in the presence or absence of inhibitors as described for radioactively labeled liposomes (Fig. 2 and Table 1). In these experiments, fluorescence of control lungs was set at 100%, and the effect of inhibitors was compared. During the 2-h perfusion period, amantadine (1 mg/ml) added to the labeled liposomes significantly reduced the net uptake of NBD-PC. Cytochalasin D (2 µg/ml in the perfusate) was not as effective as amantadine but also resulted in a statistically significant decrease in uptake of NBD-PC. The net uptake of NBC-PC in the presence of both inhibitors was reduced further to 30% of control, although this increased effect was not statistically different from amantadine alone (Fig. 2B). Under resting conditions, the relative inhibition with amantadine was significantly greater for NBD-PC- compared with [3H]DPPC-labeled liposomes, whereas the effects of cytochalasin D or the combination of inhibitors was not statistically different for the two types of liposomes (Table 1).

Fig. 3. NBD-PC-labeled liposomes are taken up by alveolar type II cells (A) and colocalize with LysoTracker Red-labeled lamellar bodies (B). A: NBD-PC-labeled liposome suspension (0.5 µmol of PC in 100 µl of PBS) was instilled intratracheally into anesthetized rats. After 30 min on the ventilator, the lungs were excised and perfused with Krebs-Ringer bicarbonate (KRBB/BSA/glucose, inflated with 5 ml 5% CO2 in air, and placed in a glass-bottomed dish on the microscope stage. NBD-PC fluorescence in subpleural alveoli was visualized, and pictures were acquired using FITC filter cubes (a). The different structures seen in a are depicted in b. B: the experiment was performed as described in A with the addition of LysoTracker Red at 0.01 µM to the liposomes. Fluorescence in subpleural alveoli was visualized, and pictures were acquired using FITC filter cubes for NBD-PC (a) and standard rhodamine filter cubes for LysoTracker Red (b). The 2 channels were merged to show cellular colocalization (c). d shows a sketch of the field. *, alveolus; #, alveolar type II cell. The image is representative of 3 independent experiments. Scale bar = 20 µm.
**NBD-PC is trafficked to alveolar type II cells in the isolated perfused lung.** To determine the cells responsible for the uptake of NBD-PC-labeled liposomes, we visualized subpleural alveoli of isolated perfused lungs microscopically at 30 min after the instillation of NBD-PC-labeled liposomes. Alveoli were clearly demarcated by the autofluorescence of the lung tissue. The fluorescent lipid was enriched in cuboidal cells localized to the corners of the alveoli compatible with alveolar type II cells (Fig. 3). Fluorescence was augmented in the perinuclear region in a punctate pattern, indicating concentration of label within specific organelles, possibly the surfactant-containing lamellar bodies. Due to their acidic pH (7), the lamellar bodies in type II cells can be labeled with the acidophilic LysoTracker dyes. LysoTracker Red instilled with the liposomes intratracheally showed the same distribution pattern as the NBC-PC, and merged images of the green (FITC/NBD-PC) and red (rhodamine/LysoTracker Red) channels showed strong cellular colocalization of the dyes (Fig. 3).

**NBD-PC localization in isolated type II cells and effect of inhibitors.** Isolated alveolar type II cells in primary culture were utilized to investigate the subcellular localization of NBD-PC. After 20 h in culture on glass coverslips, alveolar type II cells formed flattened, partly confluent groups of cells as seen in a confocal microscope transmission image (Fig. 4A). After 30 min of incubation with NBD-PC-labeled liposomes followed by thorough washing and lipid back exchange, the confocal images showed bright fluorescence in perinuclear punctate structures suggestive of perinuclear organelles. A weaker, diffuse labeling of the cells was seen as well (Fig. 4B). In addition to NBD-PC, the cells were incubated with LysoTracker Red during the last 5 min of the incubation. The fluorescence pattern of LysoTracker Red showed punctate perinuclear distribution compatible with localization to lamellar bodies (Fig. 4C). Merging the images that were separately acquired at the two different wavelengths showed strong colocalization of the two labels in most of the punctate structures as indicated by the yellow color (Fig. 4D). NBD-PC did not traffic to all acidic organelles, so that some of the punctate structures were predominately labeled by LysoTracker Red as shown by the persistence of red in the merged image.

To evaluate uptake by clathrin- or actin-mediated pathways, we incubated NBD-PC liposomes with type II cells without or with inhibitors. Preincubating isolated type II cells with amantadine (1 mg/ml) and/or cytochalasin D (2 μg/ml) for 20 min followed by a 30-min incubation with liposomes in the presence of inhibitors led to markedly reduced fluorescence intensity of intracellular organelles (Fig. 5). Compared with control, fluorescence was reduced 60.1 ± 2.8% with amantadine, 60.2 ± 2.2% with cytochalasin D, and 78.4 ± 0.7% with the combination (means ± SE for n = 3; P < 0.05). The effect of both inhibitors together was...
greater than either alone ($P < 0.05$). These concentrations of inhibitors had no cytotoxic effects as determined by the Molecular Probes Live/Dead Cell Assay.

**DISCUSSION**

In this paper, we evaluated the different uptake pathways for pulmonary surfactant in a whole lung model. This experimental system enables the study of alveolar type II cells in their native environment with intact cell-cell interactions and avoids the unpredictable effects of enzymatic digestion needed for the isolation of type II cells, as well as the phenotypic changes that occur rapidly in culture. In addition, by applying trace amounts of labeled liposomes to the natural surfactant pool in the lung, the measurements should reflect the uptake of native surfactant. Finally, this system allows for the application of secretagogues or inhibitors by alveolar and vascular routes. Inhibitors of uptake were used at the lowest possible concentration required to achieve a maximal effect, as determined by evaluation of dose response and activity.

Previous studies have indicated that alveolar type II cells play a major role in the uptake of surfactant lipids in the intact lung with a contribution from alveolar macrophages and possibly other cell types (8, 11). To investigate this issue further, we used the ex vivo model of the isolated perfused lung in combination with microscopic imaging of subpleural alveoli. We were thus able to demonstrate uptake of NBD-PC into alveolar type II cells of the intact lung. These cells were identified as alveolar type II cells on the basis of their shape, location in the corners of the alveoli, and organelar fluorescence with LysoTracker Red, a dye known to specifically fluoresce in acidic compartments such as the lamellar bodies of the granular pneumocytes (7). Because of background fluorescence caused by the autofluorescence of unfixed lung tissue, low-level uptake of NBD-PC in alveolar type I or other cells cannot be excluded. Possible uptake by alveolar macrophages could not be confirmed, since these cells were scarce in the lungs of these relatively young and specific pathogen-free rats.

We used [$^3$H]DPPC-labeled liposomes to study the pathways responsible for the uptake of lipids by alveolar type II cells (14). We assume that NBD-PC and [$^3$H]DPPC colocalize, since the uptake mechanisms as determined by the results with inhibitors were similar. PAO, a trivalent arsenical that blocks endocytosis via pathways involving clathrin, purportedly by cross-linking the clathrin coat (17, 21), was used as an inhibitor of receptor-mediated endocytosis. Inhibition of liposome uptake by 40% in the presence of PAO demonstrated the importance of clathrin-mediated endocytosis as an uptake pathway for surfactant lipids. We have previously shown that PAO also inhibits the uptake of SP-A (3), lending support to the controversial hypothesis that SP-A mediates uptake of surfactant lipids via its receptor. It was initially not clear, how-

Fig. 5. Exposure to actin and clathrin inhibitors reduces the uptake of fluorescently labeled liposomes by isolated type II cells. Type II cells in culture for 20 h on glass coverslips were washed and incubated for 30 min ± inhibitors before adding the NBD-PC labeled liposomes for another 30 min. Cells were processed as described in Fig. 4. Images are representative of 3 independent experiments. A–D: images were acquired in the transmission mode on a confocal microscope. E and F: NBD-PC fluorescence was acquired with FITC filter set. A and E: control; B and F: amantadine HCl (1 mg/ml); C and G: cytochalasin D (2 μg/ml); D and H: amantadine + cytochalasin D. All images were acquired with identical settings, and postacquisition processing was performed in parallel. Scale bar = 20 μm.
ever, whether PAO alone would completely block the clathrin-mediated pathway. Thus we used amantadine, a cationic amphiphilic drug believed to exert its effect by stabilization of clathrin-coated vesicles (30), as an independent inhibitor of clathrin-mediated endocytosis (34). Inhibition by amantadine was similar to that seen with PAO. Because PAO and amantadine are chemically dissimilar and have different modes of action, their lack of synergy suggests that each individually and completely blocked the clathrin-mediated uptake pathway.

Cytochalasin D, an inhibitor of actin-dependent endocytosis, exerts its effect by depolymerizing the microfilament actin network of the cell. It has previously been used to study uptake pathways in isolated type II cells (3, 25). Uptake was inhibited by ~50% under resting conditions and 65% when turnover was stimulated with 8-Br-cAMP. The combination of cytochalasin D with one of the clathrin inhibitors was synergistic, resulting in 70–80% inhibition. These results with inhibitors indicate that 8-Br-cAMP stimulated both the clathrin and actin pathways for uptake approximately fourfold. Because the effect of either inhibitor exceeded 50% and the effects were not additive, we deduce partial overlap of the pathways, compatible with some involvement of actin filaments in clathrin-mediated endocytosis as previously suggested (4, 16, 23).

Because the combination of inhibitors decreased lipid uptake in the lung by only 70–80%, it is possible that an unidentified pathway(s) plays a role in uptake. At this stage, the identity of possible alternate pathways is not clear. Surface binding is unlikely to have influenced these measurements of uptake, since it should be excluded by subtracting the initial 5-min lung association value. Caveolar-mediated uptake into alveolar type II cells also appears unlikely since these cells do not express caveolin-1 (5). Uptake via monomer exchange or fusion of the liposomes with the plasma membrane (27, 29) are possible explanations; although these processes are likely to occur in large part during the initial 5-min period, they could continue beyond that initial time point.

The present results with the intact lung are similar to our laboratory’s previous report of uptake pathways in isolated type II cells (25). In that study using NBD-PC-labeled liposomes on cells cultured on plastic support, uptake was reduced 49% by incubation in hypertonic medium to inhibit clathrin-mediated endocytosis and by 50% in the presence of cytochalasin D with a nearly additive effect of their combination. In another study with cells cultured on microporous membranes and radioactively labeled natural surfactant, there was only 35% inhibition with hypertonic medium, 27% inhibition with cytochalasin D, and ~45% inhibition by their combination (18). The trauma of enzymatic isolation and loss of interaction with other lung cells (1) might be responsible for differences between various studies with isolated type II cells and from results with an intact lung system. Our results reveal that a combination of cytochalasin D with a clathrin inhibitor is synergistic, suggesting the presence of two distinct endocytotic pathways.

Freshly isolated type II cells were used for subcellular localization of NBD-PC fluorescence since this was difficult with available technology in the intact lung. NBD-PC in isolated type II cells colocalized with LysoTracker Red in acidic, large, circular, perinuclear organelles consistent with lamellar bodies. This lends further support to the theory that lipids are recycled by way of the lamellar body compartment. We utilized a 30-min uptake period in the cell experiments on the basis of our previous observation that intracellular NBD-PC fluorescence reaches a plateau in isolated cells at that time (25); the plateau may reflect self-quenching of NBD-PC in higher concentrations rather than a limitation of uptake. In the present study, a plateau was not observed in the homogenate from perfused lungs, since the extracted lipids were resuspended in a relatively large volume, which would minimize self-quenching. Furthermore, the use of low-intensity transmission light for focusing and of low-intensity laser illumination for acquiring images avoided significant photobleaching. We showed, thereby, a significant decrease of total intracellular fluorescence in the cells incubated in the presence of actin inhibitors, clathrin inhibitors, or a mixture of the two.

In summary, the isolated perfused lung proved useful for evaluating lipid uptake by different endocytotic pathways in alveolar type II cells. On the basis of NBD-PC fluorescence, alveolar type II cells play a major role in alveolar lipid uptake. As previously reported from studies of isolated cells in primary culture, we confirm the presence of both clathrin- and actin-dependent pathways in alveolar type II cells in the intact lung that together account for at least 70% of lipid uptake. Because inhibition of endocytosis was incomplete, a minor portion of lipid uptake may occur through other pathways.

The authors are grateful for the excellent technical assistance of Chandra Dodia, Jian-Qin Tao, and Kathy Notarfrancesco. This research was supported by National Heart, Lung, and Blood Grant HL-19737.

A preliminary report of this study in abstract form was presented at Experimental Biology 2001 (FASEB J 15: A420.11, 2001).

REFERENCES


LUNG UPTAKE OF SURFACTANT LIPIDS


