Lysosomes from rabbit type II cells catabolize surfactant lipids

EVELYN D. RIDER,1 MACHIKO IKEGAMI,2 KENT E. PINKERTON,3 J ANICE L. PEAKE,3 AND ALAN H. JOBE2

1Department of Pediatrics, University of Arizona and Steele Memorial Children’s Research Center, Tucson, Arizona 85724; 2Division of Pulmonary Biology, Children’s Hospital Medical Center, Cincinnati, Ohio 45229; and 3Department of Anatomy, University of California School of Veterinary Medicine, Davis, California 95616

Rider, Evelyn D., Machiko Ikekami, Kent E. Pinkerton, Janice L. Peake, and Alan H. Jobe. Lysosomes from rabbit type II cells catabolize surfactant lipids. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L68–L74, 2000.—The role of a lysosome fraction from rabbit type II cells in surfactant dipalmitoylphosphatidylcholine (DPPC) catabolism was investigated in vivo using radiolabeled DPPC and dihexadecylphosphatidylcholine (1,2-dihexadecyl-sn-glycero-3-phosphocholine; DEPC), a phospholipase A1- and A2-resistant analog of DPPC. Freshly isolated type II cells were gently disrupted by shearing, and lysosomes were isolated with Percoll density gradients (density range 1.0591–1.1457 g/ml). The lysosome fractions were relatively free of contaminating organelles as determined by electron microscopy and organelle marker enzymes. After intratracheal injection of rabbits with [3H]DPPC and [14C]DEPC associated with a trace amount of natural rabbit surfactant, the degradation-resistant DEPC accumulated 16-fold compared with DPPC in lysosome fractions at 15 h. Lysosomes can be isolated from newly isolated type II cells, and lysosomes from type II cells are the primary catabolic organelle for alveolar surfactant DPPC following reuptake by type II cells in vivo.

Lauryllamellar bodies; surfactant metabolism; dipalmitoylphosphatidylcholine

PULMONARY SURFACTANT is a complex of macromolecular aggregates composed of phospholipids, neutral lipids, cholesterol, and surfactant specific proteins that is essential for normal lung function. Among the phospholipids, dipalmitoylphosphatidylcholine (DPPC) is most abundant, representing about 50% of the surfactant lipid by weight (15). DPPC is the surfactant component most often used to characterize surfactant phospholipid metabolic pathways. DPPC is rapidly turned over in the adult rabbit lung, with a turnover time of about 5 h (14, 22). About 50% of the alveolar pool is recycled back into type II cells, and the rest is catabolized. Negligible recovery elsewhere in the body indicates degradation within the lung itself (22).

Lung fractionation, cell isolation, and catabolic studies in vivo and in vitro indicate that type II cells have a primary role in clearing and catabolism of alveolar surfactant DPPC (4, 23, 24, 28). Alveolar macrophages also take up and degrade surfactant phospholipids and proteins (18, 23, 26, 29). Alveolar macrophages internalize and rapidly degrade DPPC when presented in a variety of forms in vitro (18, 26, 29). However, from cellular localization studies using a poorly degraded diether analog of DPPC (dihexadecylphosphatidylcholine, DEPC), we estimated that the contribution of alveolar macrophages to overall lung clearance of DPPC in the intact animal was about 20% in 24 h (23, 24).

Studies investigating the organelles involved in intracellular degradation are limited. Young et al. (30) demonstrated with electron-microscopic autoradiography that internalization of DPPC and surfactant protein (SP) A by type II cells was followed by time-dependent association with lamellar bodies consistent with recycling. Horowitz et al. (12) reported greater endocytosis of small-aggregate surfactant by an alveolar epithelial cell line, MLE-12, compared with large-aggregate surfactant. This is consistent with the hypothesis that the protein-depleted small-aggregate surfactant is the catabolic, inactive form of alveolar surfactant destined for cellular reuptake and clearance from the air space. Fractionation of rabbit lung homogenates demonstrated that most of the surfactant DPPC catabolism occurred in lysosomes (24). The observations with lung lysosomes together with the cellular localization studies that identified the type II cells as the primary cells involved in intrapulmonary clearance of alveolar surfactant in vivo (23) imply that lysosomes in type II cells are the primary subcellular organelle responsible for surfactant DPPC degradation in vivo. However, type II cells do not have clearly identified lysosomes by electron microscopy and have not been fractionated to directly identify the intracellular catabolic compartment. Therefore, we adapted a lysosome isolation procedure used for other tissues to recover a lysosome-like fraction from freshly isolated rabbit type II cells. Subsequently, we used DEPC, the diether analog of DPPC, to evaluate whether surfactant DPPC catabolism could be localized to this lysosome fraction from type II cells.

MATERIALS AND METHODS

Materials. Deoxyribonuclease I, trypsin, and soybean trypsin inhibitor used for cell isolation and all substrates and products.
chemicals for the enzyme and biochemical assays were from Sigma Chemical (St. Louis, MO). Elastase was from Worthington Biochemicals (Freehold, NY), and colloidal barium sulfate was a gift from Lafayette Pharmaceutical (Lafayette, IN). Nylon cloth used for filtration was from TETKO (Elmsford, NY). Percoll was from Pharmacia Biotechnology (Piscataway, NJ). Joklik minimum essential medium (JMEM) and Hanks’ balanced salt solutions were from Gibco BRL (Grand Island, NY). The New Zealand White rabbits were clinically healthy, and lung lavage performed as part of the cellular isolation procedures showed no evidence of acute or chronic lung disease.

Preparation of radiolabeled lipids. Synthetic DPPC and DEPC were prepared as previously described (22). Unlabeled 1,2-dipalmitoyl-sn-glycero-3-N,N-dimethylphosphatidylethanolamine (DMPE) and 1,2-dihexadecyl-sn-glycero-3-N,N-dimethylphosphatidylethanolamine (DMPE-ether; Serdary Research Laboratories, Port Huron, MI) were methylated to DPPC and DEPC, respectively, using potassium carbonate and 18-Crown-6 (Sigma Chemical) in benzene. DMPE was methylated with [3H]methyl iodide (14.4 Ci/mmol, Amer-sham, Arlington Heights, IL), and DMPE-ether was methylated with [3C]methyl iodide (56.6 mCi/mmol, ICN Radiochemicals, Irvine, CA). The labeled lipids were isolated by thin-layer chromatography using chloroform-methanol-acetic acid-water (65:25:8:4, vol/vol) as the solvent system. Pure unlabeled lipids (Serdary Research Laboratories) were run in parallel lanes as standards. The appropriate spots on the silica chromatogram were recovered, treated with osmium tetroxide, and processed through neutral alumina columns to isolate the radioactively labeled saturated phosphatidylcholines (17).

Isolation of type II cells. Type II cells were isolated from healthy male New Zealand White rabbits weighing 1.2 ± 0.04 kg (23). Rabbits were killed using intravenous pentobarbital sodium (200 mg/kg) containing heparin (1 U/mg pentobarbital sodium) followed by exsanguination. A tracheal tube was secured for alveolar lavage, and the lungs were perfused in situ via the pulmonary artery with 4°C buffered saline to remove residual blood. To recover alveolar macrophages and surfactant, the lungs were then lavaged six times with 4°C calcium-free balanced salt solution (BSS). The postbronchoalveolar lavage lung was removed from the chest and filled with BSS containing colloidal barium sulfate (5 mg of BaSO4 in 50 ml of BSS). After a 10-min incubation at 37°C, the lung was lavaged three more times with 4°C BSS to remove additional alveolar macrophages and residual extracellular barium sulfate. The volume of the second alveolar lavage fluid was measured and recorded. In animals used for recovery of labeled tracer lipids, the two alveolar lavage suspensions were combined and the total volumes were recorded and processed for alveolar macrophage and radiolabel recovery.

The conducting airways and large blood vessels were dissected and discarded following enzyme exposure of the lung (23). The residual lung tissue was finely minced in trypsin inhibitor solution and serially filtered through nylon cloth to remove residual debris and washed free of excess protease. The filtrate was then layered over 0.8 M sucrose in saline and centrifuged at 500 g for 15 min to remove residual extracellular surfactant. The resulting cell pellet was washed with J MEM, resuspended in J MEM to a concentration of 5 × 107 cells/ml, and designated the crude cell suspension. The cells were separated on discontinuous Percoll gradients (3 ml density = 1.08 and 8 ml density = 1.04 g/ml Percoll in J MEM) on which 5 ml of the crude cell suspension was layered. The gradient was centrifuged at 1,730 g for 20 min. The type II cell and mixed cell bands were recovered and washed free of Percoll before resuspension in 25 ml of J MEM. An aliquot from each cell isolate was saved for slide preparation and subsequent biochemical analyses. The remaining type II cell suspension was centrifuged at 1,730 g for 10 min and resuspended in Tris-buffered sucrose solution (250 mM sucrose containing 1 mM EDTA, pH 7.0) to a concentration of 5 × 107 cells/ml.

Cell disruption and lysosome isolation. The type II cell suspension was disrupted by repeated aspiration through a 1-ml plastic pipette tip attached to a 10-ml graduated plastic pipette. The lysate was centrifuged at 750 g for 10 min to recover a supernatant containing the intracellular organelles and a pellet containing nuclear debris and unbroken cells. The pellet was resuspended in one-half the original volume of Tris-buffered sucrose, and the process of cellular disruption and centrifugation was repeated three more times to achieve lysis of 90% of the type II cells in the original suspension. The cellular disruption was monitored by microscopic examination of the pellet following resuspension after each centrifugation. Attempts at using other disruption techniques, such as sonication and freeze-thawing, compromised organelle separation.

The pooled supernatants containing the organelles were centrifuged at 20,000 g for 15 min. The resulting organelle pellet was resuspended in HEPES-buffered sucrose (250 mM sucrose and 20 mM HEPES, pH 7.0) to an average protein concentration of 7 mg/ml. This suspension was mixed with isotonic Percoll (40:60 vol/vol, organelle suspension-isotonic Percoll). Organelle separation was accomplished by centrifugation at 34,000 g for 90 min to generate a shallow gradient with a density profile of 1.0591–1.1457 g/ml. The gradient was divided into 10 equal fractions, and each fraction was diluted with a 10-fold excess volume of HEPES-buffered sucrose and centrifuged at 20,000 g for 15 min to remove excess Percoll. The pellets were resuspended in HEPES-buffered sucrose.

Subcellular marker enzymes. All enzyme assays were performed in substrate excess. Lysosomal β-hexosaminidase activity was measured fluorometrically using 4-methylumbel-liferyl-2-acetamido-2-deoxy-β-D-glucosaminide as the substrate (27). Mitochondrial succinate dehydrogenase was assayed with 2-(p-nitrophenyl)-3-p-nitrophenyl-5-phenyltetrazo-lum chloride as the acceptor (20). Plasma membrane-associated 5'-nucleotidase activity was determined by the release of inorganic phosphate from adenosine monophosphate (11). Endoplasmic reticulum-associated glucose-6-phosphatase activity was measured using glucose 6-phosphate as the substrate (19). Both 5'-nucleotidase and glucose-6-phosphatase assays were performed in the presence of 12 mM sodium potassium tartrate to inhibit nonspecific acid phosphatases (8). Inorganic phosphate release was measured by the method of Chen et al. (5). Catalase was used as the peroxidase marker and assayed as previously described (21, 24).

Electron microscopy. Three separate gradients were prepared for electron microscopy. From each gradient, pooled aliquots designated as pooled fractions 1–5 were pelleted by centrifugation. Pooled fraction 1 consisted of gradient fractions 1–3 (density 1.1150–1.1459 g/ml), pooled fraction 2 contained gradient fractions 4–5 (density 1.1009–1.1108 g/ml), pooled fraction 3 represented gradient fraction 6 (density 1.0931 g/ml), pooled fraction 4 represented gradient fractions 7–8 (density 1.0757–1.0817 g/ml), and pooled fraction 5 was formed by gradient fractions 9–10 (density 1.0591–1.0654 g/ml). Each pellet was fixed in 2% glutaraldehyde–2% tannic acid in 86 mM sodium cacodylate buffer containing 3 mM EDTA and postfixed in 1% osmium tetroxide and 2% uranyl acetate. The specimens were dehydrated in a graded series of
Acetone and propylene oxide solutions at 4°C prior to being embedded in araldite. Thin sections (60–70 nm) were stained in 4% aqueous uranyl acetate and lead citrate and photographed with a Zeiss 10A transmission electron microscope.

Morphometric analysis of density gradient fractions. Electron micrographs were photographed at each pooled fraction at final magnifications of 10,700 to most consistently and fully characterize organelar contents. Each micrograph was placed under a test lattice overlay consisting of uniformly spaced 1-cm-long lines and counted a minimum of three times by two different observers. Organelles and cytoplasmic elements identified were lysosomes, mitochondria, lamellar bodies, vesicles (vacuoles), and plasma membrane. The relative volume fraction of each organelle was based on the proportion of total points that fell on that organelle. A total of three different samples prepared for each of the five different pooled fractions were analyzed at the two magnifications.

Lysosomes were identified as electron-dense, membrane-bound vacuoles of heterogeneous appearance (Fig. 1, A and B). Some lysosomes were of a uniform, electron-dense composition, whereas others contained vesicles of varying sizes and shapes. Occasional lysosomes contained electron-lucent structures within the limiting membrane of the lysosome. Mitochondria in three different configurations were identified by the presence of cristae (Fig. 1, B and C). Most mitochondria were similar to those found in an intact cell. Others were swollen, but cristae were still visible. A third form of mitochondria contained prominent cristae and a condensed appearance. Lamellar bodies were identified by the presence of lamellae. Vesicles and vacuoles were identified as structures possessing a unit membrane with granular contents. Plasma membranes were identified by their linear structure. Plasma membranes were commonly associated with cytosolic elements identified as granular, proteinaceous material (Fig. 1D).

Intratracheal injection suspensions. [3H]DPPC and [14C]DEPC were prepared as liposomes in 0.45% NaCl and associated with trace amounts of unlabeled natural rabbit surfactant that was purified from pooled alveolar washes of adult rabbit lungs (22). Liposomes prepared in this manner were shown previously to have alveolar clearance behavior similar to endogenously labeled surfactant (13). The final liposome suspension contained 1.5 µCi of [3H]DPPC, 0.3 µCi of [14C]DEPC, and 0.4 µmol of total phospholipid phosphorus in each 3-ml injection volume. The radiolabeled lipids represented no more than 2% of the phospholipid contained in the injection suspension. The 0.4 µmol total phospholipid content of the injection suspension was less than 10% of the endogenous alveolar saturated phosphatidylcholine pool (15).

Intratracheal injections. Male New Zealand White rabbits (n = 4), weighing 1.42 ± 0.11 kg, were anesthetized with a CO2–O2 gas mixture and injected intratracheally with the liposome suspension containing the labeled lipid tracers using an 8-Fr flexible bronchoscope (23). A catheter inserted through the suction port of the bronchoscope was visually guided through the vocal cords and positioned 1–2 cm above the carina and followed by injection of the liposome suspension. The animals recovered rapidly and were killed 15 h after the intratracheal injection with intravenous pentobarbital sodium containing heparin. The lungs were lavaged as above, and type II cell and subsequently lysosomes were isolated. The gradient fractions were characterized by measurements of density profile, protein content, phosphorus content, β-hexosaminidase enzyme activity, and label recovery.

Alveolar lavage for radiolabeled recovery. Alveolar macrophages were isolated by centrifugation from the combined alveolar lavages of each animal before and after the barium sulfate incubation. Aliquots (30 ml) of the lavage fluid in triplicate were layered over 10 ml of 0.8 M sucrose in saline and centrifuged at 500 g for 15 min. The alveolar macrophage pellets free of extracellular alveolar surfactant were washed free of sucrose and resuspended in Hanks’ balanced salt solution. One sample from each triplicate was used for a cell

Fig. 1. Electron micrographs of pooled gradient fractions. Scale bar, 1 µm. Shown are representative electron micrographs from 1 of 3 separate gradient preparations evaluated by electron microscopy. A: numerous lysosomes within pooled fraction 1 (density 1.1150–1.1457 g/ml). B: pellet from pooled fraction 2 (density 1.1009–1.1108 g/ml) with a similar frequency of lysosomes to pooled fraction 1 (A). C: wide variety of organelles present within pooled fractions 3 and 4 (density 1.0757–1.0931 g/ml). A greater number of mitochondria and vacuoles are noted with a reduced number of lysosomes. D: pooled fraction 5, representing gradient fractions 9–10 of the cell lysate preparation (density 1.0591–1.0654 g/ml). Lamellar bodies, mitochondria, membrane-limited vesicles, and vacuoles are present.
count and viability estimate. The other two samples were analyzed for radiolabel recoveries.

Characterization of cell isolates. Cell counts were determined using a hemocytometer counting chamber (AO Scientific Instruments, Buffalo, NY), and viability was monitored by exclusion of the vital dye trypan blue. Purity of the alveolar macrophage, mixed cell, and type II cell isolates was determined by performing 200 cell differential counts on cytocentrifuge-prepared slides stained by the modified Papa-nicolaou technique.

Biochemical analyses. Protein content was measured using bovine serum albumin as standard (3). Lipid was extracted from aliquots of alveolar washes, postlavage lung homogenates, alveolar macrophages, type II cells, organelle pellets, and each of the gradient fractions using chloroform-methanol (2:1, vol/vol) (2). In samples from animals receiving radiolabeled phospholipids, an aliquot was taken for measurement of radioactivity. Lipid phosphorus was quantified from extracts of the alveolar washes, lung homogenates, and type II cells by the method of Bartlett (1). The small quantities of lipid phosphorus in the organelle pellets and gradient fractions were measured spectrophotometrically using a modification of the malachite green assay of Hess and Derr (10). Brieﬂy, the extracted samples were digested in 50 µl of 10 N H2SO4 by heating for 1.5 h at 180°C. The samples were then cooled, and 50 µl of water, 50 µl of 10 N NaOH, and 25 µl of concentrated HCl were added to each sample, and the samples were dried in a 70°C oven. To each sample, 200 µl of distilled water were added, followed by 1 ml of color reagent [0.045% malachite green and 4.2% ammonium molybdate in 4 N HCl (3:1, vol/vol) to which was added 0.1 ml of 1% Triton X-100 per milliliter of malachite green-ammonium molybdate mixture]. The optical density at 650 nm was read after 30 min. The assay was sensitive to 0.5 nmol of phosphorus and linear to 20 nmol of phosphorus (r = 0.99).

Data analysis. All values are group means ± SE, except the morphometry data, which are given as means ± SD. Differences between groups were tested by two-tailed Student’s t-test. When more than two comparisons were made, analysis of variance followed by the Student-Newman-Keuls multiple comparison procedure was used. Significance was accepted at P < 0.05.

RESULTS

Cell isolates. Alveolar macrophages from alveolar washes were 96 ± 3% viable, and mixed cells and type II cells from Percoll gradients were 93 ± 3% viable based on vital dye exclusion. The type II cell band contained 88 ± 1% type II cells, and the mixed cell band contained 39 ± 2% type II cells and 52 ± 2% macrophages. The balance of the mixed cell band content consisted of other white blood cells. The alveolar lavage contained 97 ± 2% alveolar macrophages, with neutrophils and lymphocytes comprising the remaining cells.

Gradient fractions from type II cells. A shallow Percoll density gradient was used for subcellular organelle separation (1.0591 ± 0.001 to 1.1457 ± 0.004 g/ml). The distribution proﬁles of protein and lipid phosphorus from eight separate gradients were comparable (data not shown). The gradients from the type II cell lysates had two visibly distinct bands: 1) a thick, creamy protein and phosphorus-rich less dense band and 2) a less prominent, more dense band corresponding to the lysosome-rich fractions.

Specific activity of the lysosomal enzyme β-hexosaminidase A was maximal at density 1.1009–1.1108 g/ml (Fig. 2). Except for a small amount of mitochondrial enzyme activity at density 1.1009 g/ml, these fractions were relatively free of plasma membrane fragments and other subcellular organelles, including peroxisomes and endoplasmic reticulum. Lysosomes at density 1.1009–1.1108 g/ml were puriﬁed approximately 62-fold relative to the lung homogenate and 8-fold relative to type II cells, as indicated by enrichment of β-hexosaminidase specific activity.

Morphometric analysis of gradient fractions. Lysosomes were numerous within pooled fraction 1 (Fig. 1A, density 1.1150–1.1457 g/ml). Pooled fraction 2 (Fig. 1B, density 1.1009–1.1108 g/ml) had a similar frequency of lysosomes as seen in pooled fraction 1, as well as a number of mitochondria. The number of lysosomes decreased in pooled fractions 3 (density 1.0931 g/ml) and 4 (density 1.0757–1.0817 g/ml). Pellets from these two pooled fractions showed the greatest variability in organelle composition (Fig. 1C). In pooled fraction 5
(Fig. 1D, density 1.0591–1.0654 g/ml), lamellar bodies, mitochondria, membrane-limited vesicles, and vacuoles were present. Table 1 summarizes the relative organellar volume fractions for each pellet prepared from each sample of pooled fractions. Lysosomes predominated in fractions 1 and 2.

Localization of radiolabeled DPPC and DEPC. Recoveries of the radiolabeled phospholipids in the alveolar lavages, macrophages, and postlavage lung homogenates 15 h after intratracheal injection are summarized in Table 2. The alveolar wash recoveries, when corrected for macrophage contribution, were similar for DPPC and DEPC. The lung tissue contained a significantly higher amount of DEPC relative to DPPC. Of the lung tissue-associated DEPC radiolabel, 82.9 ± 5.8% was recovered in the postdigestion crude cell suspension. Of the crude cell suspension-associated DEPC radiolabel, 73.4 ± 2.5% was recovered in type II cells.

The radiolabel recoveries in lipid extracts of the lysosomal gradient fractions prepared from type II cells 15 h after the intratracheal injection are shown in Fig. 3. The highest specific activities for DEPC were in lysosome fractions (Fig. 3A). There was very little labeled DPPC recovered from the gradient. A 16-fold accumulation of the DEPC label relative to DPPC was noted in the lysosome-enriched fractions at 15 h (Fig. 3B).

Radiolabel in the aqueous phase as a percentage of the total label (lipid phase plus aqueous phase) contained in each fraction also was measured for the lysosome-enriched and the more heterogeneous upper areas of the gradients (Fig. 4). A greater percentage of total label was recovered as water-soluble metabolites in the lysosome-enriched fractions (density 1.0931–1.1108 g/ml) compared with the lighter fractions (density 1.0654–1.0757 g/ml). Less DEPC-derived label was recovered in the aqueous phase of the lysosome-enriched fractions compared with the DPPC-derived label, consistent with greater resistance of DEPC to phospholipase. In contrast, less than 2% of the total label near the top of the gradient was recovered in water-soluble metabolites for both DPPC and DEPC labels, indicating little or no degradation in fractions that contain lamellar bodies.

Table 1. Organelle composition of Percoll gradient fraction of type II cells

<table>
<thead>
<tr>
<th>Pooled Fractions</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tr>
<td>Density, g/ml</td>
<td>1.1150–1.1457</td>
<td>1.1009–1.1108</td>
<td>1.0931</td>
<td>1.0757–1.0817</td>
<td>1.0591–1.0654</td>
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<td>Organelle, %</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Lysosomes</td>
<td>85.0 ± 6.6</td>
<td>77.0 ± 5.3</td>
<td>63.4 ± 1.4</td>
<td>2.2 ± 0.1</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>9.2 ± 3.3</td>
<td>13.4 ± 3.0</td>
<td>26.6 ± 3.9</td>
<td>10.0 ± 1.4</td>
<td>51 ± 2.9</td>
</tr>
<tr>
<td>Lamellar bodies</td>
<td>0</td>
<td>0.1 ± 0.2</td>
<td>0.3 ± 0.1</td>
<td>8.9 ± 1.5</td>
<td>4.9 ± 2.7</td>
</tr>
<tr>
<td>Vesicles/vacuoles</td>
<td>4.4 ± 2.6</td>
<td>5.1 ± 4.6</td>
<td>7.0 ± 2.4</td>
<td>14.5 ± 0.1</td>
<td>9.8 ± 5.5</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>0.8 ± 0.7</td>
<td>0.5 ± 0.2</td>
<td>1.2 ± 0.5</td>
<td>1.0 ± 0.3</td>
<td>4.4 ± 3.8</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.7 ± 0.5</td>
<td>4.0 ± 6.0</td>
<td>1.5 ± 0.5</td>
<td>63.4 ± 0.2</td>
<td>74.0 ± 3.5</td>
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Values are means ± SD for 3 separate gradient preparations.

Table 2. Recovery of radiolabeled phospholipids in lipid extracts

<table>
<thead>
<tr>
<th></th>
<th>DPPC</th>
<th>DEPC</th>
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<tr>
<td>Alveolar wash</td>
<td>12.4 ± 0.3</td>
<td>12.3 ± 0.5</td>
</tr>
<tr>
<td>Macrophages</td>
<td>3.4 ± 0.2</td>
<td>14.5 ± 0.4*</td>
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<tr>
<td>Lung tissue homogenate</td>
<td>16.6 ± 2.5</td>
<td>34.1 ± 5.0*</td>
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Values are % radiolabel recovered 15 h after intratracheal administration. Alveolar wash values exclude macrophages. DPPC, dipalmitoylphosphatidylcholine; DEPC, dihexadecylphosphatidylcholine. *P < 0.05 for higher amount of DEPC relative to DPPC.

Fig. 3. Radiolabel recoveries from gradients from type II (TII) cells. A: specific activities of labeled phospholipids are given as dpm per nanomole phospholipid phosphorus. Each rabbit received on average 1.5 µCi of [3H]DPPC and 0.2 µCi of [14C]DEPC. Highest specific activities were in fractions containing highest lysosomal enzyme activities. B: specific activity ratios of dihexadecylphosphatidylcholine (DEPC) to dipalmitoylphosphatidylcholine (DPPC) in gradient fractions demonstrate a 16-fold accumulation of ether analog within lysosomal fractions relative to DPPC. Ratio of lipids was low in lighter fractions containing lamellar bodies and other organelles (density 1.0591–1.0654 g/ml).
DISCUSSION

Surfactant DPPC catabolism was localized to this lysosomal fraction from type II cells following alveolar uptake of DPPC in vivo. A shallow density gradient very similar to the one used for isolation of lysosomes from whole lung homogenates was utilized (24). The results of the marker enzyme analyses indicated that the fractions with peak activity for the lysosomal marker enzyme, β-hexosaminidase A, were relatively free of other contaminating organelles. The midpeak density of 1.11 g/ml was consistent with what was reported for lysosomes from liver, fibroblasts, and lung (24, 25, 27). Electron microscopy of the gradient fractions verified that lysosome-like organelles were isolated. No isolation of lysosomes from freshly isolated type II cells has been reported previously to our knowledge. Of note, lysosomes have not been definitely identified in type II cells by electron microscopy. The sizes and densities of the organelles recovered from the gradients may be modified as a result of the isolation procedures. Previous work by Gibson and Widnell (9) characterized two different organelle fractions from rat type II cells that contained lamellar aggregates of surfactant. Only one of the organelle fractions contained markers for lysosomes. The potentially degradative function of the organelles containing the lysosomal matrix enzyme β-N-acetylglucosaminidase was suggested by identification of proteins within the compartment that reacted with antibodies to surfactant protein A but with molecular mass less than 28 kDa. That study did not further evaluate the role of these organelles in degradation of other surfactant components.

Because DEPC is a poorly degraded analog of DPPC, it was used to localize the intracellular compartment involved in degradation of DPPC. Radiolabeled DPPC served as a marker for movement of the naturally occurring surfactant phospholipid through the various intracellular compartments. The metabolic behavior and in vivo function of DEPC relative to DPPC were previously shown to be comparable (7, 22–24). Both lipids have similar alveolar clearance kinetics, initial lung tissue and cellular association kinetics, turnover times, and reutilization efficiencies despite accumulation of the ether analog within the lung tissue. The accumulation was nontoxic to the animal and presumably occurred secondary to inadequate degradation resulting from resistance of the ether bonds to phospholipases A₁ and A₂ and a relative resistance to acid hydrolysis (6). We used high-specific activity phospholipids associated with trace amounts of unlabeled natural surfactant to minimize the changes in the endogenous surfactant phospholipid pools of the animals.

Radiolabel distribution across the lysosome gradient fractions of type II cell was very similar to the distribution previously demonstrated across lysosome gradient fractions for whole lung. There was a 16-fold relative accumulation of the ether analog compared with DPPC at 15 h in the fractions with the highest lysosomal enzyme activity. Despite this accumulation, there was a minimal concentration of DEPC relative to DPPC in the upper fractions containing the lamellar bodies, a result similar to that seen for lung lysosomes (24). These results are consistent with findings where accumulation of the ether analog within the lung tissue did not influence the recoveries from the alveolar space and the recycling efficiency (22). These observations support the concept that phospholipids within the degradative compartment cannot reenter the recycling compartment. Any contribution of phospholipids from the catabolic compartment would be indirect, occurring by incorporation of degradation products into newly synthesized phospholipids destined for lamellar bodies.

When the data for accumulation of DEPC relative to DPPC in the present study were compared with the relative accumulations measured in lysosome preparations from lung homogenates, the levels found in type II cells at 15 h were similar to values for lung lysosomes at 12 and 24 h (24). This result was consistent with the type II cells being the cellular population primarily responsible for the lysosomal degradative activity. This information together with the cellular localization and lung subfractionation studies indicates a primary role for type II cells in intrapulmonary surfactant phospholipid catabolism in vivo.

Ultrastructural studies by Kalina and Socher (16) demonstrated time-dependent localization of gold-labeled surfactant to intracellular organelles of type II cells following internalization via clathrin-coated pits. Initial deposition was localized to electron-lucent multivesicular bodies that lack lysosomal enzymes. Subsequently, localization was to lamellar bodies and acid phosphatase-containing organelles such as dense multivesicular bodies and homogeneous vesicles consistent with lysosomes. Young et al. (30) documented internalization of radiolabeled DPPC and SP-A by type II cells followed by time-dependent deposition in lamellar bodies.
both DPPC and SP-A labels appeared in light multivesicular bodies, only DPPC label was found in dense multivesicular bodies. This selective segregation of the lipid label into dense multivesicular bodies that are known to contain lysosomal enzymes supports a role for this cellular compartment as a degradation pathway.

Recovery of labeled water-soluble choline metabolites as a fraction of total label (in lipid and aqueous phases of the extracts) was highest in the lysosome fractions. The DPPC-derived label recoveries were low in these fractions relative to the total DEPC-derived label. This result was consistent with rapid disappearance of DPPC-derived label from the lysosome-enriched fractions, suggesting loss of degradation products from the lysosomal compartment. The percents of total label partitioning into the aqueous phases of the less dense fractions were very low, indicating that minimal degradation of either phospholipid occurred in the organelles contained within these fractions. This is consistent with the noncatabolic nature of lamellar bodies, endoplasmic reticulum, and mitochondria.

In summary, lysosomes relatively free of other contaminating organelles were recovered from freshly isolated type II cells. Fractionation of freshly isolated type II cells is another technique for extending in vivo investigations of surfactant metabolic processes to the cellular level without using cultured cells that can interfere with surfactant metabolic studies. Type II cells is another technique for extending in vivo investigations of surfactant metabolic processes to the cellular level without using cultured cells that can interfere with surfactant metabolic studies.

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