Inhibition of Cellular Processing of Surfactant Protein C by Drugs Affecting Intracellular pH Gradients*

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Surfactant protein C (SP-C) is a hydrophobic protein synthesized and secreted exclusively by alveolar type II cells through proteolysis of a 21-kDa proprotein (SP-C21) to produce the 3.7-kDa surface active form. Previous studies from this laboratory have demonstrated that early processing of proSP-C involves extensive intracellular proteolysis of the COOH terminus of proSP-C21 in subcellular compartments, which include the acidic type II cell-specific subcellular organelle, the lamellar body. (Beers, M. F., Kim, C. Y., Dodia, C., and Fisher, A. B. (1994) J. Biol. Chem. 269, 20318–20328). The role of intracellular pH gradients in SP-C processing was studied in freshly isolated rat type II cells. Using vital fluorescence microscopy, the pH indicator acridine orange (AO) identified intense fluorescence staining of acidic cytoplasmic vesicles within fresh type II cells. The AO vesicular staining pattern was similar in cells labeled with the lamellar body marker phosphine 3R and the phospholipid dye Nile red. AO fluorescence was quenched by the addition of a membrane-permeable weak base, methylamine. Immunoprecipitation of cell lysates with anti-proSP-C antisera following pulse-chase labeling (0–2 h) with 35S-Translabel demonstrated rapid synthesis of 35S-proSP-C21, with a time-dependent appearance of 16- and 6-kDa intermediates (SP-C16 and SP-C6). Tricine polyacrylamide gel electrophoresis analysis of organic extracts of cell lysates showed time-dependent appearance of mature SP-C3.7. The addition of 5 mM methylamine significantly blocked the post-translational processing of proSP-C resulting in disruption of normal precursor-product relationships and inhibition of SP-C3.7 formation. Methylamine-treated cells exhibited slow accumulation of SP-C16 and SP-C6, a persistence of SP-C21, and an absence of SP-C3.7 for the duration of the chase period. The lysosomotropic agent chloroquine, the proton ionophore monensin, and bafilomycin A1, a specific vacuolar H+-ATPase inhibitor, each caused inhibition of proSP-C processing in a similar manner. These results demonstrate that normal post-translational proteolysis of proSP-C occurs in acidic intracellular compartments, which include the lamellar body, and that complete processing to SP-C3.7 is dependent upon maintenance of transmembrane pH gradients by a vacuolar H+-ATPase.

Pulmonary surfactant is a heterogeneous surface active complex composed of approximately 80% phospholipid, 10% other lipids, and 10% proteins synthesized and secreted exclusively by the alveolar type II cell (Beers and Fisher, 1992; Hawgood, 1989). The surface tension lowering properties of surfactant are primarily due to the phospholipid components (mainly dipalmitoylphosphatidylcholine); however, enhancement of the biophysical properties of these lipids has been attributed to the presence of specific surfactant-associated proteins, SP-A, SP-B, and SP-C (Hawgood, 1989; Mathalgian and Possmayer, 1990; Weaver and Whitsett, 1991). Significant evidence exists to support the notion that the type II cell plays a central role in the metabolism of all surfactant components participating in synthesis, secretion, and clearance of both lipids and proteins (Baritussio et al., 1992; Breslin and Weaver, 1992; Weaver and Whitsett, 1991; Young et al., 1989).

Morphologically, type II cells are readily distinguished from other lung cell types using transmission electron microscopy to show the presence of characteristic osmiophilic, concentric lamellar organelles 1–2 μm in diameter located in the cytoplasm (Phizackerley et al., 1979). At the light microscopy level, these lamellar bodies demonstrate intense fluorescence when isolated type II cells or lung tissue slices are incubated with phosphine 3R (Mason et al., 1977).

Lamellar bodies can be recovered intact from whole lung as well as from isolated type II pneumocytes and contain all of the surfactant phospholipids as well as SP-A, SP-B, and SP-C (Chander, 1989; Oosterlaken-Dijkstra et al., 1991; Phizackerley et al., 1979). Using ultrastructural morphology, immunocytochemistry, autoradiography, and biochemical methodologies, it has been demonstrated that surfactant protein and lipid components are stored in lamellar bodies prior to release into the alveolar space (Oosterlaken-Dijkstra et al., 1991; Phelps and Flores, 1991; Voorhout et al., 1993; Weaver and Whitsett, 1989). Conversely, by the same methods, alveolar surfactant components have been shown to be endocytosed and routed back to lamellar bodies (Baritussio et al., 1992; Breslin and Weaver, 1992; Young et al., 1989). Isolated lamellar bodies also contain lysosomal acid hydrolases, and using immunological techniques, the lysosomal glycoprotein marker CD63 has been detected on the limiting membrane (Voorhout et al., 1993).

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†The abbreviations used are: SP-A, pulmonary surfactant protein A (26–36 kDa); SP-B, pulmonary surfactant protein B (9 kDa); SP-C, pulmonary surfactant protein C (3.7 kDa); Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; v-ATPase, vacuolar H+-ATPase; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; AO, acridine orange; BFA, brefeldin A.
et al., 1993). Collectively, these data show that the lamellar body functions both as a classic secretory granule and also interacts with the endocytic pathways of the central vacuolar system.

Organic extracts of surfactant contain the two smaller lipophilic proteins, SP-B and SP-C, which alone or in combination are sufficient to confer properties of rapid surface adsorption and surface tension lowering to mixtures of synthetic phospholipids (Mathalilgian and Possmayer, 1990). SP-C is a 33–35-amino acid extremely hydrophobic peptide migrating with a M$_r$ of 3700 under reducing conditions. Alveolar SP-C ("mature" SP-C$_{21}$) is generated by synthesis of a larger primary translation product, proSP-C$_{21}$ (M$_r$ = 21,000), post-translational addition of covalent palmitic acid residues, and intracellular proteolysis involving cleavage of NH$_2$- and COOH-terminal flanking domains of the precursor molecule to yield the secreted surface active form (Beers and Fisher, 1992; Beers et al., 1992, 1994a; Beers and Lomax, 1995; Fisher et al., 1989; Glasser et al., 1988; Vorbroke et al., 1992, 1995b). Previously, this laboratory had reported the production and characterization of several epitope-specific polyclonal antibodies directed against rat proSP-C using synthetic peptides corresponding to spatially distinct regions of the proSP-C primary sequence (Beers et al., 1992, 1994a; Beers and Lomax, 1995). In two different experimental models labeled with $^{35}$S-methionine, immunoprecipitations of both homogenates from a perfused rat lung preparation and lysates from freshly isolated type II cells with these antisera identified the $^{35}$S-labeled 21-kDa SP-C$_{21}$ primary translation product (Beers et al., 1994a; Beers and Lomax, 1995). Pulse-chase experiments demonstrated processing of proSP-C$_{21}$ through 16- and 6-kDa intermediate forms (proSP-C$_{16}$ and proSP-C$_{6}$). In both experimental systems, proteolysis of proSP-C$_{21}$ was blocked by brefeldin A, demonstrating that intracellular processing of proSP-C in the exocytic pathway was occurring in subcellular compartments located distal to the trans-Golgi network. Western blotting of lamellar bodies isolated from adult rat lungs showed that proSP-C$_{6}$ was enriched in this subcellular fraction, and immunoprecipitation analysis of $^{35}$S-labeled lamellar bodies showed time-dependent appearance of SP-C$_{6}$ and production of mature SP-C$_{3.7}$ within this compartment (Beers et al., 1992, 1994a; Beers and Lomax, 1995).

In other cell systems, it is well recognized that there is a progressive decrease in pH along the exocytic pathway, whereas some organelar components of the vacuolar systems (both endo- and endocytic) contain a membrane proton pump (H$^+$/ATPase) that is responsible for generation and maintenance of the internal acidic environment (Beers et al., 1982; Mellman et al., 1986). The pH of most secretory organelles (including chromaffin granules, mast cell granules, and islet cell granules) as well as endocytic vesicles and lysosomes has been measured in the range of 4.5–6.5 and can be neutralized with the use of basic amines and proton ionophores (Beers et al., 1992; Mellman et al., 1986). Chander et al. (1986) have used $^{14}$Cmethylamine accumulation by isolated lamellar bodies to estimate that the pH of the lamellar body matrix is 5.6–6.1. Additionally, the transmembrane pH gradient ($\Delta$H) was dependent upon external energy (ATP), and both lamellar body acidification and ATPase activity in isolated limiting membranes was inhibited by N-ethylmaleimide and dicyclohexylcarbodiimide, indicative of the presence of a vacuolar H$^+$/ATPase (v-ATPase) (Chander, 1992).

Although the role of acidification of the central vacuolar system is not well understood, recent experiments in a variety of cell types have used novel, cell-permeable nontoxic specific inhibitors of the v-ATPase (bafilomycin A$_1$ and concanamycin A) to show that $\Delta$H can play a role in the regulation of sorting, transport, and degradation of proteins as well as in modulation of ligand-receptor interactions and cell effector functions such as cytolytic killing (Bidani and Henning, 1995; Bowman et al., 1988; Mellman et al., 1986; Ori et al., 1986).

In the type II cell, there have been no studies to date defining the role of the $\Delta$H in metabolism of surfactant components. Based on the emerging understanding of the SP-C biosynthetic pathway, we hypothesized that normal acidification of the secretory pathway including the lamellar body is crucial for proper proteolytic processing of proSP-C. In this study, we utilized an epitope-specific proSP-C antiserum (Beers et al., 1994a) and a well characterized, metabolically labeled type II cell system (Beers and Lomax, 1995) to examine the role of organelar acidification in the processing of SP-C. Using both lipophilic basic amines and carboxylic ionophores, the disruption of transorganelar pH gradients resulted in a blockade of proSP-C processing that could be mimicked with bafilomycin A$_1$, a specific inhibitor of v-ATPases. Collapse of the intracellular pH gradient was also associated with complete blockade of SP-C$_{3.7}$ production. Together, the data show that proteolytic processing of SP-C proroprotein involves multiple proteolytic cleavages occurring in acidic subcellular compartments dependent upon vacuolar ATPase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**

Methylamine, acridine orange (3,6-bis(dimethylamino)acridine), monensin, chloroquine, and bafilomycin A$_1$ were obtained from Sigma. Trans-$^{35}$S-label (70% L-Met 15% L-Cys; 1100 mCi/ml as Met) was purchased from ICN/Flow, Inc. (Irvine, CA). Phosphine 3R was obtained from Pfaltz and Bauer, Inc. Nile red was purchased from Molecular Probes, Inc., (Eugene, OR). Protein A-agarose and $^{14}$C molecular weight markers were obtained from Bethesda Research Labs (Gaithersburg, MD). Electrophoretic reagents were purchased from Bio-Rad.

**Anti-NPROSP-C Antiserum**

For these studies, we utilized an epitope-specific rat proSP-C antisem, anti-NPROSP-C, previously produced in rabbits using a synthetic peptide antigen (Beers et al., 1994a). Anti-NPROSP-C recognizes a spatially distinct region on the linearized proSP-C molecule near the amino terminus (Met$^2$–Glu$^3$). This domain forms the adjoining flavin of the nule. The primary structure of proSP-C has been previously been shown to recognize all major synthetic precursors, and proSP-C intermediates but does not recognize mature SP-C and does not cross-react with SP-A, SP-B, proSP-B, or rat serum proteins.

**Isolation of Type II Cells**

Type II pneumocytes were isolated using elastase digestion of lungs from adult Sprague-Dawley rats by the method of Dobbs et al. (1986). The preparation obtained after panning on IgG-coated plates (i.e. fresh type II cells) contained greater than 80% type II cells.

**$^{35}$S Metabolic Labeling**

For metabolic labeling studies, we utilized freshly isolated cells maintained in suspension as previously published (Beers and Lomax, 1995). Cells harvested from the IgG plates (3–5 $\times$ 10$^6$ cells/ml) were resuspended in serum-free, methionine-free Dulbecco’s modified Eagle’s medium (DMEM-Met), aliquoted into 15-ml conical centrifuge tubes, gassed with 5% CO$_2$/95% air, capped, and equilibrated in a temperature controlled shaker bath at 37 °C for 1 h prior to the addition of radiolabel.

For the duration of the metabolic labeling (pulse-chase) studies, the isolated type II cells were maintained in suspension culture by continuous shaking as previously published (Beers and Lomax, 1995). At the start of the equilibration/starvation period in DMEM-Met, inhibitors or control vehicle were added as indicated. Following substrate depletion (1h), suspended cells were metabolically labeled by the addition of 100 $\mu$Ci/ml Trans-$^{35}$S-label for a 30-min pulse-labelling period. Labeled cells were pelleted by centrifugation at 130 x g for 10 min and immediately resuspended in prewashed mammalian replete medium (time = 0) for varying lengths of chase time.

Harvesting of cells and media was achieved by centrifugation at 130
containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 6 mM EDTA, 2% absolute ethanol (1 mM) was subsequently diluted directly into fresh methylamine. At the completion of the incubation period, 5 g of cells were identified using two fluorescent dyes. Using the method of Mason et al. (1977), type II cells suspended in DMEM were incubated with phosphine 3R (1 μg/ml) and viewed through a fluorescence microscope (λex = 488 nm and λem = 520 nm). Alternatively, some preparations were stained with the hydrophobic lipid dye, nile red (Brown et al., 1992). A stock solution prepared in absolute ethanol (1 mM) was subsequently diluted directly into fresh type II cell suspensions to a final concentration of 1 μM. Viewed through fluorescence microscopy (λex = 563 nm and λem = 625 nm, long pass), nile red fluorescence is quenched, but upon interaction with cellular phospholipid inclusions, orange-red fluorescent bodies appear (Brown et al., 1992). Cells labeled with either dye were each photographed in black and white using TMAX ASA3200 black and white film using 2-s exposures.

RESULTS

Vital Fluorescence Microscopy: Vesicular Acidification in Intact Type II Cells—Freshly isolated type II cells showed characteristic staining of their lamellar bodies with phosphine 3R. Suspensions of cells harvested by panning of the IgG-coated culture plates consistently contained greater than 80% type II cells, which exhibited bright yellow fluorescent cytoplasmic phospholipid inclusions (Fig. 1A, arrow). In contrast, with this compound, macrophages showed a faint, diffuse green fluorescence pattern (Fig. 1A, arrowhead).

The neutral lipid dye, nile red, has also been shown to be useful in identifying phospholipid containing vesicles from a variety of nonpulmonary tissues (Brown et al., 1992). With an aliquot of the same type II cell preparation, nile red staining was performed. When viewed in the fluorescence microscope at 563 nm excitation and 625 nm emission, orange-red inclusions (arrows) of similar size and location as the phosphine 3R vesicles were observed exclusively within type II cells and indicated the presence of phospholipid-rich cytoplasmic vesicles in the freshly isolated cell population (Fig. 1B). Using in situ techniques, quantitative measurement of the accumulation of 14C-radiolabeled methylamine has previously shown that isolated lamellar bodies maintain a transmem-

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brane pH gradient (acidic interior) (Chander et al., 1986). Freshly isolated type II cells were incubated with the pH-sensitive dye acridine orange (AO) and examined by fluorescence microscopy. As shown in Fig. 2A, type II cells were found to contain AO fluorescent cytoplasmic vesicles (arrow). The appearance and distribution of AO vesicles in the cytoplasm was similar to that of the phosphine 3R/nile red positive vesicles. No AO-stained vesicles were observed in alveolar macrophages. When type II cells were preincubated for 30 min with 5 mM methylamine, the acridine orange fluorescence was quenched consistent with a collapse of the organelar ΔpH-mediated by the accumulation of the weak base (Fig. 2B).

Taken together, fluorescence microscopy using specialized stains indicated that preparations of freshly isolated type II cells used in these studies contained a phospholipid-rich compartment that maintained a transmembrane pH gradient (acidic interior) and that could be collapsed by incubation with methylamine. The data are consistent with the identification of at least a significant portion of these vesicles as lamellar bodies.

ProSP-C Processing by Type II Cells: Effect of Intracellular pH Gradients—The effect of intracellular pH gradients on synthesis and early processing events for proSP-C was studied using freshly isolated type II cells in suspension culture labeled with [35S]methionine in a pulse-chase protocol. Autoradiographs of immunoprecipitates of control cell lysates with an epitope specific proSP-C antiserum previously shown to recognize all major proSP-C forms (anti-NPROSP-C) (Beers et al., 1992) demonstrated the appearance of the SP-C primary translation product (21-kDa band) with processing of proSP-C_{21} to SP-C_{16} (arrowheads) and proSP-C_{6–10} (bracketed intermediates). 3C Low molecular mass markers (lane MW; M_{r} given in kDa at left) and control immunoprecipitation of a 1-h sample using nonimmune serum (lane NIS) are also shown. Both methylamine (B) and monensin (C) inhibit proteolysis of SP-C_{21} and disrupt the normal progression of appearance of proSP-C intermediates.

When type II cells were preincubated with 5 mM methylamine for 30 min prior to pulse labeling, normal intracellular processing of proSP-C was markedly altered. Immunoprecipitation of lysates from methylamine-treated cells showed a persistent appearance of the SP-C_{21} primary translation product and a delayed accumulation of the SP-C 16-kDa and SP-C 6-kDa intermediates (Fig. 3B). The SP-C_{6–10} forms in methylamine-treated cells were only clearly detectable after 2 h of the chase using prolonged exposures of the film (not shown). Analysis of
the medium of methylamine-treated cells by immunoprecipitation failed to detect the presence of proSP-C forms (not shown), indicating that neutralization of organellar pH did not induce constitutive secretion of proSP-C precursors.

In addition to methylamine, carboxylic ionophores are another class of "acidotropic" agents that have been used to elevate vacular pH (Bidani and Heming, 1995; Mellman et al., 1986). Monensin is a cell-permeable ionophore shown to mediate the exchange of monovalent cations for protons (i.e., cytoplasmic K⁺ for organellar H⁺). Monensin was tested for its effects on SP-C processing by preincubation with type II cells for 30 min prior to metabolic labeling. At a concentration of 2 μM, monensin treatment markedly attenuated the proteolysis of SP-C21 and SP-C16 (Fig. 3C).

The effect of methylamine and monensin on proSP-C processing was quantitated using direct β-counting of the major bands. The net counts in the 35S-labeled SP-C21, SP-C16, and SP-C6 forms appearing in the gels from the immunoprecipitated cell lysates were counted and then normalized for the number of methionine residues in each form as previously published (Beers and Lomax, 1995). Correction for interexperimental variability in absolute counts in the bands was done by expressing the sum of the total counts immunoprecipitated in the 3 major SP-C forms at the conclusion of the 30-min pulse (time 0) for each experiment as 100%. At subsequent time points within the chase, counts in each individual proSP-C band were then expressed as a percentage of the initial post-pulse total. The normalized data for each time point from the individual experiments were combined and expressed as the means ± S.E.

As Fig. 4 demonstrates, at the start of the chase period (time = 0 h), almost 95% of the total counts found in immunoprecipitated proSP-C bands were in SP-C21 in both control and treated cells. During the chase (0–2 h), control lysates showed rapid disappearance of 35S-SP-C21 with a commensurate appearance of SP-C16 and SP-C6 forms and then subsequent disappearance of the SP-C6 band consistent with conversion of proSP-C to the immunologically undetectable mature SP-C3.7 (Fig. 4A). Under these conditions, only about 20% of the initial post-pulse amount of proSP-C21 remained after 1 h, which was consistent with significant proteolytic processing. In contrast, administration of methylamine significantly blunted the proteolytic processing of SP-C21. As Fig. 4B shows, during the chase, methylamine-treated cells retained large amounts (90–95% of post-pulse levels) of proSP-C21 and exhibited a delayed but significant accumulation of SP-C16 and SP-C6.

The effect of monensin on the inhibition of initial proteolysis of the SP-C primary translation product was somewhat intermediate (Fig. 4C) with the amount of 35S-proSP-C21 recovered after 1 h of the chase period approaching 70% that of the starting material. However, as for methylamine-treated cells, proSP-C16 and proSP-C6 forms accumulated in the monensin lysates at later time points, and the normal precursor-product kinetic relationships appeared to be significantly altered.

The effect of these acidotropic agents on proSP-C processing was not due to a generalized effect on proSP-C21 translation and synthesis. At the end of the 30-min pulse-labeling period, the net raw counts incorporated into the 35S-SP-C21 primary translation product band were unchanged by the addition of methylamine as compared with matched controls (methylamine = 102 ± 3% of net counts in control; p = 0.59; n = 4). Likewise, monensin administration did not alter the initial rate of proSP-C synthesis (post-pulse monensin = 90.6 ± 7.2% net counts versus control; p = 0.32, n = 3). In addition, there was no difference in the relative amount of proSP-C21 (as a percentage of total proSP-C forms) at the start of the chase period for methylamine-treated, monensin-treated, or control preparations (94.5 ± 1.2, 95.0 ± 1.0, and 93.9 ± 0.8%, respectively).

Inhibition of SP-C3.7 Production by Methylamine—Although the kinetic profiles in Fig. 4 demonstrate precursor-product relationships between proSP-C21 and other proSP-C intermediates, full processing to SP-C3.7 cannot be directly shown using immunological methods. In order to demonstrate SP-C3.7 production by this experimental system and to determine if
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In a previous study, we had shown that Tricine PAGE of lamellar body proteins could precisely resolve peptides of 3.7 kDa (Beers et al., 1994b). By Western blot, the 7–8-kDa band was identified as SP-C, and the 6-kDa band was identified as SP-B, and the 4-kDa band was identified as SP-A (Beers et al., 1994a). By Coomassie Blue staining, the 4-kDa band co-migrates with a synthetic SP-C3.7 peptide standard, and using lamellar body proteins could precisely resolve peptides of 3.7 kDa. By Western blot, the 7–8-kDa band was identified as SP-C, and the 6-kDa band was identified as SP-B, and the 4-kDa band was identified as SP-A.

To exclude these factors and to more specifically define the effect of organellar pH on SP-C processing, experiments were carried out using a cell-permeable inhibitor of the v-ATPase, the H⁺-ATPase previously shown to be responsible for acidification of secretory organelles. Freshly isolated type II cells were preincubated for 1 h with 0.2 mM bafilomycin A1 and analyzed for proSP-C synthesis and processing. As Fig. 7A demonstrates, bafilomycin A1 produced a processing profile for proSP-C that was similar to that seen using methylamine, monensin, and chloroquine. On quantitation of individual intermediates (Fig. 7B), there was marked attenuation of proSP-C21 proteolysis and a delayed appearance and accumulation of proSP-C16 and SP-C6. Preincubation with vehicle alone (0.1% v/v Me2SO) had no effect of proSP-C processing profiles (data not shown).

DISCUSSION

In the present work, synthesis and post-translational processing of the SP-C primary translation product was studied in isolated type II cells using pulse-chase labeling. Previously, we had utilized epitope-specific antisera and the macrolide antibiotic brefeldin A (BFA) in both a perfused lung preparation and isolated type II cells to characterize the initial post-translational proteolytic processing events for proSP-C3.7. These steps include cleavage of COOH-terminal propeptide domains in subcellular compartments distal to the trans-Golgi network followed by subsequent proteolysis of a vestigial NH2-terminal flanking domain in a particulate lamellar body fraction prior to secretion of SP-C3.7 into the alveolar space (Beers et al., 1994a; Beers and Lomax, 1995). The present study extends these observations and demonstrates the qualitative kinetics of SP-C3.7 production. To correct for interexperimental variability, the net counts measured in the 4-kDa band at each time point in both control and methylamine-treated lysates were normalized by setting the SP-C3.7 counts in the 1 h control as 100%. Under control conditions, a small amount of SP-C3.7 appeared as early as 30 min, but a significant increase in band intensity occurred at 1–2 h following the pulse. When compared with Figs. 3 and 4, SP-C3.7 production was preceded by the synthesis of SP-C21 as well as the appearance and disappearance of proSP-C6, consistent with a precursor-product relationship for proSP-C6-mature SP-C3.7. In the presence of methylamine, commensurate with disruption of proSP-C processing and accumulation of intermediate forms, the production of SP-C3.7 is completely blocked (<5% versus control at 1 h).

Chloroquine Also Inhibits proSP-C Metabolism—Chloroquine, a well known lysosomotropic agent, is a tertiary amine shown to accumulate in acidic organelles (Orci et al., 1986). As illustrated by the autoradiograph in Fig. 6A, chloroquine also caused inhibition of proSP-C processing. Pretreatment of type II cells with 10 μM chloroquine resulted in disruption of the normal proteolysis of proSP-C21 with an accumulation of significant amounts of SP-C16 intermediate and a lesser accumulation of the lower molecular weight forms, which were detected on prolonged exposure of the blots to film. Processing kinetics from quantitation of individual bands from several experiments performed in the presence of chloroquine are shown in Fig. 6B. As for the other acidotropic agents, sequential proteolysis of each proSP-C form was blocked with chloroquine pretreatment. The use of chloroquine did not alter the initial rate of proSP-C synthesis (Post-pulse chloroquine = 84.0 ± 17.0% net counts versus control; p = 0.63, n = 3).

Inhibition of Vacuolar ATPase Blocks SP-C Processing—Although the effect of acidotropic agents on intracellular pH gradients is predicted to be most profound on the organelles with the highest degree of acidification (e.g. lamellar bodies, lysosomes, or multivesicular bodies), the additional effects of some of these reagents such as the alteration of the pH in other compartments (i.e., Golgi) (Orci et al., 1986) or the nonspecific inhibition of the proSP-C proteases by these drugs is possible. To exclude these factors and to more specifically define the effect of organellar pH on SP-C processing, experiments were carried out using a cell-permeable inhibitor of the v-ATPase, the H⁺-ATPase previously shown to be responsible for acidification of secretory organelles. Freshly isolated type II cells were preincubated for 1 h with 0.2 μM bafilomycin A1 and analyzed for proSP-C synthesis and processing. As Fig. 7A demonstrates, bafilomycin A1 produced a processing profile for proSP-C that was similar to that seen using methylamine, monensin, and chloroquine. On quantitation of individual intermediates (Fig. 7B), there was marked attenuation of proSP-C21 proteolysis and a delayed appearance and accumulation of proSP-C16 and SP-C6. Preincubation with vehicle alone (0.1% v/v Me2SO) had no effect of proSP-C processing profiles (data not shown).

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observations with new data demonstrating that in this isolated type II cell model, proSP-C is processed completely to SP-C3.7. Because of its poor immunogenicity, detection of SP-C3.7 was exploited to quantify SP-C intermediate production. The modification of intracelluar pH on SP-C processing by acidotropic agents could be mimicked by bafilomycin A1, a cell-permeable specific inhibitor of vacuolar ATPases.

The experiments presented in this study were performed by metabolic labeling of freshly isolated type II cells in suspension in which acidotropic agents were used to disrupt proSP-C processing. This laboratory has previously shown that the use of this model for the dissection of the synthetic pathway for SP-C is physiologically relevant to events occurring in vivo. The proSP-C processing profile (time course and major intermediates) obtained by immunoprecipitation of fresh type II cells (Fig. 3A) was similar to that observed in a previous published analysis of lung homogenates from a 35S-labeled isolated perfused lung (Beers et al., 1994a). Subcellular fractionation of the metabolically labeled perfused lung preparation showed that the initial proteolysis of SP-C21 yielding a proSP-C16 intermediate appears to occur within a vesicular compartment of the secretory pathway at a yet undefined site located between the trans-Golgi network and the lamellar body compartment (Beers et al., 1992, 1994a; Beers and Lomax, 1995). Based on immunoelectron micrographic data from Voorhout et al., the multivesicular body or a noncathrin-coated Golgi budding vesicle appears to be the most likely site for this initial cleavage step (Voorhout et al., 1993). The second step in proSP-C processing, the conversion of SP-C16 to SP-C6, is most likely also occurring in a late multivesicular body. Both Western analysis of subcellular lung fractions (which has shown a marked enrichment in SP-C6 in lamellar bodies) as well as the metabolic labeling profile observed in 35S-labeled lamellar bodies indicate that the lamellar body fraction is the subcellular compartment for the final removal of the N-flanking domain in SP-C6 (Beers et al., 1994a; Beers and Lomax, 1995).

A fundamental assumption of studies using epitope-specific proSP-C antibodies has been that under control conditions, proSP-C21, and the intermediates that are generated in a precursor-product kinetic relationship lead to production of SP-C3.7. Because of its poor immunogenicity, detection of SP-C3.7 has not been possible using conventional immunoprecipitation (Beers and Fisher, 1992). Using an alternative approach, the unique solubility and low molecular weight of SP-C3.7 were exploited to quantify the intracellular production of 35S-labeled SP-C3.7 in organic extracts of type II cell lysates. In a previous report, we had shown the utility of Tricine PAGE to separate 35S-radialabeled bands of 7 (SP-B), 5.5 (proSP-C6), and 4 (mature SP-C) kDa in lamellar body fractions (Beers et al., 1994b). This technique was modified for cell lysates by using the method of Bligh and Dyer (1959) to extract low molecular weight hydrophobic surfactant proteins into organic solvents followed by analysis with Tricine SDS-PAGE. The
kinetic data generated by direct quantitation of the gels (Fig. 5) demonstrate time-dependent appearance of SP-C3.7 during the chase period that follows both the initial proteolysis of proSP-C21 and the appearance/disappearance of SP-C16 and SP-C6. This is consistent with a precursor-product relationship for proSP-C-mature SP-C.

A direct quantitative comparison of the counts in SP-C21 in the organic extracts with those counts in proSP-C forms obtained by immunoprecipitation of the lysates is not possible because of the differences in technique and the uncertainty in the total recovery. The efficiency of immunoprecipitation and the partition co-efficient for mature SP-C in the organic phase of the Bligh-Dyer extract make the calculation of the exact percentage of full conversion of SP-C21 impossible. However, although stoichiometric quantitation is not possible, based on two independent investigations, it is unlikely that much of the synthesized SP-C21 is degraded. Previously, we used brefeldin A to block anterograde transport of proSP-C from the endoplasmic reticulum-Golgi compartment of isolated type II cells (Beers and Lomax, 1995). In pulse-chase analysis using BFA, we saw no significant degradation of proSP-C21 in immunoprecipitates of these cell lysates up to 4 h after labeling, indicating that proSP-C21 retained in the endoplasmic reticulum in the BFA-treated state is not metabolized. Recently, work by Vorbroker et al. (1995b) has confirmed this finding using type II cells cooled to 20°C to mimic the BFA phenotype. Cells treated in this fashion also do not degrade proSP-C21 (Vorbroker et al., 1995b). These results differ from those obtained for secretion of apolipoprotein B in HepG2 cells in which significant amounts of apo B are degraded in the endoplasmic reticulum rather than processed to mature lipoprotein (Dixon et al., 1991). Taken in toto, the BFA results, the documentation of SP-C3.7 in cell lysates, and the precursor kinetics suggest that a degradative path in the exocytic pathway is minor.

Incubation of type II cells with acidotropic agents results in disruption of the normal processing sequence for proSP-C. Methylamine (Fig. 3B) altered the observed normal precursor-product relationship between proSP-C21 and proSP-C16 as well as the kinetics of conversion of SP-C16 to SP-C6 and proteolysis of SP-C6 (Fig. 4). Commensurate with the accumulation of intermediates, production of SP-C3.7, indicative of the final anterograde processing of SP-C6, was also completely blocked by methylamine (Fig. 5). Based on these results, it appears that the disruption of the normal precursor-product curves (control) leading to the accumulation of SP-C16/SP-C6 (methylamine) is associated with a total blockade of SP-C3.7 production. In addition to methylamine, other classes of acidotropic agents including chloroquine (Fig. 6) and the ionophore monensin (Fig. 3C) as well as bafilomycin A1, a selective inhibitor of H+ ATPases, all effectively disrupted much of the proteolytic processing of proSP-C21 and resulted in the characteristic accumulation of SP-C16 and varying amounts of SP-C6. Therefore, it is likely that all of these agents completely block production of SP-C3.7.

Concurrent with the observed alterations in metabolic processing of SP-C, fluorescence microscopy indicated that acidotropic agents also affect the normal acidification of type II cell subcellular organelles. Staining of freshly isolated type II cells with acridine orange demonstrated the presence of acidic subcellular vesicles (Fig. 2A). Methylamine administration resulted in total quenching of the fluorescent signal indicative of complete collapse of all intracellular transmembrane pH gradients by the membrane-permeable basic amine (Fig. 2B). The distribution and location of the acidic type II cell vesicles visualized with acridine orange were similar to yellow-gold cytoplasmic fluorescent vesicles identified with phophine 3R (Fig. 1A) and orange-red vesicles stained with nile red (Fig. 1B). Phosphine-stained vesicles were shown by Mason et al., 1977 to correspond to lamellar bodies in electron micrographs of both isolated type II cells and intact lung, whereas nile red has been used to demonstrate intracellular phospholipid vesicles in other cells (Bidani and Heming, 1995; Greenspan et al., 1985).

Although the results obtained using fluorescent dyes are consistent with acridine orange staining of lamellar bodies, co-localization of the dyes within the same cells using double labeling would yield more definitive characterization of vesicular staining. However, the physical properties of nile red and acridine orange preclude their use with selective filter packages for such studies. Nile red is actually a dual purpose lipid dye. In the cytoplasm, nile red exhibits both yellow-gold fluorescence (λm = 550–580 nm), indicative of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescence (λm = 515–565 nm; λs = 528 nm) indicating of neutral lipids as well as the orange-red fluorescenc...
The precise subcellular localization of the proton ATPase is not known but has been functionally demonstrated on lamellar body limiting membranes (Chander et al., 1986). Brefeldin A has previously been shown to completely block proSP-C processing through inhibition of Golgi-budded vesicle formation (Beers and Lomax, 1995).

Section 3: ProSP-C Processing in Alveolar Type II Cells

Secretory granules in the neurohyphophysis and pancreas each utilize an acidic intragranular pH that provides optimal conditions for the proteolytic processing enzymes that cleave prohormones into mature species (Loh et al., 1984; Ori et al., 1986). Chloroquine has previously been shown to directly inhibit cathepsin B activity in rat fibroblasts (Wibo and Poole, 1974). At present, none of the proteases that process SP-C are currently defined, nor are the pH profiles for these events known; however, the potential roles for pH in the processing of another hydrophobic surfactant protein SP-B has been partially elucidated. Processing of the 42-kDa SP-B primary translation product has been shown to mediate by a cathepsin D-like protease, which has a pH optimum of 5.0. At pH 7.0, enzyme activity is inhibited over 85% (Weaver et al., 1992). Further studies characterizing specific SP-C proteases, their enzymatic properties, and rates of proSP-C intermediate packaging will be required.

On the basis of the data presented, disruption of SP-C processing at the level of the conversion of SP-C11 to mature SP-C by acidotropic agents could account for the absence of mature SP-C and accumulation of SP-C5 seen with administration of all the drugs tested. However, the accumulation of SP-C11 consistently induced by each reagent as well as variations in the relative amount of proSP-C (e.g., methylamine and monensin versus chloroquine) and in levels of SP-C seen in the cell lysates treated with different classes of reagents also indicates that these compounds may be having effects in addition to changing vacuolar pH (Figs. 3, 4, and 5). Both lipophilic amines as well as carboxylic ionophores have also been shown to have other secondary cellular effects including alterations in targeting of proteins in the secretory pathway, changes in vesicular membrane trafficking, and induction of vacuolar and Golgi swelling (Mellman et al., 1986; Radons et al., 1994). We have previously shown that chromaffin granules utilize transmembrane pH gradients to regulate the uptake and storage of biogenic amines (Beers et al., 1982). Additionally, the use of methylamine and chloroquine was consistently associated with a small increase in total label incorporation in these samples during the chase, which was somewhat higher than at the end of the pulse, and raises the possibility that these agents could also affect the efficiency of the chase.

To exclude some of these nonspecific effects, additional experiments were done using an inhibitor of the vacuolar H^+ ATPase. It has previously been shown that the lamellar body limiting membrane is markedly enriched in H^+ ATPase activity, which was inhibited by known blockers of vacuolar ATPases and distinguishable from other H^+ ATPases (F_{0} F_{1}, E_{1} E_{2}, and P type) (Chander, 1992). Bafilomycin A, a cell-permeable, specific v-ATPase inhibitor disrupted the processing profiles resulting in a secondary accumulation of SP-C16 and SP-C12 in the cell lysates (Fig. 7). This result suggests that the observed impairments in proSP-C processing are not due solely to a direct inhibition of protease activity by reagents such as chloroquine and methylamine. Because azide insensitive H^+ ATPase activity has been found in clathrin-coated vesicles isolated from the brain (Xiao-Song and Stone, 1986), the role of pH in premembrane cell compartments will require the localization of v-ATPase in type II cells and intracellular pH measurements using ultrastructural techniques.

Recently, additional evidence for the importance of proper intracellular targeting and proteolysis in the biosynthesis and post-translational processing of SP-C has been underscored by the phenotypic characterization of congenital SP-B deficiency resulting from mutational disruption of the SP-B gene (Nogee et al., 1993). In both the J121insn2 (d’Mello et al., 1994) and R236C mutations (Ballard et al., 1995), the SP-B-deficient phenotype is associated with abnormal post-translational processing of SP-C manifested as an abnormal accumulation of a 6–12-kDa proSP-C intermediate with a concomitant lack of another hydrophobic surfactant protein SP-B. Further studies characterizing specific SP-C proteases, their enzymatic properties, and rates of proSP-C intermediate packaging will be required.

In conclusion, synthetic processing of SP-C studied in metabolically labeled adult alveolar type II cells using epitope-specific proSP-C antisera has demonstrated extensive proteolytic remodeling of the SP-C primary translation product leading to production of mature SP-C3,7. The normal post-translational cleavage pattern of the propeptide-flanking regions, which can be blocked by basic amines and ionophores, leads to inhibition of SP-C3,7 formation and accumulation of metabolic intermediates. Together these results indicate that complete processing is dependent, in part, upon maintenance of intracellular transmembrane pH gradients in the exocytic pathway by a vacuolar proton ATPase in vesicular compartments, which include the lamellar body.

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