Methylamine decreases trafficking and packaging of newly synthesized phosphatidylcholine in lamellar bodies in alveolar type II cells

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Lung lamellar bodies, the storage organelles for lung surfactant phosphatidylcholine (PC), maintain an acidic pH that can be increased with weak bases. This study investigates the effect of a weak base, methylamine, on the pH in lamellar bodies and on the trafficking and packaging of newly synthesized PC in lamellar bodies. Methylamine increased the pH of isolated lung lamellar bodies and of lamellar bodies in intact cells. Metabolic labelling of isolated type II cells with [*methyl*-³H]choline showed that although methylamine (2.5–10 mM) did not alter the labelling of cellular or microsomal PC and disaturated PC, it decreased the labelling of the PC and disaturated PC in lamellar bodies. The packaging of PC in lamellar bodies (the specific activities ratio between the PC in lamellar bodies and the

microsomal PC) also decreased in a time- and concentration-dependent manner. The cellular synthesis of PC or its packaging into lamellar bodies was unaltered by brefeldin A, suggesting that the Golgi was not involved in PC packaging. Although methylamine also increased surfactant secretion, the inhibition of PC packaging in lamellar bodies seems unrelated to the secretagogue effect, (1) on the basis of metabolic consequences of increased secretion and (2) because ATP, another secretagogue, did not inhibit PC packaging. Methylamine seems to inhibit PC packaging by inhibiting trafficking of PC to lipid-rich light subcellular fractions. Together our results suggest that the trafficking of surfactant PC into lamellar bodies might be sensitive to changes in the pH of lamellar bodies.

INTRODUCTION

Lung surfactant, a phospholipid-rich lipoprotein-like substance, lines the alveolar epithelium and lowers surface tension at the air–liquid interface during end-expiration (reviewed in [1]). Phosphatidylcholine (PC), the major phospholipid in lung surfactant, is synthesized and secreted by lung epithelial type II cells (reviewed in [2,3]). Pulse-labelling kinetics in lung tissue *in vivo* indicate that maximum labelling of PC is first achieved in the microsomal fraction, then in the fraction containing lamellar bodies, and subsequently in the alveolar surfactant [4]. Thus the newly synthesized PC is transported from endoplasmic reticulum to alveolar lumen via the lamellar bodies.

Although the synthesis of PC is regulated by metabolic enzymes [2], and the secretion of lung surfactant is regulated by a variety of agents [3], it is not clear whether the transport of PC from its site of synthesis and subsequent packaging in the lamellar bodies is a regulated process. The following observations suggest a possible regulation of surfactant PC packaging. Pulse-chase studies show slower labelling of PC in lamellar bodies relative to microsomal PC [4], and type II cells in culture lose the ability to transport and package surfactant PC in lamellar bodies [5]. We have previously demonstrated that the lamellar bodies can be isolated from both the lung or type II cell homogenates in high purity, as determined by their biochemical and ultrastructural characteristics [6]. These organelles maintain an acidic interior via an ATP-dependent process [7], possibly an ATPase that may be present in the limiting membrane of lamellar bodies [8]. The acidic interior of lamellar bodies can be dissipated with a weak

base, ammonium chloride [7]. The present study examines the effect of methylamine on the packaging of newly synthesized surfactant PC in the lamellar bodies. For this study, packaging is defined as the ratio of specific activity (nmol of choline incorporated/ μ g of lipid P_i) of lamellar body PC to that of the microsomal PC. Because the internal pH of the Golgi is also acidic [9], we examined surfactant packaging in the presence of brefeldin A and monensin. These two agents have previously been used to evaluate the role of the Golgi in processing proteins in various cell types including lung and type II cells [10–13]. Our results suggest that PC packaging in lamellar bodies is selectively modulated by methylamine and that the pH in lamellar bodies may play a role in such modulation. Parts of these studies have been presented in abstract form [14].

MATERIALS AND METHODS

[*methyl*-³H]Choline, [³H]leucine and [¹⁴C]dipalmitoyl PC (DPPC) were obtained from Amersham (Arlington Heights, IL, U.S.A.). Methylamine, brefeldin A, monensin, phorbol 12-myristate 13-acetate (PMA), rat serum IgG, Acridine Orange, quinacrine hydrochloride, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetomethoxy ester (BCECF-AM) and other standard biochemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Pig elastase was obtained from Worthington Biochemicals (Freehold, NJ, U.S.A.). All tissue-culture plastic ware was obtained from Co-Star (Cambridge, MA, U.S.A.). Bacteriological culture dishes (Falcon 1029) were obtained from Beckton and Dickinson (Franklin Lakes, NJ, U.S.A.).

Abbreviations used: BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetomethoxy ester; DPPC, dipalmitoyl PC; DSPC, disaturated PC; MEM, minimum essential medium; PC, phosphatidylcholine; pH_i, intracellular pH; PMA, phorbol 12-myristate 13-acetate; TSE, 10 mM Tris/150 mM NaCl/1 mM EDTA.

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Isolation of type II cells

Alveolar type II cells were isolated from lungs of adult rats (180–220 g body weight) after digestion with elastase [15] as described previously [16]. For overnight (20–22 h) culture, type II cells were plated in 35 or 100 mm tissue-culture plastic dishes in minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum. At the end of this culture period, the cells attached to the plastic showed more than 95% viability, as judged by exclusion of the vital dye Erythrosin B, and approx. 93% of these cells were type II cells, on the basis of the fluorescence of Phosphine 3 R.

Synthesis of PC

Isolated type II cells were cultured for 20–22 h, washed with serum-free MEM and incubated for the indicated periods of time in MEM containing 100 μ M [*methyl*- 3 H]choline (5 mCi/mmol). The indicated concentrations of various agents in less than 0.1% volume were added during the pulse or chase period to some of the culture dishes. Methylamine and ammonium chloride solutions were prepared in 6 mM Hepes or MEM with pH adjusted to 7.4. Brefeldin A and monensin were added from stock solutions in ethanol. At the end of each incubation the cells were washed with MEM, harvested by scraping on ice with a rubber 'police-man', and sedimented by centrifugation at 300 g for 10 min at 4 °C.

Isolation of subcellular fractions

All processing for isolation of subcellular fractions was performed at 4 °C. Cells (approx. 2×10^7) from three to five plates were pooled, suspended in 1 M sucrose and disrupted by sonication (three times for 15 s each) with a probe sonicator set at 10% of maximum output (Sonic Dismembrator 50; Fisher Scientific, Philadelphia, PA, U.S.A.). The cell homogenate (2 ml) was placed under a discontinuous sucrose gradient (0.8–0.2 M) and the fraction rich in lamellar bodies was recovered at the 0.4/0.5 M sucrose interface (average 0.42–0.47 M sucrose) by upward flotation after centrifugation, and the lamellar bodies were isolated as described previously [6]. Lamellar bodies isolated by this protocol contain less than 2% microsomal contamination as determined from the specific activity of NADPH:cytochrome *c* reductase [6,17]. After removal of the band rich in lamellar bodies [6], the remaining gradient was centrifuged for 16 h at 80000 g_{av} . The pellet containing all membranes was suspended by sonication (three times for 15 s each) in 2 ml of 0.32 M sucrose in 10 mM Tris/150 mM NaCl/1 mM EDTA (TSE) buffer, pH 7.4, and the suspension was sequentially centrifuged for 20 min at 7700 g , and then through 0.8 M sucrose in TSE buffer for 3 h at 80000 g_{av} to obtain the microsomal fraction in the pellet [4,17]. The microsomal fraction was enriched 4.8 ± 0.5 -fold (mean $\pm$ S.E.M., $n = 14$) over the cell homogenate in NADPH:cytochrome *c* reductase activity.

Secretion of surfactant PC

Isolated type II cells were labelled with [*methyl*- 3 H]choline (0.5 μ Ci per dish; specific radioactivity 86 Ci/mmol) during the 20–22 h incubation and then studied for basal and stimulated secretion as described previously [16]. Secretagogues in less than 1% of the final volume were added at indicated concentrations from stock solutions for ATP in water, PMA in dimethyl-formamide, and methylamine in 6 mM Hepes buffer, pH 7.4.

Extracellular matrix protein synthesis and secretion

Synthesis and secretion of extracellular matrix protein were studied in 20–22 h cultured type II cells as described previously [18]. Cells were incubated for 6 h in leucine-deficient Dulbecco's MEM containing 80 μ M [3 H]leucine (65–128 d.p.m./pmol) in the absence or presence of 10 μ M brefeldin A. At the end of each incubation, cellular proteins and extracellular matrix proteins were sequentially extracted at 4 °C in 0.25 M ammonium hydroxide containing 1 μ M PMSF and in 1 M NaOH respectively; they were then precipitated with trichloroacetic acid and processed for radioactivity measurement by scintillation counting.

Measurement of intracellular pH

The intracellular pH (pH_i) of type II cells was measured by ratio fluorimetry [19] as described elsewhere (S. Wadsworth, A.-M. Wu, A. Spitzer and A. Chander, unpublished work). Cells were cultured for 20–22 h on glass coverslips, washed and then loaded for a 30 min incubation at 37 °C with 5 μ M BCECF-AM, a pH-sensitive dual-excitation-wavelength probe. The coverslips were washed with fresh MEM, loaded on to a sample holder, and placed in a standard quartz fluorescence cuvette in a temperature-controlled (37 °C) sample chamber in a spectrofluorimeter (Aminco Bowman Series 2; Milton Roy, Rochester, NY, U.S.A.). The fluorescence ratio of BCECF (excitation 440 and 500 nm, emission 530 nm) was monitored as a function of time before and after addition of the indicated concentrations of methylamine. The fluorescence ratio was calibrated by equilibrating BCECF-loaded cells in high- K^+ /2 μ M nigericin buffers (5 mM NaCl, 130 mM KCl, 30 mM Hepes, 1.3 mM $CaCl_2$ and 1.2 mM $MgSO_4$) of various pH values (6.0–8.0).

pH of lamellar bodies

Uptake of fluorescent weak base, quinacrine, by isolated lung lamellar bodies was followed as a measure of acidic pH of lamellar bodies. Isolated lung lamellar bodies [6] were suspended in 0.25 M sucrose in 10 mM Tris/Hepes buffer, pH 7.0, containing 130 mM potassium gluconate (solution A). The baseline fluorescence (excitation 470 nm, emission 530 nm) of 8 μ M quinacrine in solution A and containing 10 mM $MgATP^{2-}$ was established. The suspension of lamellar bodies (approx. 5 μ g of protein) was equilibrated for 10 min at 37 °C with or without various concentrations of methylamine and then added to the ATP-containing quinacrine solution. The decrease in fluorescence (due to sequestration of quinacrine in lamellar bodies) was then measured as a function of time.

Analytical

For secretion studies, the cell-free medium and the cells were extracted for lipid [20] after the addition of egg PC (0.4 mg) as carrier lipids and [14 C]DPPC (800–3000 d.p.m.) as a tracer lipid. The secretion of PC was calculated as described previously [16]. For synthesis studies, PC in the lipid extracts was separated by TLC on boric acid-impregnated silica gel G plates [21]. Disaturated PC (DSPC) in lipid extracts was separated after osmication of lipids and chromatography on neutral alumina columns [22]; recovery from such columns ranged between 85% and 100%, as monitored by elution of [14 C]DPPC (approx. 500 d.p.m.) that was added to each sample. Phospholipid phosphorus was measured by the method of Marinetti [23] as described previously [24]. In pulse-chase studies, radioactivity on TLC plates was quantified with a radioactivity scanner (Automatic TLC Linear Analyzer, Model Tracemaster; Berthold, Wallac,

Gaithersburg, MA, U.S.A.) (efficiency for ^3H 1–2%), and is expressed in c.p.m. For all other experiments, radioactivity was measured by scintillation counting. Results on PC and DSPC synthesis were transformed into nmol of choline on the basis of the specific radioactivity (d.p.m./nmol) of choline in the medium.

Proteins were measured [25] after reaction with protein-binding dye reagent (Bio-Rad Laboratories, Richmond, VA, U.S.A.) with bovine γ -globulin as standard. Lactate dehydrogenase was measured by following the reduction of NAD at 340 nm [26]. NADPH:cytochrome *c* reductase was measured by following the reduction of cytochrome *c* at 550 nm [27].

Data handling and statistics

All secretion experiments were conducted in duplicate and the results averaged to yield single data points. Results are expressed as means $\pm$ S.E.M. for experiments conducted in separate cell preparations as indicated. Results were evaluated for differences by Student's *t*-test for unpaired observations, or by ANOVA followed by Tukey's *post hoc* test for experiments with multiple groups, and were considered significantly different at $P < 0.05$.

RESULTS

The incorporation of choline into cellular, microsomal and lamellar body PC increased with the time of incubation (Figure 1A). The packaging of PC (the ratio of specific radioactivity of lamellar body PC to that of microsomal PC) in lamellar bodies also increased with the time of incubation (Figure 1B). After 3 h the packaging was approx. 10%, suggesting that 10% of microsomal PC was translocated to the lamellar bodies during this period.

Choline incorporation into cellular or microsomal PC was unaffected during 1 h (results not shown) or 3 h incubations with 10 mM methylamine (Table 1). In the presence of 10 mM methylamine, choline incorporation into PC in lamellar bodies was decreased by almost 40% after 1 h of incubation (results not shown), and by 55% after 3 h when compared with the controls (Table 1). In parallel, methylamine also decreased the packaging of PC in lamellar bodies by 65% at 3 h. This slightly accentuated effect on packaging is due to a 10% increase ($P > 0.05$) in the specific radioactivity of microsomal PC. The recoveries of proteins in the fraction containing lamellar bodies was 1.02 ± 0.06 and $0.87 \pm 0.20\%$ ($n = 6$, $P > 0.05$) of cellular proteins, and the phospholipid-to-protein ratio (by weight) was 5.5 ± 0.7 and 6.2 ± 0.6 ($n = 6$, $P > 0.05$) in the absence or presence of 10 mM methylamine respectively.

Next, type II cells were pulsed for 2 h with $100 \mu\text{M}$ [$\text{methyl-}^3\text{H}$]choline, and the label in PC was chased by a 2 h incubation in fresh MEM containing $100 \mu\text{M}$ choline and in the absence or presence of 10 mM methylamine (pulse-chase protocol). In control cells, compared with approx. 6% of microsomal PC being packaged in lamellar bodies during 2 h of pulse-labelling (Figure 1B), almost 15% of microsomal PC was packaged at the end of the chase period (Table 1). This increase in packaging is possibly due to a slow turnover of PC in lamellar bodies, as previously demonstrated during studies *in vivo* [4]. Continued transfer of microsomal PC (the precursor pool with higher specific radioactivity) to the lamellar bodies during the chase period further increased, and not decreased, the specific radioactivity of PC in lamellar bodies. Compared with control cells, such trafficking of microsomal PC to the lamellar bodies was inhibited in methylamine-treated cells.

At low concentrations (1 mM), methylamine did not affect PC labelling in either the cell homogenate or any of the subcellular

Figure 1 Phosphatidylcholine synthesis in 20–22 h cultured type II cells

Isolated type II cells were incubated with $100 \mu\text{M}$ [$\text{methyl-}^3\text{H}$]choline for indicated periods. (A) Specific radioactivity of PC in cells (●), microsomes (◆) and lamellar bodies (◇) as a function of time. (B) Packaging of PC (specific radioactivity ratio of lamellar body PC to microsomal PC) in lamellar bodies as a function of time. Results are means $\pm$ S.E.M. for three experiments.

Table 1 Effect of methylamine on choline incorporation into PC in type II cells

Isolated type II cells were incubated for 3 h with $100 \mu\text{M}$ [$\text{methyl-}^3\text{H}$]choline in the absence or presence of 10 mM methylamine (pulse-labelling protocol) or were pulse-labelled for 2 h with $100 \mu\text{M}$ [$\text{methyl-}^3\text{H}$]choline and washed, after which the label was chased for 2 h with $100 \mu\text{M}$ choline in the absence or presence of 10 mM methylamine (pulse-chase protocol). Packaging is the specific radioactivity of PC in lamellar bodies divided by the specific radioactivity of microsomal PC, expressed as a percentage. Results are means $\pm$ S.E.M. for the number of experiments in parentheses. * $P < 0.05$ compared with the corresponding control.

	Choline incorporation			
	Cells	Microsomes	Lamellar bodies	Packaging
Pulse-labelling protocol (nmol/μg of PC phosphorus)				
Control (7)	3.16 $\pm$ 0.43	7.46 $\pm$ 1.16	0.64 $\pm$ 0.09	9.3 $\pm$ 1.3
Methylamine (7)	4.06 $\pm$ 0.50	8.38 $\pm$ 0.62	0.29 $\pm$ 0.06*	3.3 $\pm$ 0.6*
Pulse-chase protocol (c.p.m./μg of PC phosphorus)				
Control (3)	240 $\pm$ 24	612 $\pm$ 7	92 $\pm$ 2	15.1 $\pm$ 0.5
Methylamine (3)	281 $\pm$ 14	646 $\pm$ 50	56 $\pm$ 2*	8.8 $\pm$ 0.8*

Figure 2 Concentration dependence of the effect of methylamine on labelling of PC and its packaging in lamellar bodies

Cultured type II cells were incubated for 3 h with 100 μ M [*methyl*- 3 H]choline in the absence or presence of the indicated concentrations of methylamine. Symbols: (A) $\blacklozenge$, microsomes; $\bullet$, cells; (B) $\diamond$, lamellar bodies. Results are expressed as the specific radioactivity of PC (A, B) and specific radioactivity ratio (C), and are means $\pm$ S.E.M. for five to seven experiments under each condition. * $P < 0.05$ compared with control, by ANOVA.

Table 2 Effect of methylamine on labelling of DSPC in type II cells

Type II cells were incubated for 3 h with 100 μ M [*methyl*- 3 H]choline in the absence (control) or presence of 10 mM methylamine. Results are means $\pm$ S.E.M. for the numbers of experiments in cell preparations shown in parenthesis. 'Unsaturated' PC was calculated from the differences between the labelling and the mass of total PC and DSPC. * $P < 0.05$ compared with the corresponding controls.

	Choline incorporation (nmol/ μ g of phosphorus)		
	Cells (9)	Lamellar bodies (8)	Microsomes (5)
PC			
Control	2.34 $\pm$ 0.28	0.58 $\pm$ 0.06	5.40 $\pm$ 0.49
Methylamine	2.99 $\pm$ 0.39	0.24 $\pm$ 0.05*	5.61 $\pm$ 0.67
Disaturated PC			
Control	2.59 $\pm$ 0.22	0.68 $\pm$ 0.08	6.19 $\pm$ 0.18
Methylamine	2.71 $\pm$ 0.30	0.38 $\pm$ 0.08*	5.65 $\pm$ 0.17
'Unsaturated' PC			
Control	2.68 $\pm$ 0.27	0.64 $\pm$ 0.06	6.51 $\pm$ 0.36
Methylamine	3.55 $\pm$ 0.48	0.23 $\pm$ 0.05*	7.24 $\pm$ 0.44

fractions. In four separate experiments, choline incorporation into PC in lamellar bodies in control and 1 mM methylamine-treated cells was 0.63 ± 0.07 and 0.52 ± 0.09 nmol/3 h per μ g of PC phosphorus ($P > 0.05$). At higher concentrations, however,

Table 3 Effect of ammonium chloride on PC synthesis in type II cells

Cells were labelled for 3 h with 100 μ M [*methyl*- 3 H]choline in the absence (control) or presence of 5 mM ammonium chloride. Results are means $\pm$ S.E.M. for experiments in four separate preparations. * $P < 0.05$ compared with the control.

Fraction	Choline incorporation (nmol/ μ g of PC phosphorus)	
	Control	Ammonium chloride
Cells	2.58 $\pm$ 0.35	2.14 $\pm$ 0.36
Microsomes	7.77 $\pm$ 0.75	6.43 $\pm$ 0.83
Lamellar bodies	0.63 $\pm$ 0.07	0.39 $\pm$ 0.04*

Figure 3 Quinacrine uptake by isolated lung lamellar bodies

The ATP-dependent uptake of quinacrine (8 μ M) was followed as a measure of acidic pH of lamellar bodies (approx. 5 μ g of protein). The rate and maximum uptake of quinacrine by lamellar bodies were decreased in the presence of methylamine. Results are from a representative of three experiments with separate preparations. Lamellar bodies were added at the indicated time ($\downarrow$). F, fluorescence.

methylamine inhibited the labelling and packaging of PC in lamellar bodies in a concentration-dependent manner (Figure 2). The inhibitory effect on labelling and packaging appears to be maximal (65–70%) at 5 mM.

In another series of experiments we determined the effects of methylamine on the labelling of PC, DSPC and 'unsaturated' PC (Table 2). In these studies, labelling of 'unsaturated' PC was calculated from the difference between the labelling (and pool size) of total PC and DSPC, and therefore should be interpreted with caution. Methylamine did not alter the labelling of microsomal PC, DSPC or 'unsaturated' PC, but caused a large decrease in the labelling of lamellar body PC, DSPC and 'unsaturated' PC. In these studies, DSPC accounted for the same percentage of labelled PC in the cell homogenates, and in the microsomal or lamellar-body fractions of control and methylamine-treated cells (results not shown). Another weak base, ammonium chloride, also decreased the labelling of lamellar

Figure 4 Uptake of Acridine Orange in type II cells

Cultured type II cells were incubated for 3 h in the absence (A) or presence (B) of 10 mM ammonium chloride or (C) 10 mM methylamine. Acridine Orange (0.1 μ M) was added to each cell sample and cells were viewed with an inverted-fluorescence microscope. Cells were photographed for 2 s in each case except for those stained with Phosphine 3R (D), which were photographed with the automatic setting. Magnification $\times 162$.

body PC and DSPC by 37% and 41% respectively compared with the controls (Table 3), suggesting that other weak bases also inhibit surfactant PC packaging.

Methylamine also decreased both the rate and maximum uptake of quinacrine by isolated lung lamellar bodies (Figure 3). In three separate preparations, methylamine decreased the maximum uptake of quinacrine by $42 \pm 3\%$ at 2.5 mM, $76 \pm 3\%$ at 5 mM, and $90 \pm 5\%$ at 10 mM, compared with the control uptake. Next, using intact cells, we evaluated Acridine Orange uptake in acidic vesicles that seem to be lamellar bodies [7]. The cells were treated with 0.1 μ M Acridine Orange for 15–30 s and viewed with an inverted-phase microscope (Olympus IMT-2; Olympus, New York, NY, U.S.A.) with a fluorescence attachment and fitted with a filter cube (excitation 425–490 nm; emission above 520 nm). The punctate fluorescence was similar to that observed with Phosphine 3R, which is routinely used to stain the lipid-rich lamellar bodies (Figure 4D). Compared with control cells (Figure 4A), preincubation of cells for 3 h with 10 mM ammonium chloride (Figure 4B) or 10 mM methylamine (Figure 4C) decreased Acridine Orange fluorescence in lamellar bodies. Because these cells were photographed for the same period (2 s), we suggest that the decreased fluorescence of Acridine Orange is due to increased pH of lamellar bodies.

Methylamine also increased the pH_i of type II cells, albeit transiently. Compared with the steady-state pH_i of 7.17 ± 0.04 ($n = 9$) in control cells, the pH_i was 7.24 ± 0.03 , 7.42 ± 0.06 and 7.46 ± 0.09 ($n = 3$ in each case) within 1 min of addition of 2.5, 5.0 and 10.0 mM methylamine respectively. In each case the pH_i returned to the base level within 5 min.

Next we evaluated the effects of monensin and brefeldin A on PC packaging in lamellar bodies. Both brefeldin A and monensin have been used to assess the role of the Golgi in processing lipids

Table 4 Effect of methylamine on PC secretion in type II cells

Results are means $\pm$ S.E.M. for experiments in four cell preparations. *Significantly different from all other groups by ANOVA.

Addition	PC secretion in 150 min (%)
None	$1.64 \pm 0.18^*$
ATP, 1 mM	4.52 ± 0.42
PMA, 80 nM	5.12 ± 0.52
Methylamine:	
2.5 mM	2.77 ± 0.23
5.0 mM	3.85 ± 0.16
10.0 mM	4.96 ± 0.36

and proteins [10–13]. In these experiments, choline incorporation (nmol/3 h per μ g of PC phosphorus) in control cells was 2.91 ± 0.30 in the cell homogenate, 8.90 ± 0.75 in the microsomal fractions and 0.61 ± 0.09 in the lamellar body fractions ($n = 5$). Neither brefeldin A nor monensin inhibited the labelling of any of these fractions. The corresponding choline incorporations were 2.35 ± 0.15 , 8.08 ± 0.72 and 0.59 ± 0.05 ($n = 4$) in the presence of 25 μ M brefeldin A, and 2.56 ± 0.29 , 8.05 ± 0.99 and 0.48 ± 0.05 in the presence of 1 μ M monensin ($n = 4$) respectively (all $P > 0.05$ compared with controls). Brefeldin A (10 μ M), however, decreased [^3H]leucine incorporation by 37% into cellular proteins (5.7 ± 0.6 compared with 3.3 ± 0.2 nmol/6 h per plate; $P < 0.05$) and by 61% into extracellular matrix proteins (57 ± 9 compared with 22 ± 7 pmol per 6 h per plate; $P < 0.05$). These studies suggest that, as in other cell types [28],

Table 5 Effects of ATP on choline incorporation in type II cells

Cells were labelled for 3 h with 100 μ M [*methyl*- 3 H]choline in the absence (control) or presence of 1 mM ATP. Results are means $\pm$ S.E.M. for experiments in four separate preparations.

Fraction	Choline incorporation (nmol/ μ g of PC phosphorus)	
	Control	ATP
Cells	2.08 $\pm$ 0.27	2.95 $\pm$ 0.28
Microsomes	6.04 $\pm$ 1.26	7.72 $\pm$ 1.00
Lamellar bodies	0.54 $\pm$ 0.07	0.67 $\pm$ 0.10
Packaging (%)*	9.52 $\pm$ 0.92	8.76 $\pm$ 0.94

* Specific radioactivity of PC in lamellar bodies divided by specific radioactivity of microsomal PC, expressed as a percentage.

Figure 5 Phospholipid-to-protein ratio and relative distribution of 3 H-labelled lipids on the gradient of lamellar bodies

Cultured type II cells were incubated for 3 h with 100 μ M [*methyl*- 3 H]choline in the absence (filled symbols) or presence of 10 mM methylamine (open symbols). Fractions (approx. 0.3 ml) were analysed for proteins, total phospholipids and total 3 H label in lipid. (A) Phospholipid-to-protein ratio. Fraction 7 could not be analysed reliably owing to its very low content of phospholipids and proteins. (B) The distribution of 3 H-labelled lipids is expressed as a percentage of the total 3 H label in lipid recovered in 7 fractions. Results are means $\pm$ S.E.M. for three experiments. * P < 0.05 compared with the corresponding fractions in the control cells.

in type II cells the Golgi apparatus does not significantly affect the trafficking of PC and its packaging in the lamellar bodies.

In type II cells, PC secretion could be stimulated with ATP and PMA, as previously demonstrated (reviewed in [3]), and with methylamine (Table 4). Secretagogue-induced change in volume density [29] and pool size of lamellar bodies will probably increase the specific radioactivity of PC in lamellar bodies during pulse-labelling. However, such a change in ATP-stimulated cells does not seem to be enough to alter the specific radioactivity of PC in lamellar bodies. During a 3 h incubation the labelling of PC in lamellar bodies was equivalent in the absence or presence of 1 mM ATP (Table 5).

The lamellar bodies originate as small lamellar bodies and grow into large (mature) lamellar bodies [30]. The lipid-rich large lamellar bodies are isolated with lighter fractions owing to changes in their buoyancy [31]. Analysis of various fractions (between 0.8 and 0.2 M sucrose) from the gradient for the isolation of lamellar bodies from homogenates of cells labelled for 3 h with [3 H]choline, showed that the phospholipid-to-protein ratio increased with increasing buoyancy of each fraction (Figure 5A). The lamellar-body-rich interface was routinely recovered in fractions 4 and 5. In control cells, these fractions accounted for approx. 50 % of 3 H-labelled lipids recovered on the 0.8–0.2 M gradient (Figure 5B). In cells treated with 10 mM methylamine the relative distribution of 3 H-labelled lipids was decreased in these fractions, and increased in dense fractions (1 and 2). These results suggest that methylamine inhibits trafficking of PC during the formation of large lamellar bodies.

Methylamine, brefeldin A and monensin were not toxic to type II cells because none of these agents adversely affected either PC synthesis (Table 1 and Figure 2) or cell viability, as determined by Erythrosin B exclusion (results not shown). Furthermore, the release of lactate dehydrogenase during a 2 h incubation was similar in the absence or presence of each of these agents (control, 0.9 ± 0.1 %; 10 mM methylamine, 0.85 ± 0.06 %; 25 μ M brefeldin A, 1.00 ± 0.04 %; 1 μ M monensin, 0.86 ± 0.17 %; all $n = 4$).

DISCUSSION

The results of this study support the concept that packaging of surfactant PC in lamellar bodies is a regulated process. Previous extensive investigations have demonstrated that the synthesis, secretion and clearance (recycling) of lung surfactant PC are regulated processes [1–3,32]. The packaging of surfactant lipids, however, has remained relatively uninvestigated since the early autoradiographic studies [30] delineating the intracellular pathways of synthesis and trafficking of PC. Pulse-labelling kinetic studies [4] implied that the PC synthesized *de novo* is slowly transported from endoplasmic reticulum to the lamellar bodies. Another study demonstrated that in cultured type II cells such trafficking of DSPC to the lamellar bodies decreases with the time of cell culture [5]. Our study demonstrates that methylamine inhibits PC trafficking to lamellar bodies. Because methylamine raises the lamellar bodies' pH, we suggest that the latter might regulate surfactant PC storage in these organelles.

Besides the lamellar bodies [7], lysosomes, endosomes and the Golgi also maintain an acidic interior [9,33,34]. As with lamellar bodies, methylamine increases the pH of these compartments also. The catabolic nature of lysosomes most probably excludes these organelles from affecting the biosynthesis and packaging of secretory products. The endosomes are involved in internalization of exogenous substances and may therefore not directly affect the processing of biosynthesized products. The Golgi is involved in the processing of proteins synthesized *de novo* in various cell

types, including type II cells [10–13,35]. Our results with brefeldin A support a role for the Golgi in protein synthesis and secretion, but not in the trafficking of newly synthesized PC from endoplasmic reticulum to the lamellar bodies in type II cells. These findings are in agreement with reports in other cell types indicating that the Golgi is not involved in the trafficking of PC, phosphatidylethanolamine and cholesterol [12,28,36,37]. In our hands, however, monensin slightly decreased the PC packaging in lamellar bodies (control $7.8 \pm 1.1\%$, monensin $6.2 \pm 0.8\%$; $P = 0.04$, $n = 4$), which may be related to alkalization of acidic compartments, as previously demonstrated for lysosomes in peritoneal macrophages [38]. Thus the effect of methylamine and of resulting changes in intra-organelle pH seems unrelated to Golgi function and to be selective for PC packaging in lamellar bodies.

Methylamine, like other weak bases, is membrane-permeant in an undissociated state. Based on a pK_a of 10.65 [39], the undissociated form of methylamine is probably present in the micromolar range at physiological pH. At such intracellular concentrations, methylamine does not cause a sustained increase in the pH_i in type II cells (this study) or in peritoneal macrophages [38]. In particular, 2.5 mM methylamine did not increase the pH_i , but decreased the labelling of PC in lamellar bodies by approx. 50% (Figure 2), suggesting that the two parameters are not directly related. Thus it is unlikely that a transient increase in pH_i is the underlying cause of the decreased packaging of PC.

The secretagogue effect of methylamine suggests that the pH of lamellar bodies or the pH_i might also be involved in the secretory process. The cytosolic alkalization is more probably the cause of increased PC secretion, because the maximal effect on packaging was observed at approx. 5 mM methylamine, but the effect on secretion was not maximal at this concentration (Figure 2 and Table 4). In contrast with our results, a previous report suggested that lysosomotropic weak bases did not affect surfactant secretion [40]. We cannot directly compare our results with this previous study that measured the percentage of PC remaining in the cells, rather than the percentage of PC released into the medium. A treatment with lysosomotropic weak bases results in apparent swelling of lamellar bodies and disruption of their internal lamellar structures in type II cells [23,40]. Such changes, however, were associated only with decreased transfer of PC to the lamellar bodies but not with the PC synthesis (Table 1 and Figure 2).

Our study suggests that effects of methylamine are not selective for trafficking of newly synthesized DSPC because the specific activities of total PC, DSPC and 'unsaturated' PC in the lamellar bodies were similarly decreased (Tables 1 and 2). The labelling results with microsomal DSPC and 'unsaturated' PC suggest that methylamine does not inhibit the synthesis of PC. Although a slight decrease (10%, $P > 0.05$) in labelling of microsomal PC could theoretically account for the decreased labelling of DSPC in lamellar bodies, it could not account for labelling of 'unsaturated' PC unless the PCs targeted for lamellar bodies were synthesized in specialized domains of endoplasmic reticulum. We are not aware of the presence of such target-specific domains in the endoplasmic reticulum of type II cells or of other cell types. Also, PC synthesis in such domains must comprise a small proportion of the total because it would otherwise be reflected in the specific radioactivity of the microsomal PC or 'unsaturated' PC. Taken together, our results suggest that methylamine inhibits the trafficking and packaging of both saturated and unsaturated species of PC in the lamellar bodies.

Various mechanisms for the transport of phospholipids from their site of synthesis to the intended destination have been suggested [12]. One of these involves bulk transfer of phospho-

lipids through budding of vesicles from donor membranes and their fusion with acceptor membranes [41]. In the postulated scheme of formation and enlargement of lamellar bodies through addition of PC and other surfactant phospholipids [30] bulk transfer of phospholipid might occur, because certain Ca^{2+} - and lipid-binding proteins, such as synexin [42] and other annexins [43], promote fusion of lipid vesicles with lung lamellar bodies. Although synexin is postulated to promote membrane fusion primarily during exocytosis [42,44], a role for synexin and other annexins in lipid transfer to intracellular organelles cannot be ruled out. Membrane fusion between vesicles is shown to be sensitive to the internal pH of lipid vesicles [45]. It is likely that the trafficking of PC from dense to light fractions (mature lamellar bodies) (Figure 5) proceeds through vesicle fusion. In such a case, the methylamine-induced increase in the pH of lamellar bodies would be inhibitory for PC packaging in lamellar bodies.

In summary, we have shown that the treatment of type II cells with lysosomotropic weak base inhibits the packaging of surfactant PC in lamellar bodies. Such inhibition of packaging is not related to the secretagogue effect of methylamine. The inhibitory effect seems to be related to growth (formation) of lamellar bodies owing to decreased fusion of surfactant-lipid-carrying vesicles with the growing lamellar bodies. However, the latter needs to be confirmed by experiments *in vitro*.

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