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Altered lipid synthesis in type II pneumonocytes exposed to lung surfactant

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When type II pneumonocytes were exposed to purified lung surfactant that contained 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine, radiolabelled surfactant was apparently taken up by the cells since it could not be removed by either repeated washing or exchange with non-radiolabelled surfactant, but was released when the cells were lysed. After 4 h of exposure to [^{3}H]surfactant, more than half of the ^{3}H within cells remained in disaturated phosphatidylcholine. Incorporation of [^{3}H]choline, [^{14}C]palmitate and [^{14}C]acetate into glycerophospholipids was decreased in type II cells exposed to surfactant and this inhibition, like surfactant uptake, was half-maximal when the extracellular concentration of surfactant was approx. 0.1 μmol of lipid P/ml. Inhibition of incorporation of radiolabelled precursors by surfactant occurred rapidly and reversibly and was not due solely to dilution of the specific radioactivity of intracellular precursors. Activity of dihydroxyacetone-phosphate acyltransferase, but not glycerol-3-phosphate acyltransferase, was decreased in type II cells exposed to surfactant and this was reflected by a decrease in the ^{14}C/^{3}H ratio of total lipids synthesized when cells incubated with [U-^{14}C]glycerol and [2-^{3}H]glycerol were exposed to surfactant. Phosphatidylcholine, phosphatidylglycerol and cholesterol, either individually or mixed in the molar ratio found in surfactant, did not mimic purified surfactant in the inhibition of glycerophospholipid synthesis. In contrast, an apoprotein fraction isolated from surfactant inhibited greatly the incorporation of [^{3}H]choline into lipids and this inhibitory activity was labile to heat and to trypsin. It is concluded that the apparent uptake of surfactant by type II cells *in vitro* is accompanied by an inhibition of glycerophospholipid synthesis via a mechanism that involves a surfactant apoprotein.

INTRODUCTION

Lung surfactant is a surface-active lipoprotein that is secreted by type II cells of the alveolar epithelium. Surfactant is essential for normal respiration and an inadequate production of surfactant at birth results in respiratory distress syndrome of the newborn [1]. Secreted surfactant exists in the alveoli in various forms that differ in structure, composition, and surface activity [2]. It has been proposed that less than half of alveolar surfactant is functionally active and that the remainder consists of pools from which active surfactant is derived, and pools from which surfactant is recycled by type II cells [2]. Ikegami *et al.* [3] demonstrated that radiolabelled 1,2-dipalmitoyl-glycero-3-phosphocholine, when mixed with natural surfactant and instilled into the lungs of neonatal rabbits, was a valid marker for monitoring the movement of alveolar surfactant. Radiolabelled surfactant was found to disappear rapidly from the alveoli [4] and some became associated with lamellar bodies (the organelles of surfactant storage) within type II cells [5]. Later, some of the radiolabelled surfactant reappeared in the alveoli [5].

The process of surfactant reutilization has been only partially characterized. Reutilization of disaturated phosphatidylcholine (the most abundant component of surfactant) is not stereospecific and does not appear to require degradation and resynthesis of phosphatidylcholine molecules [5,6]. A requirement for surfactant apoprotein in the uptake phase of reutilization was suggested on the basis of the finding that uptake of liposomal phosphatidylcholine by type II cells *in vitro* was stimulated by an apoprotein (approx. M_r 10000) isolated from rat lung surfactant [7]. Uptake of surfactant by isolated type II cells has only recently been demonstrated [8,9]. The mechanism of uptake of surfactant is undefined and the effect of uptake on the synthesis *de novo* of surfactant is unknown. Knowledge of the effect of extracellular surfactant on the synthesis of new surfactant is necessary not only for an understanding of the production and turnover of this lipoprotein by type II cells, but also because the therapy for respiratory distress syndrome of the newborn has included the intratracheal administration of surfactant [10], and the effect of this treatment on the production of new surfactant by neonatal lungs is unknown. The objective of this investigation was to characterize lipid synthesis in isolated type II cells that are exposed to extracellular surfactant.

MATERIALS AND METHODS

Materials

Animals used in this investigation were male Sprague–Dawley rats (200–225 g) free from respiratory pathogens and were obtained from Sasco, Omaha, NE, U.S.A. Rats were housed inside a laminar-flow hood and were fed

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ad libitum until they were killed. [1-^{14}C]Palmitic acid (56 Ci/mol) was obtained from ICN Radiochemicals. [U-^{14}C]Glycerol (30 Ci/mol) and di[1-^{14}C]palmitoyl-glycero-3-phosphocholine (120 Ci/mol) were from Amersham International. D-[U-^{14}C]Glucose (258 Ci/mol), sodium [2-^{14}C]acetate (56 Ci/mol), [2-^{3}H]glycerol (5 Ci/mmol), L-[U-^{14}C]leucine (340 Ci/mol) and 1-palmitoyl-2-[9,10-^{3}H]palmitoyl-glycero-3-phosphocholine (57 Ci/mmol) were obtained from New England Nuclear. The sources of all other materials that were employed have been described previously [11,12].

Isolation and incubation of cells

Type II cells were isolated from the lungs of adult Sprague–Dawley rats by use of a modification of the procedure of Mason *et al.* [13] as described elsewhere [11,12] except that density gradients were prepared from solutions of Metrizamide [14]. Approx. 6×10^7 cells, of which more than 85% were type II cells, were isolated routinely from the lungs of six rats. Cells were incubated in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%, v/v) and antibiotics [11]. A population of cells enriched with alveolar macrophages was obtained during the course of purifying type II cells by differential adherence [13]. Differentiated 3T3-L1 cells (adipocytes) [15], RLC cells (rat liver epithelial cells) [16], human amnion cells [17] and A431 cells (epidermoid carcinoma cells) [18] were isolated and maintained *in vitro* by use of established procedures. Between 2 h and 15 h before initiation of experiments, the various media employed in the maintenance of the different cell types were replaced with Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10%, v/v) and antibiotics [11].

Isolation of lung surfactant and surfactant apoprotein

Rats were anaesthetized with Nembutal, and tracheal cannulae were inserted surgically and tied in place. Lungs were then lavaged *in situ* using three 4 ml amounts of a solution that contained: NaCl (125 mM), KCl (5 mM), Na_2HPO_4 (2.5 mM), Hepes (17 mM), D-glucose (5.5 mM), gentamycin (10 μg/ml) and Fungi-Bact solution (Irvine Scientific, Irvine, CA, U.S.A.) (1%, v/v) pH 7.4. Alveolar lavage fluids were combined and centrifuged (200 *g* for 10 min) to remove cells. Surfactant was isolated from the supernatant fluid by use of the procedure of Katyal *et al.* [19]. The dry weight ratio of lipid to protein in the purified surfactant was 15.7 ± 1.1 (mean ± S.E.M. from 16 surfactant preparations). In some experiments, the pellet of surfactant was resuspended in sterile Dulbecco's modified Eagle's medium by mixing gently. In experiments in which surfactant apoproteins were isolated, the pellet of surfactant was resuspended in water and dialysed overnight against EDTA (5 mM, pH 7) (Spectrapor 3, M_r 3500 pore exclusion). The dialysed suspension of surfactant was lyophilized and the resultant white powder (15–20 mg) was extracted with diethyl ether/ethanol (3:1, v/v) (3×30 ml) for 36 h at 4 °C [7,20]. After extraction, the insoluble residue was removed by centrifugation (1000 *g* for 15 min) and the supernatant fluid was clarified by filtration [Whatman No. 1 filter paper prewashed with diethyl ether/ethanol (3:1, v/v)]. The extract was evaporated to dryness under N_2, redissolved in chloroform (1 ml), and subjected to chromatography on a column (15 mm × 120 mm) of Unisil (Clarkson Chemical Co., Williamsport, PA, U.S.A.). The column was eluted sequentially with chloroform (50 ml), chloroform/methanol (20:1, v/v) (63 ml), chloroform/methanol (9:1, v/v) (50 ml), chloroform/methanol (4:1, v/v) (60 ml), chloroform/methanol (3:2, v/v) (50 ml), chloroform/methanol (1:4, v/v) (50 ml), and chloroform/methanol (1:9, v/v) (50 ml) [7,20]. Non-esterified fatty acids and neutral lipids were eluted from the column with the chloroform. More than 80% of recovered protein, together with approx. 10% of recovered lipid phosphorus, was eluted with chloroform/methanol (20:1, v/v). The remainder of the recovered lipid phosphorus (> 85%) was eluted with chloroform/methanol (1:4, v/v). Each column fraction was evaporated to dryness under N_2, redissolved in chloroform (1 ml) and stored at −20 °C for further analysis. Proteins that were eluted from the column with chloroform/methanol (20:1, v/v) were separated electrophoretically [21]. The major protein eluted from the column with chloroform/methanol (20:1, v/v) had an apparent M_r of approx. 6000 based on electrophoretic mobility. This protein was detected with silver stain [22], but was stained poorly with Coomassie Brilliant Blue [21].

Measurement of surfactant uptake

Type II cells (approx. 3×10^6 in 2.5 ml of medium) were incubated overnight to allow attachment to 35 mm plastic culture dishes (Nunclon, Intermed, Roskilde, Denmark). In standard experiments, attached cells were exposed for 4 h to purified surfactant that had been mixed with radiolabelled disaturated phosphatidylcholine as follows. 1-Palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine (approx. 20 μCi) was mixed under sterile conditions with purified surfactant (approx. 10 μmol of lipid P) by use of gentle vortex-mixing and ultrasound (2×10 s in a Bransonic 125 W bath-type sonicator). The validity of mixing radiolabelled phosphatidylcholine with surfactant to monitor the movement of alveolar surfactant *in vivo* has been established [3]. In standard experiments, cells were exposed to radiolabelled surfactant (0.2 μmol of lipid P/ml), the surfactant was then removed, and the cells were washed three times with Dulbecco's modified Eagle's medium. Washed cells were detached from culture dishes by scraping three times into ice-cold ethanol (1×1 ml, 2×0.5 ml). Suspensions of broken cells in ethanol were evaporated to dryness under N_2 and lipids were extracted from the residues as described previously [23,24]. In some experiments, after the cells were exposed to radiolabelled surfactant and then washed, further attempts were made to remove radioactive surfactant that remained associated with the cells. For this purpose, washed cells were scraped gently into Dulbecco's modified Eagle's medium (1 ml), and layered onto 2 ml of Metrizamide solution [12.15% (w/v) in buffer solution that contained NaCl (125 mM), KCl (5 mM), Na_2HPO_4 (2.5 mM), Hepes (17 mM), gentamycin (10 μg/ml), Fungi-Bact solution (1%, v/v) and fetal bovine serum (2.8%, v/v), pH 7.4, *d* 1.039]. After centrifugation (200 *g* for 10 min at 4 °C), the resultant pellet of type II cells was harvested. Harvested cells were washed twice with phosphate-buffered saline and lipids were then extracted from cells as described. Some washed cells were lysed before the extraction of lipids. Cells were lysed by suspension in water followed by treatment with ultrasound (10 s in a Bransonic 125 W bath-type sonicator). Lysed cells were centrifuged at 2500 *g* for 20 min and lipids were extracted from the resultant pellet

and supernatant fluid. The amount of radiolabelled lipid that became associated with type II cells was measured and then corrected by subtraction of a blank value which was the amount of radioactivity associated with cells that were exposed to radiolabelled surfactant for only 1 min at 4 °C. Blank values were determined at each concentration of surfactant employed. Prolonged incubation (up to 60 min) at 4 °C did not result in an increase in blank values. The amount of radiolabelled surfactant that adhered to cells after 1 min at 4 °C was never more (and usually much less) than 30% of the amount that was taken up at 37 °C. Essentially all radiolabelled surfactant that could not be dissociated from type II cells by washing was released when the cells were lysed. Ultrasonic lysis of cells, however, did not appear to remove surface-adsorbed surfactant since blank values remained unchanged. Because blank incubations were performed at 4 °C and apparent uptake was measured at 37 °C, it is possible that temperature-dependent binding of surfactant could result in over-estimation of surfactant uptake. For such binding to be a complication, however, it would have to be irreversible because washing of cells to remove adsorbed surfactant was performed at 4 °C.

Analysis of effects of surfactant on precursor utilization by type II cells

In standard experiments, type II cells (approx. 3×10^6 cells/35 mm dish with 2.5 ml of medium) were exposed to surfactant (0.2 μmol of lipid P/ml) for 4 h at 37 °C. During the final 2 h of exposure to surfactant one of the following radiolabelled precursors was also present: [^{3}H]choline (0.03 mM, approx. 14 Ci/mol); [^{14}C]palmitate (0.02 M, approx. 50 Ci/mol); [^{14}C]glycerol (0.13 mM, approx. 30 Ci/mol); [2-^{3}H]glycerol (0.13 mM, approx. 100 Ci/mol); [^{14}C]acetate (0.15 mM, approx. 56 Ci/mol); or D-[^{14}C]glucose (5.5 mM, approx. 1 Ci/mol). Cells were harvested and lipids were extracted as described above. Lipids were separated by use of t.l.c. [25] and incorporation of radiolabelled precursor into various lipids was measured. In experiments where incorporation of L-[^{14}C]leucine was measured, cells were exposed to surfactant (0.2 μmol of lipid P/ml) for 5 h with L-[^{14}C]leucine (0.8 mM, approx. 1.5 Ci/mol) present during the final 4 h. Cells were then harvested in water instead of ethanol and homogenates (0.5 ml) were prepared by using a Teflon/glass Potter–Elvejhem homogenizer. Portions (0.05 ml) of each homogenate were spotted onto filter paper discs (Whatman No. 1). The dried filter paper discs were treated with trichloroacetic acid (6%, w/v) for 10 min at 100 °C and then rinsed sequentially in ice-cold water (twice), methanol (twice), acetone (twice), and diethyl ether (once). The dried filter paper discs were placed in scintillation fluid that contained Triton X-100 [23] and radioactivity was assayed. For measurement of the oxidation of D-[^{14}C]glucose, type II cells (approx. 6×10^6) were incubated in tissue-culture flasks with 5 ml of Dulbecco's modified Eagle's medium. After 2 h of exposure to surfactant (0.2 μmol of lipid P/ml), D-[^{14}C]glucose (5.5 mM, approx. 0.5 Ci/mol) was added to each flask. Flasks were sealed and the production of $^{14}CO_2$ during the following 60 min at 37 °C was measured by trapping CO_2 in Hyamine hydroxide (1 M in methanol) [26]. At the end of the incubation period, the concentration of glucose in the medium was measured and found to be unchanged from that present initially (5.5 mM).

Measurement of the specific radioactivity of intracellular choline

Type II cells (approx. 10^7 in 6 ml) were exposed to surfactant (0.2 μmol of lipid P/ml) for 4 h. During the last 2 h of exposure to surfactant, [^{3}H]choline (0.03 mM, approx. 14 Ci/mol) was present. Cells were then harvested by scraping into Dulbecco's modified Eagle's medium. Harvested cells were washed once with choline-free Dulbecco's modified Eagle's medium, once with phosphate-buffered saline, and then lysed in water (0.5 ml) by use of ultrasound. Cell lysates were heated (100 °C for 10 min), and denatured protein was removed by centrifugation (3000 g for 5 min). Some of the supernatant fluid that resulted was subjected to t.l.c. for the separation of choline [27]. Choline on chromatograms was visualized with I_2 vapour and identified on the basis of its co-migration with authentic choline standard. Radioactivity associated with choline was measured [27]. The remainder of the supernatant fluid derived from cell lysates was used for the enzymic determination of choline [27,28].

Measurement of intracellular triacylglycerol

Type II cells (approx. 10^7 in 6 ml) were incubated for 15 h in the absence or presence of surfactant (0.24 μmol of lipid P/ml). Cells were harvested and lipids were extracted as described. Neutral lipids in extracts were separated on silica gel G thin-layer plates (Analtech, Newark, DE, U.S.A.) developed in heptane/diethyl ether/acetic acid (7:25:4, by vol.). Triacylglycerol on chromatograms was visualized with I_2 vapour and identified on the basis of its co-migration with authentic triolein. After I_2 had evaporated, the area of the chromatogram containing triacylglycerol was scraped and triacylglycerol was eluted from the silica gel with chloroform (2×2 ml), followed by chloroform/methanol (2:1, v/v) (2×2 ml). Chloroform and methanol were removed from the eluted triacylglycerol by evaporation under N_2. The residue of triacylglycerol was suspended in 0.2 ml of sodium taurocholate solution (1.2% w/v, in water) at 50 °C by use of ultrasound (30 s in a Bransonic 125 W bath-type sonicator). Triacylglycerol was hydrolysed and the glycerol that was released was quantified enzymically (Sigma kit 335D). The concentration of sodium taurocholate in the assay mixture was 0.2%, and triolein was used for calibration.

Assay of glycerol-3-phosphate acyltransferase and dihydroxyacetone-phosphate acyltransferase activities

Activity of glycerol 3-phosphate acyltransferase (EC 2.3.1.15) in 700 g supernatant fractions isolated from homogenates of type II cells was assayed as described by Nimmo & Nimmo [29] using [^{3}H]glycerol 3-phosphate (0.5 mM, approx. 10 Ci/mol) and palmitoyl-CoA (0.04 mM) as substrates, in a total assay volume of 0.1 ml. Reaction rates with a fixed amount of protein were constant for at least 15 min. Reaction rates increased linearly as the amount of protein was increased (up to at least 0.075 mg).

Activity of dihydroxyacetone-phosphate acyltransferase (EC 2.3.1.42) in 700 g supernatant fractions isolated from homogenates of type II cells was assayed as described by Declercq *et al.* [30] using [^{14}C]dihydroxyacetone phosphate [generated from [U-^{14}C]fructose 1,6-bisphosphate (0.5 mM, approx. 2 Ci/mol)] and palmitoyl-CoA (0.065 mM) as substrates,

in a total assay volume of 0.5 ml. The molar ratio of palmitoyl-CoA to bovine serum albumin in the assay mixture was 0.65. Reaction rates increased linearly with the amount of protein, and for a fixed amount of protein (up to at least 0.5 mg) the rate was constant for 30 min. The addition of glycerol 3-phosphate (5 mM) to assay mixtures did not change the measured rate of dihydroxyacetone-phosphate acyltransferase activity.

Other methods

Glycerophospholipids in lipid extracts were separated by use of two-dimensional t.l.c. [25] and radioactivity associated with individual lipids was measured as described previously [23,24]. When disaturated phosphatidylcholine was analysed, total lipid extracts were treated with OsO_4 to oxidize unsaturated lipids [31], and disaturated phosphatidylcholine was then separated by use of t.l.c. on silica gel G plates (Analtech) developed in chloroform/methanol/acetic acid/water (50:25:7:3, by vol.).

Electron micrographs of type II cells were prepared as described by Snyder *et al.* [32]. Total cellular protein was measured using the procedure of Lowry *et al.* [33] calibrated with bovine serum albumin. For the measurement of surfactant protein, this assay was modified as follows: samples were dissolved in NaOH (0.5 M) that contained SDS (1%, w/v), and chymotrypsin was used as the protein standard.

Statistical significance of differences between mean values was assessed by use of Student's *t*-test [34].

RESULTS

Before investigating the influence of surfactant uptake on the synthesis of new surfactant, the possibility that type II cells take up extracellular surfactant *in vitro*, as they do *in vivo*, was investigated. Lung surfactant was purified from lung lavage of adult rats and mixed with 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine. Cells were exposed to the radiolabelled surfactant at a concentration of 0.2 μmol of lipid P/ml for various periods at 37 °C. Surfactant and cells were then separated by either washing extensively or by density-gradient centrifugation followed by washing. Radiolabel became associated with the cells in a time-dependent manner and could be removed by lysing the cells, but could not be removed by either extensive washing or exchange with non-radiolabelled surfactant. Association of radiolabel with the cells was maximal after approx. 2 h, but was increased further when the cells were washed and then re-exposed to radiolabelled surfactant. Furthermore, when cells were first exposed to surfactant radiolabelled with 1,2-di[^{14}C]palmitoyl-glycero-3-phosphocholine for 3 h, washed, and then exposed to surfactant radiolabelled with 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine for various periods, there was a time-dependent accumulation of ^{3}H without significant loss of ^{14}C (Fig. 1). The finding of non-exchangeable, essentially irreversible association of radiolabelled surfactant with type II cells, together with the observation that cell-associated radiolabel was released when the cells were lysed, is indicative that radiolabelled surfactant which cannot be removed from cells by washing most likely is not adsorbed to the cell surface but is inside the cells. This conclusion is consistent with observations of the uptake of surfactant

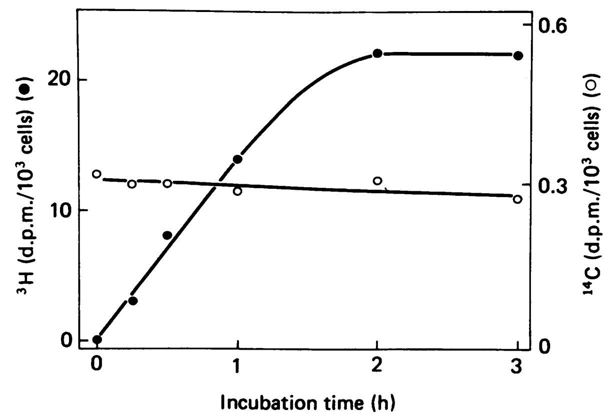

Fig. 1. Time-dependent association of [^{3}H]surfactant with type II cells that previously were exposed to [^{14}C]surfactant

Type II cells (3.2 × 10^6 cells/dish with 2.5 ml of medium) were exposed at 37 °C to surfactant (0.2 μmol of lipid P/ml) radiolabelled with 1,2-di[^{14}C]palmitoyl-glycero-3-phosphocholine (0.1 Ci/mol of total lipid P) until association of [^{14}C]surfactant with cells reached a maximum (3 h). After the medium containing the radiolabelled surfactant was removed, cells were washed three times with Dulbecco's modified Eagle's medium and then exposed to surfactant (0.2 μmol of lipid P/ml) radiolabelled with 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine (4 Ci/mol of total lipid P) for various periods at 37 °C. Cells were again washed three times with Dulbecco's modified Eagle's medium, and then lipids were extracted from the cells (Materials and methods). The amounts of ^{3}H (●) and ^{14}C (○) in total lipid extracts were measured. Incubation time (abscissa) begins with the addition of ^{3}H-labelled surfactant to the cells (after pre-exposure to ^{14}C-labelled surfactant). The data are averages of duplicate values derived from a typical experiment.

by type II cells *in vivo* [4,5]. Assuming complete mixing of radiolabelled phosphatidylcholine with phosphatidylcholine in the purified surfactant and no selective uptake of various surfactant components, the apparent uptake of surfactant by type II cells (when the extracellular concentration of surfactant was 0.2 μmol of lipid P/ml) had an initial rate of approx. 2 nmol of lipid $P \cdot h^{-1} \cdot 10^{-6}$ cells. The rate of apparent uptake subsided greatly after 1.5–3% of extracellular surfactant (0.2 μmol of lipid P/ml) had been taken up.

Radiolabelled surfactant that was taken up by the type II cells was not metabolized rapidly. After a 4 h period of exposure to surfactant radiolabelled with 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine, more than half of the ^{3}H that was non-dissociable from intact cells was recovered in disaturated phosphatidylcholine (Table 1). Essentially all the ^{3}H that was not recovered in phosphatidylcholine was recovered as non-esterified fatty acid. Some of the degradation of 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine may have occurred extracellularly since, at all times, the percentage of total [^{3}H]lipids recovered as disaturated phosphatidylcholine in type II cells was similar to that in the incubation medium (Table 1). 1-Palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine mixed with surfactant and incubated in the absence of type II cells, however, was not degraded (Table 1).

Table 1. Recovery of 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine after being mixed with surfactant and incubated either alone or with type II cells

Freshly isolated type II cells were incubated at 37 °C for 18 h and then exposed for various periods to surfactant (0.2 μmol of lipid P/ml) that had been mixed with 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine (4 Ci/mol of total lipid P). Some of the [^{3}H]surfactant was also incubated for various periods in the absence of type II cells. After incubation, cells were harvested and washed to remove surfactant. Lipids were extracted from washed cells and the percentage of ^{3}H in the total lipid extract that was still associated with disaturated phosphatidylcholine was measured (Materials and methods). Essentially all the ^{3}H that was not recovered in disaturated phosphatidylcholine was recovered as non-esterified fatty acid. Data are corrected for the recovery (83.9%) of authentic 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine throughout the procedure, and are mean values±S.E.M. derived from two experiments.

	^{3}H in disaturated phosphatidylcholine (% of total [^{3}H]lipids)		
	Cells exposed to [^{3}H]surfactant		[^{3}H]Surfactant incubated alone
Incubation Time (h)	Associated with cells	Remaining in medium	remaining in medium
0	–	–	92.4
1	74.4±4.0	76.8±1.2	95.6
2	73.8±5.4	73.3	99.9
3	61.2±1.3	69.4±0.7	97.0
4	53.9±1.9	59.4±0.8	98.2

The uptake of radiolabelled surfactant during a 4 h period of exposure was maximal at concentrations of surfactant greater than 0.2 μmol of lipid P/ml (Fig. 2). In parallel experiments, type II cells were incubated in the presence of [^{14}C]palmitate and nonradiolabelled surfactant at various concentrations. Incorporation of [^{14}C]palmitate into phosphatidylcholine by type II cells was inhibited as the extracellular concentration of surfactant was increased. Uptake of surfactant and inhibition of incorporation of [^{14}C]palmitate into phosphatidylcholine were both half-maximal at a surfactant concentration of approx. 0.1 μmol of lipid P/ml (Fig. 2). The inhibition of incorporation of [^{14}C]palmitate into phosphatidylcholine did not appear to result from non-specific effects of surfactant on type II cell viability, since surfactant did not affect several parameters including cell attachment, Trypan Blue exclusion, $^{14}CO_2$ production from D-[^{14}C]glucose, and incorporation of [^{14}C]leucine into trichloroacetic acid-precipitable material. Glucose was oxidized at a rate of 4.3 ± 0.6 nmol·h^{-1}·10^{-6} cells in the absence of surfactant and at 5.1 ± 1.1 nmol·h^{-1}·10^{-6} cells when surfactant (0.2 μmol of lipid P/ml) was present (mean values±S.E.M. from three experiments). Similarly, leucine incorporation into trichloroacetic acid-precipitable material by type II cells exposed to surfactant (0.45 ± 0.05 nmol·h^{-1}·10^{-6} cells) was not different from that of cells incubated in the absence of surfactant (0.46 ± 0.08 nmol·h^{-1}·10^{-6} cells) (mean values±S.E.M. from four experiments).

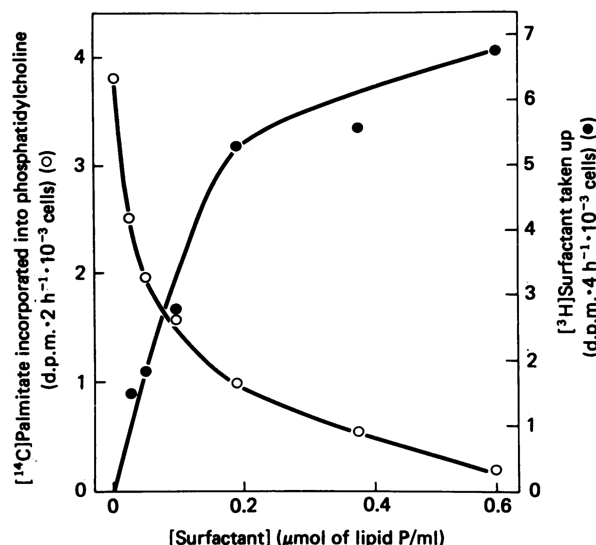

Fig. 2. Uptake of [^{3}H]surfactant and incorporation of [^{14}C]palmitate into phosphatidylcholine by type II cells exposed to surfactant at various concentrations

Type II cells (2.4 × 10^6 cells/dish with 2.5 ml of medium) were exposed to surfactant (0.02–0.6 μmol of lipid P/ml) that was radiolabelled with 1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine (1.3 Ci/mol of total lipid P). After 4 h at 37 °C, cells were harvested and washed, and the uptake of radiolabelled surfactant was determined (Materials and methods). Other type II cells (2.4 × 10^6 cells/dish with 2.5 ml of medium) were exposed for 4 h to non-radiolabelled surfactant (0.02–0.6 μmol of lipid P/ml). During the final 2 h of exposure to surfactant, [^{14}C]palmitate (0.02 mM, 50 Ci/mol) was present. Cells were harvested and the incorporation of [^{14}C]palmitate into phosphatidylcholine was measured. The data are averages of duplicate values derived from a typical experiment.

Surfactant inhibited the incorporation of [^{14}C]palmitate not only into phosphatidylcholine, but also other glycerophospholipids (Fig. 3). At any particular concentration of surfactant, incorporation of [^{14}C]palmitate into each glycerophospholipid was inhibited to a similar extent (Fig. 3). This is suggestive that surfactant inhibits an early reaction(s) common to the synthesis of all glycerophospholipids. Since phosphatidic acid is the precursor of all glycerophospholipids, the effect of surfactant on the activities of two enzymes involved in the synthesis of phosphatidic acid was investigated. Activities of dihydroxyacetone-phosphate acyltransferase and glycerol-3-phosphate acyltransferase in 700 *g* supernatant fractions prepared from type II cells incubated for 15 h in either the absence or presence of surfactant (0.2 μmol of lipid P/ml) were measured. Dihydroxyacetone-phosphate acyltransferase activity in cells exposed to surfactant was lower than in control cells (0.19 ± 0.02 versus 0.26 ± 0.02 nmol·min^{-1}·mg^{-1} of protein, mean values±S.E.M., $n = 5$, $P < 0.05$), but surfactant did not affect significantly the activity of glycerol-3-phosphate acyltransferase (0.45 ± 0.10 versus 0.39 ± 0.05 nmol·min^{-1}·mg^{-1} of protein, mean values ±S.E.M., $n = 5$, $P > 0.1$). Surfactant (0.2 μmol of lipid P/ml) added directly to assay mixtures did not affect the

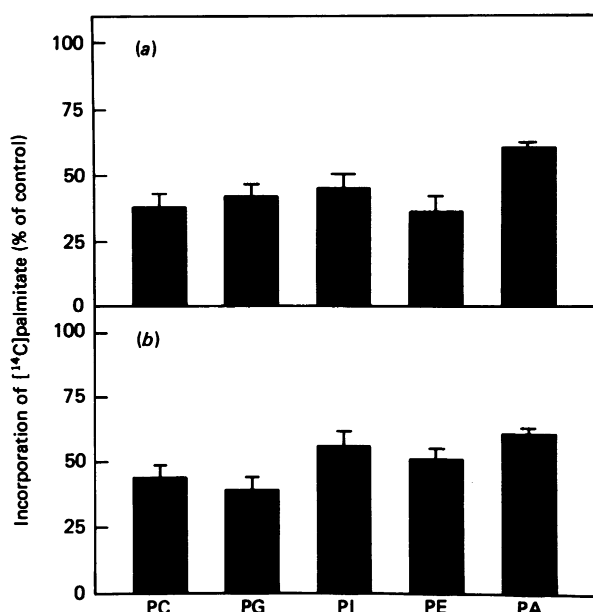

Fig. 3. Effect of surfactant on the incorporation of [^{14}C]palmitate into glycerophospholipids by type II cells

Type II cells (approx. 3×10^6 cells/dish with 2.5 ml of medium) were incubated at 37 °C for 18 h. Surfactant (0.2 μmol of lipid P/ml) was present for either the entire period (*b*) or for only the final 4 h (*a*). [^{14}C]Palmitate (0.02 mM, approx. 50 Ci/mol) was present for either the final 2 h (*a*) or 4 h (*b*) of exposure to surfactant. Cells were harvested and lipids were extracted and separated by use of two-dimensional t.l.c. (Materials and methods). The data are amounts of [^{14}C]palmitate incorporated into various glycerophospholipids by cells exposed to surfactant (expressed as a percentage of that measured in the absence of surfactant) and are mean values ± S.E.M. derived from four experiments. Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PA, phosphatidic acid.

activity of either dihydroxyacetone-phosphate acyltransferase or glycerol-3-phosphate acyltransferase. The proposition that surfactant inhibited selectively the synthesis of phosphatidic acid via the pathway that involved dihydroxyacetone-phosphate acyltransferase (but not via the pathway involving glycerol-3-phosphate acyltransferase) was supported by the results of experiments in which type II cells were exposed simultaneously to [2-^{3}H]glycerol and [U-^{14}C]glycerol and the effect of surfactant on the incorporation of ^{3}H and ^{14}C into lipids was measured. In a typical experiment, the ratio of ^{14}C to ^{3}H in total lipids extracted from control cells (0.28) was decreased to 0.22 in cells exposed to surfactant (0.2 μmol of lipid P/ml) for 4 h (radiolabelled glycerol present during the final 2 h of exposure to surfactant). Surfactant decreased the ^{14}C/^{3}H ratio in phosphatidylcholine (from 0.32 to 0.25) and in triacylglycerol (from 0.29 to 0.20), but not in phosphatidylglycerol (from 0.24 to 0.23). The influence of surfactant on the incorporation of other radiolabelled precursors into lipids was investigated. Incorporation of [^{14}C]palmitate and [^{14}C]acetate into glycerophospholipids, and of [^{3}H]choline into phosphatidylcholine, was inhibited similarly by surfactant (Table 2, and see Fig. 6). On the other hand, surfactant did not affect the incorporation of either [^{14}C]glucose or [^{14}C]glycerol into any glycerophospholipid other than phosphatidylglycerol, where there was an inhibition similar to that observed when palmitate or acetate were employed as precursors (Table 2). While surfactant inhibited the incorporation of [^{14}C]glucose and [^{14}C]glycerol into phosphatidylglycerol, it stimulated greatly the incorporation of these precursors into triacylglycerol (Table 2). The increased incorporation of precursors into triacylglycerol reflected increased synthesis of triacylglycerol. Type II cells that were exposed to surfactant (0.24 μmol of lipid P/ml) for 15 h contained more triacylglycerol than did control cells incubated in the absence of surfactant (6.0 ± 0.3 and 2.9 ± 0.1 nmol/10^6 cells respectively, mean values ± S.E.M., $n = 4$). Cells exposed to surfactant for 15 h contained cytoplasmic inclusions that stained with Oil Red O and were visible in electron micrographs (Fig. 4). These inclusions, which contained no bilayer structure, were most likely neutral lipid droplets and were observed much less frequently in cells incubated in the absence of added surfactant (Fig. 4).

Type II cells used in this investigation were isolated from a suspension of cells that was obtained by treatment of the alveolar epithelium with trypsin. Type II cells were incubated for 18 h to allow recovery from

Table 2. Effect of surfactant on the incorporation of various radiolabelled precursors into lipids by type II cells

Type II cells (approx. 3×10^6 cells/dish with 2.5 ml of medium) were incubated for 4 h at 37 °C in the absence or presence of surfactant (0.2 μmol of lipid P/ml). [^{14}C]Acetate (0.15 mM, approx. 56 Ci/mol), [^{14}C]palmitate (0.02 mM, approx. 50 Ci/mol), [^{14}C]glucose (5.5 mM, approx. 1 Ci/mol), or [^{14}C]glycerol (0.13 mM, approx. 30 Ci/mol), were present for the final 2 h of exposure to surfactant. Cells were harvested, lipids were extracted, and incorporation of precursors into various lipids was measured (Materials and methods). The data are expressed as incorporation of precursor by cells exposed to surfactant as a percentage of that measured in the absence of surfactant and are mean values ± S.E.M. derived from four experiments.

Radio-labelled precursor	Incorporation of precursor in the presence of surfactant (% of control)						
	Total lipids	Triacyl-glycerol	Diacyl-glycerol	Phospha-tidylcholine	Phospha-tidylglycerol	Phospha-tidylinositol	Phospha-tidylethanol-amine
[^{14}C]Acetate	31.6 ± 2.6	88.7 ± 1.2	35.6 ± 1.3	24.4 ± 0.6	15.7 ± 1.0	35.1 ± 1.2	25.5 ± 0.9
[^{14}C]Palmitate	68.3 ± 4.7	107.0 ± 17.3	50.1 ± 3.6	39.7 ± 0.2	47.1 ± 7.9	41.3 ± 4.6	33.7 ± 1.1
[^{14}C]Glucose	105.4 ± 7.6	1333 ± 599	297 ± 114	87.8 ± 7.3	44.5 ± 2.6	89.9 ± 2.1	66.7 ± 3.4
[^{14}C]Glycerol	160.5 ± 28.4	854 ± 318	221 ± 72	88.9 ± 5.3	44.2 ± 9.3	119.0 ± 8.8	95.3 ± 17.4

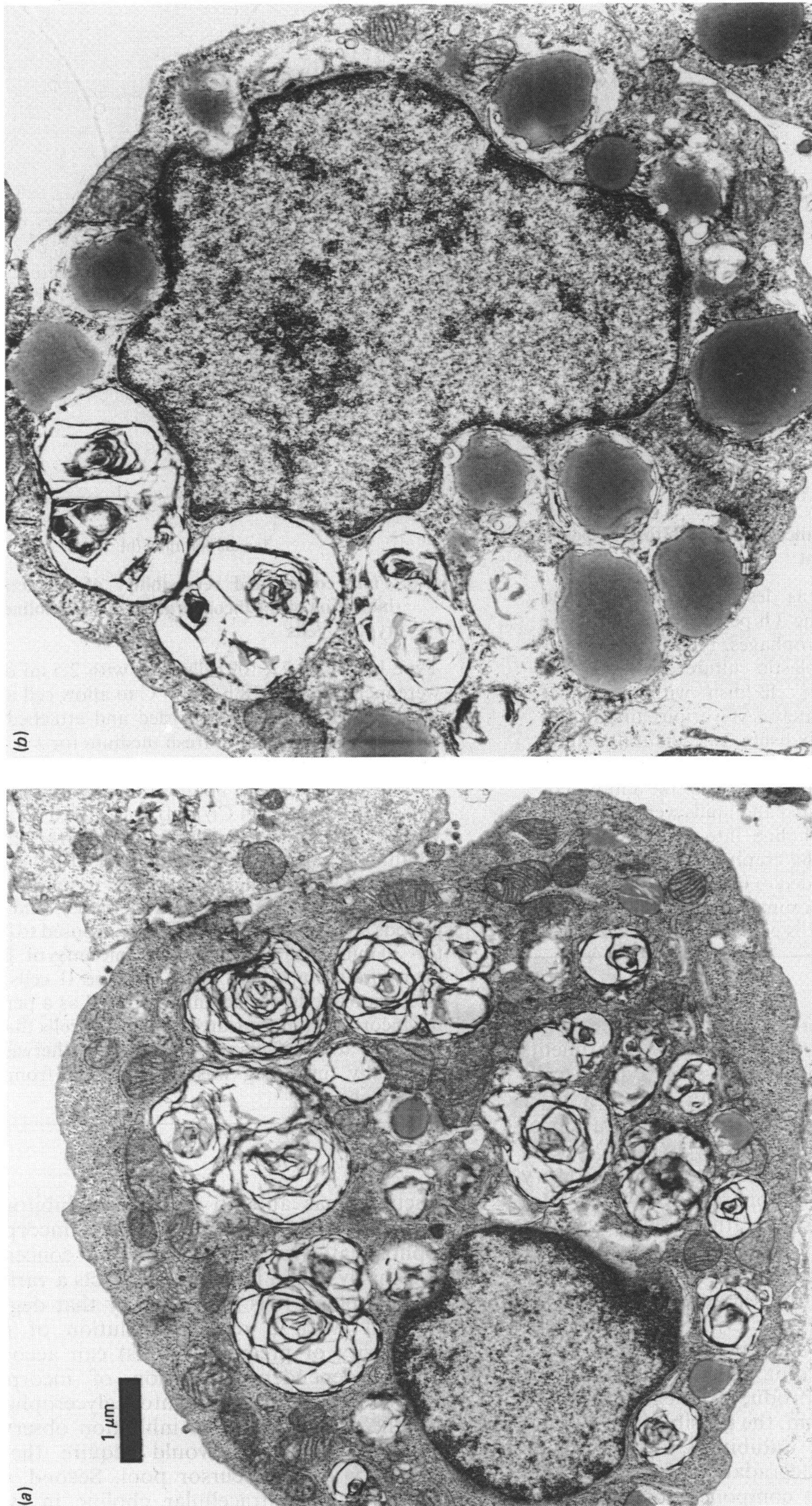

Fig. 4. Electron micrographs of type II cells incubated in either the absence or presence of surfactant

Type II cells were incubated for 15 h at 37 °C in either the absence (*a*) or presence (*b*) of surfactant (0.24 μmol of lipid P/ml). Cells were harvested, washed, and processed for electron microscopy [32].

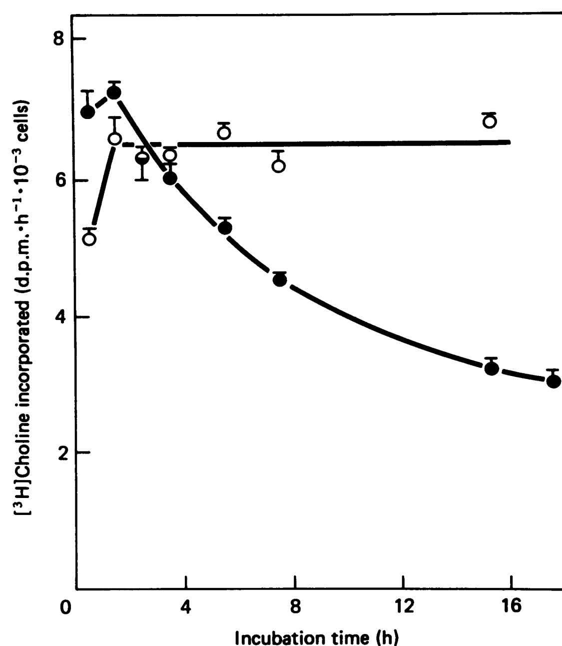

Fig. 5. Time-dependent appearance of the inhibitory response of type II cells to surfactant

Type II cells were isolated as described (Materials and methods) except that after the 3 h period for the selective attachment of alveolar macrophages, type II cells (unattached) were dispensed to plastic culture dishes (35 mm diameter) (approx. 3×10^6 cells/dish with 2.5 ml of medium) and used immediately. At various times after transfer of the type II cells to dishes, surfactant (0.2 μmol of lipid P/ml) was added, followed 1 h later by [^{3}H]choline (0.03 mM, approx. 14 Ci/mol). At 1 h after the addition of [^{3}H]choline, cells were harvested and lipids were extracted. The incorporation of [^{3}H]choline into total lipids was measured. The abscissa of the graph denotes the time at which surfactant was added. The data are mean values ± S.E.M. for three determinations. ○, Control cells (no surfactant added); ●, cells exposed to surfactant.

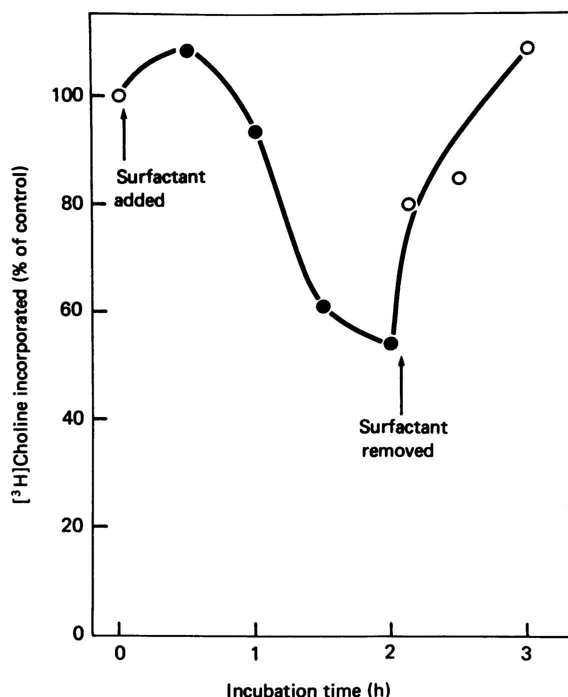

Fig. 6. Time course and reversibility of surfactant-dependent inhibition of the incorporation of [^{3}H]choline into lipids by type II cells

Type II cells (2.7×10^6 cells/dish with 2.5 ml of medium) were incubated for 18 h at 37 °C to allow cell attachment. Unattached cells were discarded and attached cells were allowed to equilibrate in fresh medium for 1 h. Surfactant (0.2 μmol of lipid P/ml) was then added (first arrow). At various times after the addition of surfactant, [^{3}H]choline (0.03 mM, approx. 14 Ci/mol) was added to some cells, which were harvested 30 min later. At 2 h after the addition of surfactant, the remainder of the cells (not yet exposed to [^{3}H]choline) were washed to remove surfactant (second arrow) (Materials and methods), and at various periods after washing, the cells were exposed to [^{3}H]choline for 30 min. The data are the amounts of [^{3}H]choline incorporated into total lipids by type II cells during or after exposure to surfactant, expressed as a percentage of the incorporation measured for type II cells that were not exposed to surfactant, but were otherwise treated identically (including washes), and are from a typical experiment.

the cell damage known to be caused by trypsin [35], before being utilized in most of the experiments described. In some experiments, however, type II cells were used immediately after isolation (i.e., 4 h after termination of the trypsin treatment). Incorporation of [^{3}H]choline into total lipids by freshly isolated type II cells was not inhibited by surfactant. The inhibitory response to surfactant was not apparent until 5–7 h after isolation (9–11 h after trypsin treatment) and was not maximal until about 15 h after isolation (Fig. 5). Inhibition in fully responsive cells (18 h after isolation), however, occurred rapidly after the addition of surfactant, and was rapidly and completely reversed when the surfactant was removed by washing the cells (Fig. 6).

Since some of the surfactant added to type II cells undergoes degradation and products of degradation can be recovered from the medium, the possible involvement of these products in the inhibitory response was investigated. Products of the degradation of phosphatidylcholine (the most abundant component of surfactant) include palmitate, lysophosphatidylcholine, choline and glycerol. None of these products, even at concentrations that would require complete degradation of added surfactant, was able to elicit the inhibitory response (Table 3). Inhibition of [^{3}H]choline incorporation by lysophosphatidylcholine occurred at concentrations at which this lysolipid is toxic and affects a variety of other cell parameters. It is also unlikely that degradation of surfactant (with a resulting dilution of the specific radioactivity of precursor pools) can account for the surfactant-dependent inhibition of incorporation of radiolabelled precursors into glycerophospholipids. First, the similar extent of inhibition observed using a variety of precursors would require the same-fold dilution for each precursor pool. Second, the specific radioactivity of intracellular choline in type II cells exposed to surfactant (0.2 μmol of lipid P/ml) for 4 h (2.0 ± 0.2 d.p.m./pmol) was not significantly different from that of cells incubated for 4 h in the absence of

Table 3. Effects of surfactant, surfactant lipids and their degradation products on incorporation of [^{3}H]choline into lipids by type II cells

Type II cells (approx. 3×10^6 cells/dish with 2.5 ml of medium) were exposed to either purified surfactant or various lipids added individually and as mixtures. Lipids were suspended in Dulbecco's modified Eagle's medium by use of ultrasound (4×20 s with a micro-tip cell disruptor, 80 W, Ultrasonics Heat Systems) at a concentration 10 times that to which cells were exposed. Exposure to surfactant or lipids was for 4 h, and during the final 2 h of exposure [^{3}H]choline was present. Incorporation of [^{3}H]choline into total lipids by type II cells was measured (Materials and methods). Data are mean values ± S.E.M. derived from five experiments. Abbreviations: DPPC, 1,2-dipalmitoyl-glycero-3-phosphocholine; DPPG, 1,2-dipalmitoyl-glycero-3-phosphoglycerol.

Experimental addition	Incorporation of [^{3}H]choline (% of control)
None (control)	100
Surfactant (0.2 μmol of lipid P/ml)	30.0 ± 3.5
DPPC (0.2 mM)	87.9 ± 1.1
DPPG (0.2 mM)	104.4 ± 5.2
DPPC (0.18 mM) + DPPG (0.02 mM)	89.0 ± 2.3
DPPC (0.18 mM) + DPPG (0.02 mM) + cholesterol (0.065 mM)	87.3 ± 2.3
Palmitate (0.04 mM)	98.5 ± 6.1
Palmitate (0.4 mM)	107.1 ± 7.5
1-Palmitoyl-glycero-3-phosphocholine (0.015 mM)	68.8 ± 1.5
1-Palmitoyl-glycero-3-phosphocholine (0.15 mM)	43.8 ± 1.8

added surfactant (2.5 ± 0.4 d.p.m./pmol) (mean values ± S.E.M., $n = 3$). Third, dilution of the specific radioactivity of a precursor pool would be minimized as the initial concentration of precursor is increased, but it was observed that when the concentration of choline in the medium was increased from 0.03 to 0.3 mM, the percentage inhibition of [^{3}H]choline incorporation in the presence of surfactant (0.2 μmol of lipid P/ml) was unaltered (results not shown).

The component(s) of surfactant that were required for the inhibition of lipid synthesis were next identified. The most abundant lipids found in surfactant (when added either individually or in mixtures with molar ratios similar to those of whole surfactant) did not elicit the full inhibitory response observed with whole surfactant (Table 3). In contrast, an apoprotein fraction isolated from surfactant (when added either alone or mixed with lipids) was a potent inhibitor of lipid synthesis by type II cells (Fig. 7). The most abundant protein in the apoprotein fraction had an M_r of approx. 6000. Half-maximal inhibition was observed at a concentration of apoprotein of 0.5 μg/ml (Fig. 7). Treatment of the apoprotein fraction with heat or trypsin resulted in partial loss of inhibitory activity (Table 4).

The effect of surfactant (0.2 μmol of lipid P/ml) on the incorporation of [^{3}H]choline into lipids by other cell types was investigated (Table 5). Although some cells (3T3-L1 cells) were unresponsive to surfactant, the response of RLC cells was equivalent to that of type II

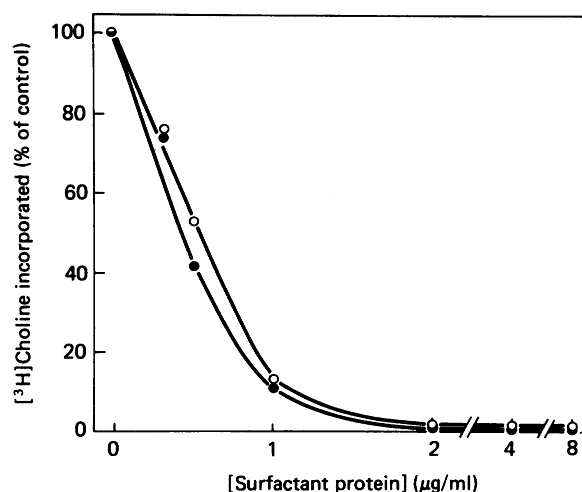

Fig. 7. Inhibition by surfactant apoprotein of the incorporation of [^{3}H]choline into total lipids by type II cells

An apoprotein fraction was isolated from purified lung surfactant (Materials and methods) and was suspended in Dulbecco's modified Eagle's medium by use of ultrasound (4×20 s with a micro-tip cell disruptor, 80 W, Ultrasonics Heat Systems). Liposomes composed of 1,2-dipalmitoyl-glycero-3-phosphocholine, 1,2-dipalmitoyl-glycero-3-phosphoglycerol, cholesterol (36:4:13, molar ratio) and surfactant apoprotein in various amounts were also prepared. Type II cells (2.7×10^6 cells/dish with 2.5 ml of medium) were exposed to apoprotein at various concentrations added either alone (○) or in combination with liposomes (●). At 2 h after the addition of apoprotein to the cells, [^{3}H]choline (0.03 mM, approx. 14 Ci/mol) was added and, after a further 2 h, lipids were extracted from the cells. The data are the amounts of [^{3}H]choline incorporated into total lipids by cells exposed to apoprotein at various concentrations, and are expressed as a percentage of the incorporation measured for cells that were not exposed to apoprotein. A typical experiment is shown.

cells. Other cells (alveolar macrophages, amnion cells and A431 cells) responded to surfactant, but the magnitude of the response was less than that of type II cells (Table 5).

DISCUSSION

Only 20–40% of the total surfactant in lungs is in the form of lamellar bodies stored within type II cells [4,5]. The remainder of the surfactant is present in the alveoli, where it is essential for normal respiration [1]. Alveolar surfactant exists in several forms that differ chemically, structurally, and in their surface activity [2]. It was estimated that, in adult rabbit lungs, as little as one-third of the alveolar surfactant is truly functional in lowering alveolar surface tension [2]. Most of the alveolar surfactant, therefore, is comprised of pools that are involved in either the formation of functional surfactant or in the reutilization of surfactant by type II cells. Jacobs *et al.* [5] computed that, in the neonatal rabbit, more than 90% of alveolar surfactant is reutilized by type II cells. Since the pool of functional surfactant appears to be relatively small and turns over rapidly, maintenance of

Table 4. Effect of trypsin and heat on the ability of surfactant apoprotein to inhibit the incorporation of [^{3}H]choline into lipids by type II cells

At 15 h after isolation, type II cells were exposed to surfactant apoprotein (0.5 μg/ml) for 4 h at 37 °C. During the final 2 h of exposure to apoprotein, [^{3}H]choline (approx. 14 Ci/mol) was present. Cells were harvested and the incorporation of [^{3}H]choline into total lipids was measured. In some cases, the apoprotein was either heated (100 °C, 5 min), or treated with trypsin before being added to cells. Trypsin (0.5 mg, type III S; Sigma Chemical Co.) was added to 0.006 mg of apoprotein in Dulbecco's modified Eagle's medium (0.5 ml) and incubated at 37 °C for 30 min. Trypsin treatment was terminated with trypsin inhibitor (1 mg, type 1 S; Sigma Chemical Co.). Mean values ± S.E.M. for two experiments are shown.

Addition to cells	[^{3}H]Choline incorporation (d.p.m. · h^{-1} · 10^{-6} cells)
None	5912 ± 152
Apoprotein	2205 ± 172
Heated apoprotein	4773 ± 120
Apoprotein treated with trypsin (plus trypsin inhibitor)	3136 ± 157
Trypsin and trypsin inhibitor	5534 ± 249

Table 5. Effect of extracellular surfactant on the incorporation of [^{3}H]choline into lipids by various cell types

Cells were isolated and maintained *in vitro* as described (Materials and methods). Between 2 h and 15 h before initiation of the experiment, medium was removed from the cells and replaced with Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Purified surfactant (0.2 μmol of lipid P/ml) was added to half of the dishes of cells, and after 2 h of incubation at 37 °C, [^{3}H]choline (approx. 14 Ci/mol) was added to each dish. After a further 2 h at 37 °C, cells were harvested and lipids were extracted. The data are the amounts of [^{3}H]choline incorporated into total lipids by cells exposed to surfactant (expressed as a percentage of the incorporation measured in the absence of surfactant) and are mean values ± S.E.M. derived from the number of determinations given in parentheses.

Cell type	[^{3}H]Choline incorporation (% of control)
Alveolar type II cells	30.0 ± 3.5 (5)
Alveolar macrophages	52.3 ± 2.5 (4)
3T3-L1 (adipocytes)	93.2 ± 4.0 (5)
A431 (epidermoid carcinoma cells)	53.8 ± 4.2 (7)
RLC (rat liver epithelial cells)	29.4 ± 1.7 (5)
Amnion cells	48.7 ± 2.7 (4)

this pool must involve strict regulation of the synthesis, secretion and reutilization of surfactant.

Previous investigations of surfactant reutilization were performed *in vivo* (e.g., [4,5]). The conclusion that type II cells reutilize alveolar surfactant was based on the observations that when radiolabelled surfactant was instilled intratracheally some disappeared from the alveoli and became associated with lamellar bodies of type II cells, but subsequently could be recovered from the alveoli again. The kinetics and some characteristics of this process have been described [4–6]. In the present investigation, it was found that isolated type II cells accumulate radiolabelled surfactant that is non-exchangeable and can be dissociated from the cells only after the cells have been lysed. This process is saturable. The conclusion that these data reflect uptake of surfactant is supported also by the recent observation of Moxley *et al.* [9] that type II cells exposed to surfactant labelled with the fluorophore 16-(9-anthroyloxy)palmitate exhibited intracellular fluorescence after 2 h. Endocytosis of other materials by type II cells has been reported [7,36,37]. Nevertheless, the possibility that the present data reflect slow, temperature-dependent binding of surfactant to the cell surface that is difficult to reverse (except by cell lysis), and which is increased by washing the cells, cannot be precluded.

Type II cells exposed to surfactant exhibit a decreased incorporation of choline, palmitate and acetate into glycerophospholipids. This inhibitory response was rapid, reversible, and occurred half-maximally at a concentration of surfactant of about 0.1 μmol of lipid P/ml. Freshly isolated type II cells did not take up extracellular surfactant and did not exhibit surfactant-dependent inhibition of lipid synthesis. Although the concentration-dependence for apparent uptake and inhibition are similar, and both processes were observed only after cells were allowed to recover after isolation, it has not been proven that uptake is a necessary prerequisite for inhibition. It is possible that interaction of surfactant with the cell surface is sufficient to cause inhibition of glycerophospholipid synthesis. The observations that (*a*) surfactant inhibits lipid synthesis without affecting other metabolic parameters, (*b*) freshly isolated cells become responsive to surfactant only after a recovery period, and (*c*) the inhibitory response can be reproduced by an apoprotein fraction at low concentrations, are suggestive that specific binding of the apoprotein to the cell surface may be involved in the inhibitory response. Such putative binding sites may not be restricted to type II cells, however, since some other cells exhibited a partial or complete inhibitory response to surfactant. Interestingly, apoprotein-stimulated uptake of liposomal phosphatidylcholine also is not restricted to type II cells [7]. Characterization of the interaction of the apoprotein with the plasma membrane and identification of apoprotein uptake by type II cells will be facilitated when this apoprotein has been purified and radiolabelled.

Surfactant lipids alone were insufficient to elicit the inhibitory response and apoprotein, either alone or in combination with lipids, was required. The possibility, however, that the small amount of residual lipid in the apoprotein fraction is required for the inhibitory response cannot be discounted. Recently, other investigators have examined the effects of surfactant lipids on lipid synthesis by type II cells *in vitro*. Miles *et al.* [38] reported that incorporation of [^{14}C]palmitate into phosphatidylcholine by type II cells freshly isolated from rat lungs was inhibited by phosphatidylglycerol (40 μM). In contrast, Gilfillan *et al.* [39] found that incorporation of [^{3}H]choline into phosphatidylcholine by rat type II cells

(18–20 h after isolation) was stimulated by phosphatidylglycerol (10 μM), and the stimulation appeared to result from an increase in activity of CTP:phosphocholine cytidylyltransferase [39]. Other glycerophospholipids also stimulated the incorporation of choline into phosphatidylcholine [40], but the effect of surfactant was not examined. In the present investigation, a small but reproducible surfactant-dependent stimulation of choline incorporation was observed in freshly isolated type II cells. When cells were allowed to recover from the isolation procedure, however, exposure to surfactant resulted in pronounced inhibition of choline incorporation. This inhibition was not a reflection of a decrease in CTP:phosphocholine cytidylyltransferase activity. Total activity of CTP:phosphocholine cytidylyltransferase in type II cells remained unchanged in the presence of surfactant even though the subcellular distribution of the enzyme was altered [41].

Miles *et al.* [38] exposed freshly isolated type II cells to pulmonary lavage fluid and observed a decreased incorporation of [^{14}C]palmitate and [^{3}H]choline into disaturated phosphatidylcholine. Inhibition was mimicked completely when cells were exposed to only the lipids extracted from pulmonary lavage fluid, and was mimicked partially when the cells were exposed to unsaturated phosphatidylcholine, disaturated phosphatidylglycerol, or cholesterol. Interpretation of these data is complicated not only because pulmonary lavage fluid contains materials other than surfactant, but also because the lipids in rat pulmonary lavage fluid are very rapidly degraded by lipases present in the fluid [42]. On the basis of the results of the present investigation, however, the inhibitory factor in pulmonary lavage fluid detected by Miles *et al.* [38] was likely surfactant apoprotein and not surfactant lipids. Their recovery of inhibitory activity in lipid extracts of surfactant is consistent with the known solubility of surfactant apoproteins in organic solvents [7,20,43].

The apoprotein fraction that inhibited choline incorporation was comprised principally of a protein (approx. M_r 6000) that was shown previously to stimulate the uptake of liposomal phosphatidylcholine by type II cells *in vitro* [7]. There is indirect evidence that this protein may be involved *in vivo* in the uptake of alveolar surfactant by type II cells. King *et al.* [44] monitored the alveolar turnover of canine lung surfactant that was prelabelled *in vivo* with [^{3}H]leucine and [^{14}C]palmitate. The kinetics of clearance of radiolabelled surfactant lipids from the alveoli were similar to those for a small apoprotein but different from those of the most abundant apoprotein (M_r 35000). The biochemical relationship between these two apoproteins is unknown [44–46].

The mechanism by which surfactant inhibits the incorporation of precursors into glycerophospholipids by type II cells was not defined. Definition of this mechanism is complicated by the degradation of some of the surfactant, leading to reduction of the specific radioactivities of some radiolabelled precursors. The alteration of incorporation of radiolabelled precursors by surfactant, however, cannot be attributed entirely to changes in precursor specific radioactivities, and the following evidence supports the conclusion that surfactant alters rates of lipid synthesis. First, surfactant inhibits greatly the incorporation of [^{3}H]choline into phosphatidylcholine without altering significantly the specific radioactivity of intracellular choline. Second, the large surfactant-induced increases in the incorporation of [^{14}C]glucose and [^{14}C]glycerol into triacylglycerol are reflected by an increase in mass of intracellular triacylglycerol. Third, surfactant-induced inhibition of incorporation of radiolabelled precursor by reduction of specific radioactivity alone would be inversely proportional to the initial concentration of precursor and this was not observed. Fourth, surfactant-induced changes in the ratio of ^{14}C to ^{3}H incorporated into total lipids when type II cells are incubated in the presence of [2-^{3}H]glycerol plus [U-^{14}C]glycerol are independent of dilution of precursor specific radioactivity. Fifth, reduction of precursor specific radioactivity is due largely to the breakdown of surfactant lipids, but the inhibition of radiolabelled precursor incorporation can be reproduced by an apoprotein fraction that contains insufficient residual lipid to contribute significantly to precursor dilution. Finally, at a particular concentration of surfactant, the similar extent of inhibition of incorporation of a variety of radiolabelled precursors would require the same-fold dilution of each precursor if the only effect of surfactant was to reduce the specific radioactivities of precursors.

The present data are consistent with the proposition that exposure to surfactant results in inhibition of an early reaction(s) in the synthesis of glycerophospholipids, possibly at the level of phosphatidic acid synthesis. The apparently selective inhibition of dihydroxyacetone-phosphate acyltransferase activity in type II cells exposed to surfactant is consistent with the observation that the incorporation into lipids of [2-^{3}H]glycerol relative to [U-^{14}C]glycerol is increased in type II cells exposed to surfactant. The observation that total incorporation of radiolabelled glycerol into phosphatidylglycerol is increased in the presence of surfactant, but the ratio of ^{14}C to ^{3}H is unchanged, is consistent with the finding that, after short incubation periods, most of the glycerol is incorporated into the head group of this lipid [24] and so does not involve dihydroxyacetone-phosphate acyltransferase. The finding that inhibition of dihydroxyacetone-phosphate acyltransferase activity in type II cells exposed to surfactant is small may be indicative that only one of the subcellular forms of this enzyme [30] is involved in surfactant lipid synthesis and is inhibited when type II cells are exposed to surfactant. Alternatively, inhibition of lipid synthesis by surfactant may involve additional reactions in the biosynthetic pathway. Also unexplained is the finding that triacylglycerol synthesis is increased even though phosphatidic acid synthesis is apparently decreased. On the other hand, there is no reason to believe that triacylglycerol and glycerophospholipids must be derived from a common pool of phosphatidic acid. Indeed, various forms of phosphatidate phosphohydrolase have been identified in lung tissue, but the functions of the various forms remain unknown [47,48].

Because of the variety of physical forms in which surfactant exists in alveoli, it is difficult to compare the concentration of surfactant used in this investigation with those likely to exist in alveoli. On the basis of results of ultrastructural investigations, however, local high concentrations of surfactant may exist in the alveoli [49]. Furthermore, several investigators have administered surfactant intratracheally to surfactant-deficient neonatal animals and the minimal effective concentration of

surfactant administered *in vivo* is typically much higher than that employed in the present investigation *in vitro* (e.g., [50]). It may be that *in vivo* the synthesis of surfactant by type II cells is chronically repressed as long as there is surfactant in the alveoli available for reutilization. Alternatively, the procedure utilized in this investigation for isolating surfactant may generate inhibitory components (e.g., M_r 6000 apoprotein). The effects of the isolation conditions and the physical form of the purified surfactant on inhibitory activity warrant further investigation because purified surfactant is used to treat infants suffering from respiratory distress syndrome (10). Treatment with an inappropriate form of surfactant may have the undesirable effect of suppressing synthesis of surfactant by the neonatal lungs.

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