Role of an acidic compartment in synthesis of disaturated phosphatidylcholine by rat granular pneumocytes

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A possible role for an acidic subcellular compartment in biosynthesis of lung surfactant phospholipids was evaluated with granular pneumocytes in primary culture. Incubation with chloroquine (100 μM) was used to perturb this compartment. With control cells, incorporation of [9,10- ^3H]palmitic acid into total lipids and into total phosphatidylcholines increased linearly with time up to 4 h. Total incorporation into phosphatidylcholine during a 1 h incubation was 999 ± 85 pmol of [9,10- ^3H]palmitic acid, 458 ± 18 pmol of [1- ^{14}C]oleic acid and 252 ± 15 pmol of [U- ^{14}C]glucose per μg of phosphatidylcholine phosphorus. The cellular content of either disaturated phosphatidylcholine or total phosphatidylcholines did not change during a 2 h incubation with chloroquine. In the presence of chloroquine, the specific radioactivity of [^3H]palmitic acid in disaturated phosphatidylcholine increased by 40%, and that of disaturated-phosphatidylcholine fatty acids from [U- ^{14}C]glucose increased by 125%. Incorporation of [1- ^{14}C]oleic acid into phosphatidylcholine was decreased by chloroquine by 79% and 33% in the presence or absence of palmitic acid respectively. Chloroquine stimulated phospholipase activity in intact cells, and in sonicated cells at pH 4.0, but not at pH 8.5. The observations indicate that chloroquine stimulates synthesis of disaturated phosphatidylcholine in granular pneumocytes from fatty acids, both exogenous and synthesized *de novo*, which can be due to stimulation of acidic phospholipase. This stimulation of acidic phospholipase A activity by chloroquine appears to be coupled to the synthesis of disaturated phosphatidylcholine, thereby enhancing remodelling of phosphatidylcholine synthesized *de novo*. Our findings, therefore, implicate the involvement of an acidic subcellular compartment in the remodelling pathway of disaturated phosphatidylcholine synthesis by granular pneumocytes.

The major component of the surface-tension-reducing material present in lung alveoli is phosphatidylcholine with both fatty acyl moieties saturated ('disaturated phosphatidylcholine') (King, 1979). This material is synthesized and secreted by granular pneumocytes, one of the cell types present in the pulmonary epithelium. With the availability of methods for isolation of metabolically intact cells with high purity, it is possible to study the control of specific metabolic pathways in the synthesis of surfactant phospholipids (Batenburg *et al.*, 1978; Smith & Kikkawa, 1979; Fisher *et al.*, 1980; Mason & Dobbs, 1980; Brown & Longmore, 1981).

The disaturated species of phosphatidylcholine in lung constitutes 50% of total phosphatidylcholine, which is a relatively high proportion compared with other tissues. It has been postulated that the

disaturated species would constitute only 17%, if the fatty acids were randomly distributed (Moriya & Kanoh, 1974). Therefore, lung tissue apparently specifically synthesizes disaturated phosphatidylcholine. Several investigators have provided evidence that phosphatidylcholine synthesized *de novo* in lung by the CDP-choline pathway undergoes further remodelling by deacylation–reacylation/transacylation pathways to form disaturated molecular species of phosphatidylcholine (Van Golde, 1976). These remodelling pathways require sequential activation of a phospholipase A_2 and an acyl-transferase. With respect to lung phospholipase A_2 , two types have thus far been identified, which can be characterized by their pH optima around 4.0 (Heath & Jacobson, 1976, 1980a) and 9.0 (Longmore *et al.*, 1979). These phospholipases A_2 have different Ca^{2+}

requirements for activation and a different intracellular localization. Whereas the acidic enzyme has been localized to a lung lysosomal fraction, the alkaline enzyme has been shown to be associated with the microsomal fraction. It should be noted that the lung lamellar bodies have an enzyme profile suggesting that they are modified lysosomes (Di Augustine, 1974; Heath & Jacobson, 1980a). One group of investigators has speculated that the acidic phospholipase A₂ is involved in the remodelling mechanism for production of disaturated phosphatidylcholine (Heath & Jacobson, 1980a), whereas another group favours the enzyme with an alkaline pH (Longmore *et al.*, 1979).

Chloroquine is a well established lysosomotropic drug; it is accumulated within acidic compartments by protonation, consequently raising the intra-organellar pH and/or injuring the organelle by osmotic effects. Chloroquine has also been reported to affect lipolytic enzymes, including phospholipase A of liver lysosomes (Markus & Ball, 1969; Matsuzawa & Hostetler, 1980). In this study, we employed chloroquine to probe the involvement of the acidic compartment of granular pneumocytes in the synthesis and/or remodelling of phosphatidylcholine to form disaturated species.

Materials and methods

Specific pathogen-free male albino rats (Charles River) were used to isolate type II cells. [9,10-³H]Palmitic acid, [1-¹⁴C]oleic acid and [U-¹⁴C]-glucose were purchased from New England Nuclear, Boston, MA, U.S.A. These radiolabelled substrates were diluted with unlabelled substrates to yield appropriate specific radioactivities. [9,10-³H]-Palmitic acid and [1-¹⁴C]oleic acid were complexed to fatty acid-poor bovine serum albumin (Pentex, Miles Labs. Inc., Elkhart, IN, U.S.A.) in a 6:1 molar ratio before use. Chloroquine diphosphate and dipalmitoylglycerophosphocholine were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of granular pneumocytes

Granular pneumocytes were prepared by a modification of methods of Kikkawa & Yoneda (1974) and Mason & Dobbs (1980). It involved the principle of differential adherence after trypsin treatment of lung as described in a report from this laboratory (Fisher *et al.*, 1980). In brief, the method involved mincing lungs that have been cleared of residual blood by perfusion through the pulmonary artery. The lung minces were then subjected to five trypsin treatments, each of 10 min duration, at 37°C with 0.25% trypsin. The free cells thus obtained were suspended in minimum essential medium (MEM)

containing 10% newborn calf serum and were plated into plastic flasks gassed with CO₂/air (1:19). After a 3 h incubation, the suspended cells were transferred to fresh culture flasks and incubated for the next 21 h, after which type II cells were the predominant cell adhering to the dishes. The supernatant was discarded and attached cells were washed with fresh medium before use. After incubation studies (described below) were completed, cells were removed from the flasks with trypsin; 85 ± 3% of these cells were judged to be granular pneumocytes by morphology and phosphine 3R staining for lamellar bodies, as previously described (Fisher *et al.*, 1980). Exclusion of erythrosin B by these cells was 89 ± 4%.

Synthesis of disaturated phosphatidylcholine by granular pneumocytes

For incorporation studies, cells were incubated at 37°C for up to 4 h in an atmosphere of CO₂/air (1:19) in MEM (without added serum), containing 5.6 mM-glucose and 7 μM-choline. Concentrations of palmitic acid and oleic acid, when added to the medium, were 0.1 mM. Specific radioactivities of palmitic acid, oleic acid and glucose were 40, 20 and 0.45 Ci/mol respectively.

The effect of chloroquine on phosphatidylcholine synthesis by granular pneumocytes was studied by pre-incubating the cells in the presence of chloroquine diphosphate (100 μM) for 1 h. The control cells were incubated without chloroquine diphosphate. After 1 h of pre-incubation, the radioactive substrate was added and incubation was continued for another hour. The reaction was stopped by washing the cells, which were then removed from the plates and extracted (Folch *et al.*, 1957) for lipid analyses.

Assay of phospholipase activity

Phospholipase activity was measured in intact cells incubated in MEM (pH 7.4). The cells were pre-labelled for 2 h with [1-¹⁴C]oleic acid. At the end of 2 h, the incubation medium containing radioactive substrate was removed and the cells were washed three times. Cells were then incubated in fresh medium for up to 120 min. At the end of the incubation, cells were scraped from the plates and processed for lipid and protein determinations. Radioactivity in phosphatidylcholine was measured and compared with radioactivity in this fraction at zero time.

Phospholipase activity in sonicated-cell preparations was assayed at pH 4.0 and 8.5. Cells were sonicated for 30 s in 0.9% NaCl by using a sonic dismembrator at 40% of maximum power (model 150; Artek, Farmingdale, NY, U.S.A.). For assay of activity at pH 4.0, the incubation medium described by Heath & Jacobson (1976) was used. In a final volume of 0.2 ml were present 300 μg of cell protein

in the form of sonicated cells, 40 mM-sodium acetate buffer, 5 mM-disodium EDTA and $34.2 \mu\text{M}$ -[1- ^{14}C]-oleoylglycerophosphocholine (sp. radioactivity $147 \times 10^3 \text{ d.p.m./}\mu\text{mole}$). Since the phosphatidylcholine was biosynthesized, the relative proportions of mono-oleoylglycerophosphocholine and dioleoylglycerophosphocholine were not known. For assay of activity at pH 8.5, the incubation was carried out as described by Longmore *et al.* (1979). In a final volume of 0.2 ml was present $40 \mu\text{M}$ -[1- ^{14}C]-oleoylglycerophosphocholine (sp. radioactivity $121 \times 10^3 \text{ d.p.m./}\mu\text{mol}$), 50 mM-Tris buffer, pH 8.5, 1 mM-EGTA, 10 mM- CaCl_2 and $300 \mu\text{g}$ of cell protein in the form of sonicated cells. Chloroquine was added to some reaction tubes at a final concentration of $100 \mu\text{M}$.

Incubations were carried out at 37°C for 60 min in a shaking water bath and the reaction was stopped by addition of 0.75 ml of chloroform/methanol (1:2, v/v). Lipids were extracted (Bligh & Dyer, 1959) and radioactivity in phosphatidylcholine and lysophosphatidylcholine was measured to analyse phospholipase A activity. [^{14}C]Oleoylglycerophosphocholine for these assays was prepared by incubating [1- ^{14}C]oleic acid with isolated type II cells. Unlabelled phosphatidylcholine was isolated from normal rat liver. Phosphatidylcholine from lipid extracts of cells and liver was separated by t.l.c. (see below), eluted once from silica gel with chloroform/methanol (1:2, v/v), followed by two elutions with methanol. The labelled and unlabelled phosphatidylcholines were mixed and dispersions were prepared in 40 mM-acetate buffer (pH 4.0) or 50 mM-Tris buffer (pH 8.5) (DiCorleto *et al.*, 1977). Percentage loss of radioactivity in the phosphatidylcholine fraction was used as an index of phospholipase activity. In one experiment, phospholipase A activity was specifically measured by the formation of lysophosphatidylcholine from [1- ^{14}C]-oleoylglycerophosphocholine (sp. radioactivity $130 \times 10^3 \text{ d.p.m./}\mu\text{mol}$).

Lipid analyses

Lipid extracts were fractionated into individual phospholipid classes by t.l.c. on silica-gel G plates with chloroform/methanol/ NH_3 /water (92:36:3:3, by vol.) (as solvent system) (Abramson & Blecher, 1964). Phospholipid spots, identified by exposing the plates to I_2 vapour, were scraped and assayed for phospholipid phosphorus and radioactivity.

Disaturated phosphatidylcholine was isolated by using OsO_4 for oxidation of unsaturated lipids (Mason *et al.*, 1976). Neutral Al_2O_3 columns were employed to isolate disaturated phosphatidylcholine. Neutral lipids were eluted with chloroform/methanol (20:1, v/v) followed by elution of disaturated phosphatidylcholine with chloroform/methanol/7 M- NH_3 (35:15:1, by vol.) as solvent.

The eluate was concentrated and portions were taken for scintillation counting and phospholipid phosphorus assays. ^{14}C -labelled disaturated phosphatidylcholine was used to assess recovery of the disaturated species and recovery was found to be greater than 90%.

Incorporation of glucose into fatty acyl moieties of phosphatidylcholine and disaturated phosphatidylcholine was determined by mild alkaline hydrolysis of the phospholipid fractions in ethanolic 1 M-KOH for 2 h at 70°C . The hydrolysates were cooled, diluted with water, acidified with 0.5 M- H_2SO_4 to pH 2, and extracted three times with 5 ml of diethyl ether to collect fatty acids. Radioactivity in fatty acids was then analysed.

Chemical analyses

Phospholipid phosphorus was assayed by the method of Bartlett as modified by Marinetti (1962). The method was adapted for lipid extracts of granular pneumocytes by proportionately reducing the volume of all reagents, thus permitting the assay of $0.1 \mu\text{g}$ of P_i . Total protein were analysed colorimetrically (Bradford, 1976). Radioactivity was counted by using a scintillation fluid containing 5.0 g of 2,5-diphenyloxazole, 0.2 g of 5-diphenyloxazole and 0.2 g of 1,4-bis-(5-phenyloxazol-2-yl)-benzene per litre of toluene in a scintillation counter. Quench corrections were made from 'auto-standard' ratios based on internal standards.

Electron-microscopic studies

For electron-microscopic studies, the cells on culture plates were fixed in glutaraldehyde (3% in 0.2 M-cacodylate buffer, pH 7.4). Cells were scraped from the plates, post-fixed with OsO_4 (1% in 0.1 M-cacodylate buffer) for 1 h at 4°C , dehydrated in ascending grades of ethanol, embedded in Epon and stained with uranyl acetate/lead citrate (Fisher *et al.*, 1980). Thin sections were examined and photographed with a Hitachi EMU 11 electron microscope.

Statistical analyses

The significance of differences between means was evaluated by using Student's *t* test for unpaired data except as noted (Croxtton, 1975). The level of significance was taken as $P < 0.05$.

Results

Phospholipid composition and synthesis of phosphatidylcholine

Phosphatidylcholine constituted 67% of the total granular pneumocyte phospholipid (Table 1). Disaturated phosphatidylcholines comprised 50% of total phosphatidylcholine. Incubation with chloroquine for 2 h did not significantly affect cell

composition of total polar lipids or total phosphatidylcholines. However, there was a 15% decrease in disaturated phosphatidylcholine.

Incorporation of fatty acids into phosphatidylcholine was studied using radiolabelled palmitic acid, oleic acid and glucose. This permitted evaluation of phosphatidylcholine synthesis from saturated, unsaturated and fatty acids synthesized *de novo*. Incorporation, on a cell protein basis, of palmitic acid into phosphatidylcholine as well as into disaturated phosphatidylcholine was linear for 4 h (Fig. 1). The percentage incorporation into the

disaturated phosphatidylcholine fraction accounted for about 65% of that into total phosphatidylcholines. Incorporation into disaturated species and total phosphatidylcholine in the presence of 100 μ M chloroquine increased in a linear fashion after the initial 30 min. Incorporation was slightly higher in the presence of chloroquine. Because of the initial lag, subsequent radioactive incorporation studies were carried out after a 1 h pre-incubation plus or minus chloroquine.

Of the three substrates investigated, palmitate incorporation into phosphatidylcholine was greatest (Table 2). Oleic acid incorporation at equimolar concentrations of the two substrates was approx. 50% of palmitate incorporation. There was preferential incorporation of palmitic acid into phosphatidylcholines over oleic acid since palmitic acid inhibited labelling of phosphatidylcholine with oleic acid when both were present in equal concentrations (Table 2). Incorporation of both these fatty acids was carried out in the presence of glucose (5.6 mM), which was present in minimum essential medium. Incorporation of glucose, on the other hand, was carried out in the absence of added fatty acids to investigate incorporation of fatty acids synthesized *de novo* into phosphatidylcholine. Glucose incorporation into the fatty acid moiety of phosphatidylcholine and disaturated phosphatidylcholine was approx. 14% and 16% respectively of the incorporation into the glycerol moiety of the

Table 1. *Effects of chloroquine on phospholipid content of granular pneumocytes in primary culture*

Granular pneumocytes in primary culture were incubated for 2 h in the presence of 100 μ M chloroquine in MEM. Cells were then harvested and lipids extracted and analysed as described in the text. Values are means $\pm$ S.E.M. from seven separate experiments. Abbreviations: PL, total phospholipids; PC, phosphatidylcholine; DSPC, disaturated phosphatidylcholine. $\dagger P < 0.05$ versus control.

Treatment	Phospholipid phosphorus (μ g/ 10^6 cells)		
	PL	PC	DSPC
Control	1.95 $\pm$ 0.06	1.32 $\pm$ 0.05	0.63 $\pm$ 0.03
Chloroquine	2.03 $\pm$ 0.15	1.25 $\pm$ 0.12	0.53 $\pm$ 0.03 $\dagger$

Fig. 1. *Effect of chloroquine on palmitic acid incorporation in phosphatidylcholine (PC) and disaturated phosphatidylcholine (DSPC) by granular pneumocytes as a function of time*

Cells were incubated with 100 μ M-[9,10- 3 H]palmitic acid and incorporation of the label into cellular lipids was determined at the indicated times by methods described in the text. Each point is a mean and the bar represents the range from two separate experiments.

Table 2. *Effect of chloroquine on incorporation of substrate into phosphatidylcholines by granular pneumocytes in primary culture*

Cells were pre-incubated for 1 h in the absence of chloroquine (0.1 mM) and then for an additional h in the presence of either 5.6 mM-[U-¹⁴C]glucose or 0.1 mM-[9,10-³H]palmitic acid and/or -[1-¹⁴C]oleic acid. At the end of incubation cells were harvested by trypsin treatment and radioactivity was analysed in phosphatidylcholine or disaturated phosphatidylcholine after lipid extraction as described in text. Results are means $\pm$ S.E.M. or individual results for *n* experiments. **P* < 0.01 versus control by *t* test for independent samples.

	<i>n</i>	Phosphatidylcholine		Disaturated phosphatidylcholine	
		Control (nmol/mg of P _i)	Chloroquine (nmol/mg of P _i)	Control (nmol/mg of P _i)	Chloroquine (nmol/mg of P _i)
[9,10- ³ H]Palmitate	6	999 $\pm$ 58	1073 $\pm$ 70	1175 $\pm$ 80	1694 $\pm$ 74*
[1- ¹⁴ C]Oleate	8	456 $\pm$ 18	333 $\pm$ 31*	—	—
[1- ¹⁴ C]Oleate + palmitate	2	192, 165	115, 101	—	—
[U- ¹⁴ C]Glucose					
In fatty acyl moiety	4	37 $\pm$ 4	58 $\pm$ 3*	40 $\pm$ 3	90 $\pm$ 6*
In deacylated fraction	4	261 $\pm$ 5	302 $\pm$ 20	253 $\pm$ 8.5	299 $\pm$ 21

acylglycerol fraction (Table 2). Thus, the predominant role of glucose is to provide the glycerol backbone for phosphatidylcholine and disaturated phosphatidylcholine synthesis by these cells.

Chloroquine significantly stimulated the incorporation of palmitic acid into disaturated phosphatidylcholine (Table 2). However, this increase was not reflected in synthesis of total phosphatidylcholines. This suggests decreased synthesis of unsaturated phosphatidylcholines and is supported by the observed decreased incorporation of oleic acid (Table 2). This inhibition of synthesis of unsaturated phosphatidylcholine was seen both in the presence and absence of palmitic acid. Incorporation of glucose into the fatty acid moiety of disaturated phosphatidylcholine was stimulated by 125%, providing additional evidence for stimulation by chloroquine of disaturated phosphatidylcholine synthesis from saturated fatty acids (Table 2). The relatively greater stimulation of incorporation into disaturated phosphatidylcholine resulted in a significant increase of incorporation into total phosphatidylcholines.

Effects of chloroquine on phospholipases

The foregoing observations show that chloroquine affects predominantly the incorporation of saturated fatty acids into disaturated phosphatidylcholines, suggesting increased activity of the remodelling pathway. We therefore studied effects of chloroquine on phospholipase activity, the initial step in the deacylation-reacylation scheme. Control cells prelabeled with [1-¹⁴C]oleate showed increasing radioactivity in the phosphatidylcholine fraction during a 2 h incubation (Fig. 2). In the presence of chloroquine, radioactivity in phosphatidylcholines declined with a maximum reduction at 1 h (Fig. 2), suggesting a stimulation of phospholipase

Fig. 2. *Phospholipase activity in intact granular pneumocytes as assessed by loss of phosphatidylcholine radioactivity as a function of time*

Cells in primary culture were pulse-labelled with [1-¹⁴C]oleic acid for 2 h and then incubated in fresh media for up to 120 min with or without 100 μ M-chloroquine. Cellular lipids were extracted and radioactivity in phosphatidylcholine fraction was analysed as described in the text. Radioactivity at zero time was taken as 100%. At zero time, cells contained 117×10^6 d.p.m./mg of protein in phosphatidylcholine. Values presented are means $\pm$ S.E.M. (represented by the bars) for the numbers of experiments shown in parentheses.

activity. By using a dispersion of biosynthesized [1-¹⁴C]oleoylglycerophosphocholine as a substrate and sonicated cells as the source of enzyme, we observed phospholipase activity at pH 4.0 and 8.5 in

Table 3. *Effects of chloroquine on phospholipase activity of granular pneumocytes*

Results are means $\pm$ s.e.m. of four experiments. Values are calculated from loss of radioactivity in phosphatidylcholine fraction at the end of 1 h of incubation at 37°C. * $P < 0.01$ versus control by paired t test.

	Phospholipase activity (nmol of phosphatidylcholine hydrolysed/min per mg of protein)	
	Assay at pH 4.0	Assay at pH 8.5
Control	0.57 $\pm$ 0.018	0.62 $\pm$ 0.10
Chloroquine	0.76 $\pm$ 0.027*	0.53 $\pm$ 0.15

control cells (Table 3). The amount of phosphatidylcholine hydrolysed representing phospholipase activity at either pH 4.0 or 8.5 was determined from loss of radioactivity from [1- 14 C]-oleoylglycerophosphocholine. Hydrolysis of phosphatidylcholine in the presence of 100 μ M-chloroquine was unaffected at pH 8.5, whereas it was stimulated by 30% at pH 4.0.

In order to confirm that hydrolysis was due to phospholipase A activity, the formation of radioactive lysophosphatidylcholine from biosynthesized [1- 14 C]oleoylglycerophosphocholine was measured in one experiment. This reaction was found to be linear with time (for 60 min) both at pH 4.0 and 8.5. Chloroquine (100 μ M) increased formation of lysophosphatidylcholine by about 30% (radioactivity in lysophosphatidylcholine was 1129 d.p.m. compared with 873 d.p.m. in controls) when activity was measured at pH 4.0, indicating enhancement of phospholipase A activity. The formation of free oleate (the other product of the phospholipase A reaction) was not evaluated.

Electron-microscopic studies

Representative micrographs of granular pneumocytes from a typical cell preparation show well preserved cells with normal cytoplasmic organelles (Plate 1a). Lamellar bodies are prominent and one appears to be unravelling in a peri-nuclear space. Granular pneumocytes exposed to 100 μ M-chloroquine for a total of 2 h show a disaggregation of lamellar structures, but the cells otherwise appear intact (Plate 1b).

Discussion

In the present investigation chloroquine, a drug that accumulates in organelles having an acidic pH (de Duve *et al.*, 1974), was used as a probe for possible involvement of an acidic compartment in the biosynthesis of disaturated phosphatidylcholine

by granular pneumocytes. Chloroquine increased incorporation of palmitic acid into disaturated phosphatidylcholine but decreased incorporation of unsaturated fatty acids into phosphatidylcholine. The increased incorporation of palmitic acid was compensated for by the decreased incorporation of unsaturated fatty acids into phosphatidylcholine, so that total phosphatidylcholine synthesis was essentially unchanged.

We further investigated the effect of chloroquine by analysing [U- 14 C]glucose incorporation into the fatty acyl and glycerol moieties of phosphatidylcholine and disaturated phosphatidylcholine. Although the incorporation of glucose into fatty acyl moieties was low, chloroquine stimulated incorporation into phosphatidylcholine by 55% and into disaturated phosphatidylcholine by 125%. At the same time, incorporation into the glycerol moiety increased by less than 16%, which was not statistically significant. Since palmitate is the primary product of synthesis of fatty acids *de novo* in the lung (Gross & Warshaw, 1974), these results are consistent with a stimulation of disaturated phosphatidylcholine synthesis from saturated fatty acids.

Since chloroquine appeared to stimulate remodeling of phosphatidylcholine to yield disaturated phosphatidylcholine, its effects on phospholipase activity were further investigated. Chloroquine has been reported to elevate levels of lysophosphatidylcholine in liver lysosomes of rats, which was attributed to increased phospholipase A₂ activity (Tjong *et al.*, 1978). Moreover, chloroquine has been found to both inhibit, at higher concentrations, and stimulate, at lower concentrations, activity of phospholipase A₂ in cells obtained from inflammatory peritoneal exudate (Authi & Traynor, 1979). In another report, chloroquine, at concentrations comparable with that used in the present study, was shown to stimulate rat liver lysosomal phospholipase A, measured at pH 4.5 (Matsuzawa & Hostetler, 1980). Our studies with intact cells indicated stimulation of phospholipase activity by chloroquine. In granular-pneumocyte sonicated preparations, chloroquine stimulated acidic (pH 4.0) phospholipase A activity, as measured by disappearance of [14 C]oleate radioactivity from the phosphatidylcholine fraction with increased formation of radioactive lysophosphatidylcholine. There was no effect of the drug on Ca²⁺-dependent phospholipase A activity measured at pH 8.5.

Since the acidic phospholipase has been shown to be inhibited at or above pH 6.0 (Heath & Jacobson, 1980b), an increase of intraorganelle pH was apparently not the mechanism for the chloroquine effect on phospholipase A activity (Seglen *et al.*, 1979). This was confirmed by the stimulatory effect of chloroquine at constant pH with the assay *in*

EXPLANATION OF PLATE 1

Electron micrograph of isolated granular pneumocytes in primary culture

The cells were fixed with glutaraldehyde, scraped off the plates and processed as described in the text. (a) Control cells showing well-preserved lamellar bodies. (b) Cells incubated for 2 h in the presence of 100 μ M-chloroquine. Disaggregation of intramellar body structures is apparent. In (a) and (b) the bar represents 1 μ m.

vitro. The mechanism may have been related to the ability of chloroquine to bind to phospholipids, particularly acidic phospholipids like phosphatidylglycerol (Harder *et al.*, 1980). Phosphatidylglycerol is present in relatively high concentrations (5–10% of phospholipids) both in the lung surfactant (King, 1979) and in isolated lamellar body fraction, and is known to inhibit the lysosomal phospholipase A₂ towards dipalmitoylglycerophosphocholine in lung (Heath & Jacobson, 1980b). Removal of phosphatidylglycerol inhibition could cause increased degradation of disaturated phosphatidylcholine and may account for the decrease in the cell content of this lipid (Table 1). However, this could not be the only result of chloroquine treatment since the present results show stimulation of phospholipase activity towards phosphatidylcholine of intact cells pulse-labelled with [1-¹⁴C]oleic acid (Fig. 2). Possibly phosphatidylglycerol also inhibits phospholipase A₂ action on 2-oleoyl-1-palmitoylglycerophosphocholine, although this substrate was not investigated by Heath & Jacobson (1980b). If chloroquine reverses the inhibition of phospholipase A by binding to phosphatidylglycerol, the result would be an increased formation of lysophosphatidylcholine, which could be either reacylated in the same compartment or transported to another subcellular compartment to form disaturated phosphatidylcholine.

The site of remodelling of phosphatidylcholine in granular pneumocytes has been a matter of controversy. Evidence put forth suggesting that this pathway is located in the endoplasmic reticulum includes the presence of acyltransferases (Tsao & Zachman, 1977) and phospholipase A₂ in isolated microsomes (Longmore *et al.*, 1979). However, a recent study on incorporation of labelled glycerol and fatty acid derived from [U-¹⁴C]glucose into phosphatidylcholine of microsomes and lamellar bodies showed that there was further enrichment of lamellar-body phosphatidylcholine with saturated fatty acids, thereby suggesting that remodelling occurred after phosphatidylcholine had moved from the endoplasmic reticulum (Engle *et al.*, 1980). Since phospholipase A₂ and possibly acyltransferase (Engle *et al.*, 1976; Heath & Jacobson, 1980a) are present in lamellar bodies these organelles could be the site of remodelling pathway. On the other hand, a somewhat modified mechanism was suggested by Baranska & Van Golde (1977), who could not detect any acyltransferase activity in lung lamellar-body fraction, but observed stimulation of microsomal lysophosphatidylcholine acyltransferase activity by the lamellar-body fraction. This led them to speculate that lamellar bodies could provide the substrate for this enzyme by the action of phospholipase A₂ on microsomal phosphatidylcholine.

Our results with chloroquine lead us to speculate

that the remodelling pathway involves an acidic compartment. This compartment is possibly the lamellar body, which, based on its content of lysosomal enzymes, appears to be a modified (secondary) lysosome (DiAugustine, 1974). Our studies with isolated lamellar bodies indicate a pH for this organelle of less than 6.4 based on uptake of [¹⁴C]methylamine (Chander *et al.*, 1982). Therefore, our results suggest a role for lamellar bodies, whether as the site for the entire remodelling pathway or in providing the enzymic activity for the microsomal substrate for the acyltransferase activity.

The method of cell isolation used in this study involved tryptic digestion of lung tissue. Since the cells were maintained in primary culture for 24 h before use, we assumed that cell damage due to tryptic treatment had been repaired in so far as phospholipid synthesis is concerned and/or that the method selected out only healthy cells, which would attach to the flask. Our assumptions are based on the observations that palmitic acid incorporation into lipids of these cells is linear with time for up to 4 h. At the same time, pulse-labelled cells selectively release radioactive disaturated phosphatidylcholine and phosphatidylglycerol into the medium in a linear fashion for up to 2 h (Chander *et al.*, 1980). Although solubilization of NADPH-cytochrome *c* reductase by tryptic treatment has been reported in freshly isolated lung cells (Finkelstein & Mavis, 1979), our cells after 24 h of incubation showed that the specific activity of this enzyme in the 105 000 *g* supernatant fraction was not elevated and was in fact comparable with that in similar fractions of whole lung (Chander *et al.*, 1982). The electron micrographs provide further evidence that the control granular pneumocytes are intact. Finally, the cells show retention of enzymes and cofactors required for glucose utilization and oxidative metabolism and normal cellular concentrations of ATP (Fisher *et al.*, 1980).

Chloroquine at the concentration used here did not appear to be toxic to granular pneumocytes. First, the cells, on electron microscopy, appeared intact, although their lamellar bodies showed a disaggregation of the lamellar structures. Secondly, incubation with chloroquine did not have a major effect on phospholipid composition of cells. Thirdly, chloroquine-exposed cells, like the controls, were capable of their physiological function of secreting disaturated phosphatidylcholine into the medium (Chander *et al.*, 1980). Finally, during the experimental period chloroquine did not promote detachment of cells from the culture flask. This lends support to our conclusion that the effect of chloroquine on lipid synthesis was a selective stimulation of phospholipase A activity rather than a non-specific manifestation of cell damage.

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