Phosphatidylcholine-Lysophosphatidylcholine Cycle Pathway Enzymes in Rabbit Lung. I. Subcellular Localization and Properties

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Summary

The *de novo* cytidine-5'-diphosphocholine (CDP-choline) pathway enzymes: choline kinase (CK); phosphorylcholine cytidyltransferase (Cyt), and phosphorylcholine glyceride transferase (PCGT), and the phosphatidylcholine-lysophosphatidylcholine (PC-lysoPC) cycle pathway enzymes: lysophospholipase (LPL), lysophosphatidylcholine-lysophosphatidylcholine acyltransferase (LAT), and acyl-CoA lysophosphatidylcholine acyltransferase (acyl-CoA LAT) were studied in the rabbit lung subcellular fractions. The purity of the fractions was examined by the marker enzymes and electron microscopy. The lamellar bodies had the highest concentration of phospholipids (10.0 $\mu\text{mol/mg}$ protein, 80% of which was phosphatidylcholine (PC), about 10-fold higher than that of mitochondria (0.8) and microsomes (1.0) (50% of which was PC in both fractions). The lamellar bodies contained no enzymic activities of either the CDP-choline pathway or the PC-lysoPC cycle pathway. The enzymic activities of CK, Cyt, LPL, and LAT were found mainly in the soluble fraction (about 40% for CK and Cyt, and 70% for LPL and LAT); PCGT and acyl-CoA LAT were microsomal enzymes.

Some general properties of PC-lysoPC cycle enzymes were also studied. The activities of LPL, LAT, and acyl-CoA LAT were not stimulated by the divalent metal ion Ca^{2+} . Their activities were inhibited by 10^{-3} M diisopropyl phosphorofluoridate (DFP). The role of the PC-lysoPC cycle pathway enzymes in remodeling the lung PC is discussed.

Speculation

The mechanisms regulating PC levels and composition in the lung are unknown. A recent idea is that regulation might occur through remodeling of PC via the PC-lysoPC cycle pathway. Isolation and characterization of the enzymes of this pathway will probe the mechanisms of remodeling of lung PC and control of this process.

The mechanisms regulating PC levels and composition in the lung are unknown, although the general pathways of synthesis and degradation have been outlined: (1) the formation of the backbone fragments of diglyceride, nitrogenous base, and glycerol; (2) the *de novo* CDP-choline pathway; (3) degradation of PC by phospholipase A_2 ; (4) acylation of lysoPC with acyl-CoA; (5) transacylation of 2 molecules of lysoPC; (6) degradation of lysoPC by lysophospholipase (for reactions 2 to 6, see Reference 34). By controlling substrate availability, remodeling preformed PC, or controlling degradation, each pathway provides possible modes of PC control that may be important in lung surfactant production.

Recently, the remodeling of preformed PC has been suggested

(1-3, 10, 12, 17, 18, 20, 36) as a regulating mechanism in which phospholipase A_2 degradation (pathway 3 above) followed by acyl-CoA acylation (pathway 4) or transacylation (pathway 5). We shall call this the "PC-lysoPC cycle pathway" (Fig. 1) in the rest of this communication. Ohta *et al.* (26) observed a soluble form of phospholipase A_2 in the rat lung and recently Garcia *et al.* (13) reported a phospholipase A_2 in the rat lung microsomal fraction. The acylation by acyl-CoA of lysoPC occurred in the microsomal fraction (12, 19), whereas the transacylation of 2 molecules of lysoPC and the degradation of this lyso compound were in the soluble fraction (2, 10).

The enzymes of the PC-lysoPC cycle pathway in the rabbit lung and in the lamellar bodies of mammalian lung have not been previously studied. We describe some general properties of these enzymes of the rabbit lung, and define the activities of the CDP-choline and the PC-lysoPC cycle pathway enzymes in lung subcellular fractions.

MATERIALS AND METHODS

CHEMICALS

1,2-[1- ^{14}C]Dipalmitoyl-sn-glycerol-3-phosphorylcholine ([1- ^{14}C]dipalmitoyl phosphatidylcholine) and 1-palmitoyl-sn-glycerol-3-phosphorylcholine (1-palmitoyl-2-lysophosphatidylcholine) were obtained from Applied Science Laboratories, Inc., State College, Penn. [methyl- ^{14}C]Choline chloride was obtained from Amersham/Searle Corp., Arlington Heights, Ill. [1,2- ^{14}C]phosphorylcholine and CDP-[1,2- ^{14}C]choline were the products of ICN Chemical and Radioisotope Division, Irvine, Calif. Palmitoyl coenzyme A (CoA), ATP, CTP, CDP-choline, cytochrome *c* (type III), NADPH (type 1), sodium borohydride, DFP, and activated charcoal were obtained from Sigma Chemical Co., St. Louis, Mo. Ammonium reineckate was purchased from Eastman Kodak Co., Rochester, N. Y. Iodoacetic acid (Eastman Kodak Co.) was recrystallized from petroleum ether (30°-60°) and diethyl ether.

PREPARATION OF LUNG HOMOGENATE

The New Zealand male adult rabbit was anesthetized with 10-15 mg sodium pentobarbital/pound; circulation and respiration were adequate as judged by mucous membrane color and pulse palpation. The animal was killed by phlebotomy during open chest surgery with immediate placement of the pulmonary artery cannula, and the lungs were perfused with cold saline for 3-5 min until essentially colorless. The perfused lung tissue was then minced with scissors after removal of the bronchi. Two grams of lung were homogenized in 6 ml 0.33 M sucrose-0.01 M Tris-HCl buffer (pH 7.4) in a Potter-Elvehjem homogenizer (clearance 0.007-0.009 inch, Arthur Thomas Co., Philadelphia, Pa.) with four strokes at the low speed and then with two strokes at the

anol-water (65:25:4) and the reference components were located with iodine vapor. The corresponding band of radioactively labeled lysophosphatidylcholine was scraped into 1 ml water with vigorous stirring. After the addition of 2 ml methanol vigorous stirring, and centrifugation, the solution was separated from the silica gel. Four milliliters of chloroform were added to the collected extract and, after stirring the centrifugation, the bottom layer was collected and the top layer was re-extracted twice with 4 ml chloroform. The silica gel was re-extracted one more time by the same procedure. The solvent of the collected bottom layer was evaporated under N_2 and residual lipid was stored at -20° until used. The substrate of enzymic reaction was prepared from the dilution of radioactively labeled palmitoyl lysoPC with commercially available unlabeled palmitoyl lysoPC in anhydrous methanol. The purity of the palmitoyl-lysoPC was examined by thinlayer chromatography (TLC) and gas-liquid chromatography, and was found to contain >97% palmitic acid and also by the phosphorous (P) to ester (E) ratio (P/E = 1.3).

Assay of LPL (EC, 3.1.1.5) and LAT: The standard assay of LPL and LAT were carried out in the same incubation mixture containing 0.4 mM 1-[1- ^{14}C]palmitoyl-2-lysophosphatidylcholine (specific activity 1×10^5 cpm μ mol), 0.1 M Tris-succinate (pH 7.4), 0.15 M NaCl, and 0.3 mg protein of the crude lung homogenate or 0.1–0.2 mg protein of the subcellular fractions in a final volume of 0.5 ml. A blank had either boiled protein solution or no protein solution in the mixture. After a 1-min preincubation of the reaction mixture at 37° , 5 μ l substrate in anhydrous methanol were added to the mixture. After 5 min the reaction was stopped and lipids were extracted with chloroform-methanol (1:2) by the method of Bligh and Dyer (5). The lower layer of chloroform was removed, evaporated to dryness under N_2 , and residue was redissolved in 0.1 ml chloroform-methanol (1:2). An aliquot was spotted on an E. Merck precoated silica gel 60 plate to separate the lipids. The free fatty acids (FFA), PC, and lysoPC spots were removed. The radioactivities of FFA and PC were determined as a measure of the activities of lyso-phospholipase and acyltransferase, respectively.

Acyl-CoA: lysophosphatidylcholine acyltransferase (EC, 2.3.1.23): The standard reaction mixture contained 0.4 mM 1-[1- ^{14}C]palmitoyl-2-lysophosphatidylcholine (specific activity 1×10^5 cpm/ μ mol), 0.16 mM palmitoyl-CoA, 0.1 M Tris-succinate (pH 7.4), 0.15 M NaCl, and 0.3 mg protein of the lung homogenate or 0.1–0.2 mg protein of the subcellular fractions in a final volume of 0.5 ml. The reaction was initiated by the addition of 5 μ l lysophosphatidylcholine in anhydrous methanol after preincubation of the protein solution and all other components for 1 min at 37° . The rest of the reaction conditions and procedures were the same in the assay of lyso-phospholipase.

Marker Enzymes. Cytochrome *c* oxidase (EC, 1.9.3.1) (31) was used as a marker enzyme for mitochondria and NADPH-cytochrome *c* reductase (EC, 1.6.2.4) (23) was used as a microsomal marker enzyme.

PROTEIN AND PHOSPHOLIPIDS ANALYSES

Proteins. The protein concentration of the lung homogenate and subcellular fractions was determined by the method of Lowry *et al.* (22). Bovine serum albumin (Fraction V, Miles Laboratories, Inc., Kankakee, Ill.) was used as a standard.

Phospholipids. A portion of lung homogenate or subcellular fractions containing approximately 1 mmol total phospholipids was extracted by the method of Bligh and Dyer (5). Phosphatidylcholine, phosphatidylethanolamine (PE), and sphingomyelin (SM) were quantitatively isolated by TLC with a developing solvent of chloroform-methanol-water (65:25:4). The samples of total phospholipids and individual phospholipids removed from TLC with the presence of silica gel were digested in 60% perchloric acid at 200° for 1 hr. The color development for phosphate was carried out in 2.5% ammonium molybdate and

10% freshly prepared ascorbic acid as described by Chen *et al.* (7). The ester bond of the substrate, lysophosphatidylcholine, was measured by the method of Rapport and Alonzo (28). Tris-tearin (Supelco, Inc., Bellefonte, Penn.) was used as a standard.

All analyses and enzyme assays were carried out in duplicate.

ELECTRON MICROSCOPY

The pellets of lamellar bodies, mitochondria, and microsomes were prepared and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4° for 2 hr, postfixed in 2% osmium tetroxide at room temperature for 1 hr, dehydrated in a series of graded ethanol solution and propylene oxide, infiltrated, and embedded in Epon. The specimens were sectioned, picked up on copper grids, stained with saturated uranyl acetate and lead citrate, and examined with a Hitachi HSH electron microscope.

RESULTS

SUBCELLULAR LOCALIZATION

The purities of the lamellar body, mitochondrial, and microsomal fractions were examined by assay for the marker enzymes (Table 1) and electron microscopy (Fig. 2). Marker enzymes assays showed that lamellar bodies and microsomes had 5% and 11% mitochondria contamination, respectively. The microsomal NADPH-Cytochrome *c* reductase activity was also found in the lamellar body fraction (16%) and the mitochondria fraction (14%). The soluble fraction had 7% microsome contamination. Electron micrograph confirmed the purity and the contamination of lamellar bodies and microsomes; however, the electron micrograph of mitochondria showed that this subcellular fraction was not homogenous and can only be considered as a mitochondria-rich fraction as shown by the activity of cytochrome *c* oxidase (Table 1).

Lamellar bodies had the highest concentration of total phospholipids (10.0 μ mol/mg protein, 80% of which was PC). Virtually no enzymic activities of either the CDP-choline pathway or the PC-lysoPC cycle pathway were found in the lamellar bodies. Only trace amounts of CyT, PCGT, and acyl-CoA LAT activity were found (Table 1).

Compared to the lamellar bodies, the other subcellular fractions had less phospholipid: crude homogenate, 0.46 μ mol/mg protein; mitochondria, 0.78 μ mol/mg protein; microsomes, 1.0 μ mol/mg protein; and soluble fraction, 0.11 μ mol/mg protein. Only 50% of the total phospholipids was found as PC in the mitochondrial fraction, microsomal fraction, and crude homogenate. The soluble fraction contained 70% of the phospholipids as PC. The amount of PE and SM was very low in both lamellar bodies and soluble fractions (Table 1).

The enzymic activities of CK and CyT were found primarily in the soluble fraction (about 40% for each). Some CyT activity was also found in the microsomal fraction. Although recovery was low, 60% PCGT activity was in the microsomal fraction, and 40% in the mitochondrial fraction.

Most of the activity (70%) of two of the PC-lysoPC cycle pathway enzymes, LPL and LAT, was found in the soluble fraction (Table 1). Neither of them was found in the lamellar body fraction and only trace amounts were observed in the mitochondrial and microsomal fractions. The acylation of lysoPC, which requires the acyl-CoA as cosubstrate, was probably a microsomal reaction. The microsomes had the highest specific activity and total activity of this enzyme (Table 1). Because of the high activity of LAT in the crude homogenate and soluble fraction, no significant enhancement of the acylation reaction was observed by adding palmitoyl-CoA to these reaction mixtures. Consequently, the reaction rate of acyl-CoA LAT in the homogenate could not be detected. If the total activity of acyl-CoA LAT in microsomes was arbitrarily designated as

Table 1. Phospholipid concentrations and activities of CDP-choline pathway enzymes, phosphatidylcholine-lysophosphatidylcholine cycle¹ pathway enzymes, and marker enzymes of rabbit lung homogenate and its subcellular fractions¹

Subcellular fractions	Phospholipids				CK		CyT		PCGT		LPL		LAT		acyl-CoA LAT		NADPH cytochrome c reductase		Cytochrome c oxidase	
	C ₂ of total																			
	Total ²	PC	PE	SM	S.A. ³	C ₂	S.A. ³	C ₂	S.A. ³	C ₂	S.A. ³	C ₂	S.A. ³	C ₂	S.A. ³	C ₂	S.A. ³	C ₂	S.A. ³	C ₂
Crude homogenate	0.46 ±0.08	57.75 ±3.32	14.00 ±0.28	7.25 ±0.64	2.09 ±0.78	100 0	0.24 ±0.02	100 0	0.20 ±0.01	100 0	25.95 ±0.83	100 0	17.46 ±0.63	100 0	0.16 ±0.04	0.31 ±0.08	0.01 ±0.10	100 ±0.06	1.05 ±0.33	100 0.13
Lamellar bodies	10.00 ±1.41	79.50 ±5.94	7.85 ±3.18	Trace ⁵	0	0	0.16 ±0.06	0.37 ±0.06	0.22 ±0.06	0.36 ±0.07	0	0	0	0	0.16 ±0.04	0.31 ±0.08	0.01 ±0.10	0.01 ±0.06	0.40 ±0.28	0.13 ±0.09
Mitochondria	0.78 ±0.14	47.85 ±4.03	23.95 ±5.44	9.55 ±0.21	0.15 ±0.06	0.40 ±0.23	0.14 ±0.04	0.21 ±0.10	0.30 ±0.10	4.75 ±1.77	3.11 ±0.37	0.25 ±0.07	0.49 ±0.25	0.04 ±0.03	3.45 ±0.59	61.20 ±1.04	0.14 ±0.09	2.67 ±1.44	8.71 ±0.53	16.16 ±5.76
Microsomes	1.00 ±0.10	49.95 ±1.91	23.90 ±2.97	9.65 ±3.47	0	0	0.24 ±0.12	1.05 ±0.64	0.82 ±0.50	7.10 ±3.39	2.89 ±0.14	0.20 ±0.14	1.54 ±0.15	0.62 ±0.08	11.75 ±2.29	100 ±0.08	1.03 ±0.10	6.85 ±0.46	0.97 ±0.13	1.06 ±0.30
Soluble	0.11 ±0.01	71.50 ±5.66	Trace ⁵	Trace ⁵	1.93 ±0.42	43.35 ±6.86	0.21 ±0.04	42.00 ±2.26	0	0	51.31 ±17.65	66.85 ±6.15	37.90 ±4.59	72.55 ±12.80	±0.03	±0.03	±0.03	±0.03	±0.03	±0.03

¹All values represent mean $\pm$ SD from duplicates of two separate experiments. CK: choline kinase; CyT: phosphorylcholine cytidyltransferase; PCGT: phosphorylcholine glyceride transferase; LPL: lysophospholipase; LAT: lysophosphatidylcholine-lysophosphatidylcholine acyltransferase; PC: phosphatidylcholine; PE: phosphatidylethanolamine; SM: sphingomyelin; S.A.: specific activity.

² Presented in micromoles per mg protein.

³ Presented in nanomoles of product per mg protein per min. Product: CK, phosphorylcholine; CyT, CDP-choline; PCGT, PC; LPL, FFA, LAT, PC; Acyl-CoA LAT, PC.

⁴ Presented in Δ O.D. per mg protein per min.

⁵ Percentage of the enzymic activity was calculated based on the 100% activity in the crude homogenate, except for the microsomal acyl-CoA LAT. Acyl-CoA LAT activity in the microsomal fraction was assigned as 100% because its activity in the crude homogenate could not be distinguished from the activity of LAT.

Fig. 2. Electron micrograph of the (A) lamellar body fraction and (B) microsomal fraction prepared from adult rabbit lung. Both $\times 12,000$.

100%, mitochondria had 61% and lamellar bodies only 0.3% activity.

ENZYMIC PROPERTIES OF PHOSPHATIDYLCHOLINE-LYSOPHOSPHATIDYLCHOLINE CYCLE PATHWAY ENZYMES

The optimal pH values for LAT activity in both crude homogenate and soluble fraction were at 6.5, for LPL were 8.0, and for the microsomal acyl-CoA LAT were between 6.0 and 7.5 (Fig. 3). Using either crude homogenate or soluble fraction at pH 7.4, both activities of LPL and LAT were linear with time for 5 min under the standard assay conditions and the microsomal enzyme activity was linear for 10 min. The enzymic activity of LPL was linearly proportional to the protein concentration below 0.5 mg for the crude homogenate and below 0.3 mg for soluble fraction under the standard assay conditions (Fig. 4). However, the microsomal acyl-CoA LAT failed to show a linear relationship between the enzymic activity and protein concentration below 0.4 mg protein (Fig. 4, *insert*). In the crude homogenate, LAT had a wider range of linear protein concentration (up to 1.5 mg) proportional to the enzymic activity than LPL. In the soluble fraction, LAT was linear only within 0.5 mg in the soluble fraction (Fig. 4). The activity of LAT in both crude homogenate and soluble fraction was greatly inhibited by the high concentration of protein and failed to show a hyperbolic curve as observed for LPL.

The activity of LPL showed a hyperbolic curve with increase of the substrate concentration (Fig. 5A). The transacylation of lysoPC failed to show normal Michaelis-Menten kinetics. The enzymic activity was inhibited by high concentration of 1-palmitoyl-2-lysoPC. Results showed that both PC and PE concentrations dropped gradually with increasing 1-palmitoyl-2-lysoPC (Fig. 5B). Palmitoyl-CoA was a substrate of the acyl-CoA de-

Fig. 3. Effect of pH on the activity of lysophospholipase (LPL) and lysophosphatidylcholine-lysophosphatidylcholine acyltransferase (LAT) in the lung homogenate (● and ○) and soluble fractions (▲ and △). The reaction mixture of LPL and LAT contained 0.4 mM 1-[1-¹⁴C]-palmitoyl-2-lysophosphatidylcholine (specific activity 1×10^5 cpm/μmol), 0.1 M Tris-succinate, and 0.15 M NaCl in a final volume of 0.5 ml. The protein concentration in the crude homogenate was 0.30 mg and in the was carried out at 37° for 5 min. The reaction of acyl-CoA LAT (□) was similar to that of LPL and LAT, except that 0.16 mM palmitoyl-CoA was added to the reaction mixture and the microsomal protein was 0.2 mg.

Fig. 4. Effect of protein concentration on lysophospholipase (LPL) and lysophosphatidylcholine-lysophosphatidylcholine acyltransferase (LAT) in the lung homogenate (● and ○) and soluble fraction (▲ and △) and acyl-CoA LAT in the microsomal fraction (□) (*insert*) at pH 7.4. The reaction conditions were the same as in Figure 3 at various concentrations of protein.

pendent acylation reaction of lysoPC, but it also inhibited the activity of LAT and LPL, especially at high concentration in both crude homogenate and soluble fraction (Table 2).

The microsomal acyl-CoA LAT activity was also inhibited by the two substrates: 1-palmitoyl-2-lysoPC (Fig. 6A) and palmitoyl-CoA (Fig. 7A). The former inhibition also decreased the amount of PC and PE at concentrations of 1-palmitoyl-2-lysoPC greater than 0.2 mM (Figs. 6B); however, the latter effect of palmitoyl-CoA on the concentrations of PC and PE was not significant (Fig. 7B).

The effect of various reagents on the reaction rates of PC-lysoPC cycle pathway enzymes was studied (Table 3). Hydrolysis and acylation of 1-palmitoyl-2-lysoPC in the soluble fraction and acylation in the microsomal fraction were inhibited by DFP. More than 70% activity was lost after the enzyme was preincubated in 10^{-3} M DFP at 37° for 10 min. None of the enzymes required Ca^{2+} ion for the activity. Iodoacetic acid (10^{-3} M) did not effect the enzymic activity significantly. Anhydrous methanol was used as the solvent to prepare the substrate of 1-palmitoyl-2-lysoPC and showed no effect on the enzymic activity. Since DFP was prepared in isopropanol, addition of isopropanol to the reaction mixture was studied. Isopropanol had no inhibition on the activities of LAT and acyl-CoA LAT, but reduced about 20% activity of LPL. The enzymes of LPL and LAT in the soluble fraction were stable at least for 1 month and the microsomal acyl-CoA LAT was stable at least for a week at -20° in 0.33 M sucrose-0.01 M Tris-HCl buffer (pH 7.4). However, some loss of the activity was observed if the protein solution was thawed several times.

PC was recovered from both the LAT reaction in the cytosol fraction and the microsomal acyl-CoA LAT reaction (starting with 1-[1-¹⁴C]palmitoyl-2-lysoPC in each reaction) and subjected to hydrolysis by snake venom phospholipase A₂. The results showed that 42% and 79% of the radioactive fatty acids were present in lysoPC and 58% and 21% in FFA for LAT and acyl-CoA LAT products, respectively.

Fig. 5. A: the enzymic activities of lysophospholipase (LPL) and lysophosphatidylcholine-lysophosphatidylcholine acyltransferase (LAT) at various concentrations of 1-palmitoyl-2-lysophosphatidylcholine (PC) in the lung homogenate (▲ and △) and soluble fraction (● and ○) at pH 7.4. The reaction conditions were the same as in Figure 3. B: the effect of 1-palmitoyl-2-lysoPC on the concentrations of PC (○) and phosphatidylethanolamine (△) recovered from the same crude homogenate reaction as in A. The concentration of each lipid was determined by measuring the content of phosphorous as described in the text.

Table 2. Effect of palmitoyl-CoA on activities of lysophosphatidylcholine-lysophosphatidylcholine acyltransferase (LAT) and lysophospholipase (LPL) in rabbit lung homogenate and soluble fraction¹

Palmitoyl-CoA, mM	LAT, nmol PC/mg protein/min		LPL, nmol FFA/mg protein/min	
	Crude homogenate	Soluble	Crude homogenate	Soluble
	17.93	35.36	25.95	34.88
0.04	21.11	34.26	15.90	
0.08	15.92	30.74	10.72	25.19
0.16	5.37		4.24	
0.24	3.67	5.17	3.12	4.54

¹ All values are means of duplicate. PC: phosphatidylcholine; FFA: free fatty acids.

DISCUSSION

Histochemical and morphologic studies have suggested that the source of the pulmonary surfactant is at least in part the alveolar type II cell. The type II cell is distinguished by the presence of lamellar bodies (30). This particular subcellular fraction contains very high concentrations of phospholipids, especially phosphatidylcholine (9, 11, 16, 29). The exact function of the lamellar body is not well understood, but it may serve as a site of surfactant synthesis, storage, and/or secretion.

A number of non-lipid hydrolases (9), several lipid pathway enzymes such as phosphatidic acid phosphohydrolase (EC. 3.1.3.4) (33) and glycerophosphate phosphatidyltransferase (EC. 2.7.8.5) (15, 16, 29), and the transacylation of PC with palmitoyl-CoA (11) have been reported in the lamellar bodies.

However, we found no enzyme activity of the CDP-choline pathway and/or the PC-lysoPC cycle pathway in this subcellular fraction, except that a trace of activities of CyT, PCGT, and acyl-CoA LAT was observed in the lamellar bodies (Table 1), probably because of a small contamination with microsomes. Our results, therefore, indicate that the lamellar bodies themselves cannot synthesize the lung surfactant PC through either the CDP-choline pathway or through the PC-lysoPC cycle pathway. A similar lack of the CDP-choline pathway enzymes in lamellar bodies was noted previously (16, 29). Recent results of Garcia *et al.* (14) also showed that the rat lung lamellar bodies lack some other key enzymes of phospholipid metabolism such as phospholipase A, acyl-CoA glycerol-3-phosphate acyltransferase, and phosphatidic acid phosphohydrolase, although 40% of the latter enzyme activity has been observed in the pig lung lamellar bodies (33).

Although a small amount of glycerophosphate phosphatidyltransferase activity was observed in the lamellar bodies reported by Hallman and Gluck (15, 16) and Rooney *et al.* (29), it was indicated (15, 16) that this was due to the contamination with microsomes. Again, our results, as well as those of other investigators, suggest that the lamellar bodies are not active in the synthesis of the surfactant through the *de novo* pathway or the remodeling PC-lysoPC cycle pathway.

Endoplasmic reticulum in the microsomal fraction is probably the main site for acyl-CoA LAT and PCGT in the rabbit lung, as also indicated by other investigators (10, 12, 16, 19, 29, 32). These biochemical studies also confirmed the electron microscopic autoradiography findings that PC was initially synthesized in endoplasmic reticulum (8). The mitochondria may have enzymes required for the synthesis of membrane phospholipids (25). The rest of the enzymes (LPL, LAT, CK, and CyT) were all found mainly in the soluble fraction, although some CyT ac-

Fig. 6. A: the enzymic activity of acyl-CoA lysophosphatidylcholine acyltransferase at various concentrations of 1-palmitoyl-2-lysophosphatidylcholine (PC) in the lung microsomes at pH 7.4 (●). Details of the reaction conditions are described in Figure 3. B: the effect of 1-palmitoyl-2-lysoPC on the concentrations of PC (○) and phosphatidylethanolamine (△) recovered from the reaction in A.

Fig. 7. A: the enzymic activity of acyl-CoA lysophosphatidylcholine acyltransferase at various concentrations of palmitoyl-CoA in the lung microsomes at pH 7.4 (●). Details of the reaction are described in Figure 3. B: the effect of palmitoyl-CoA on the concentrations of phosphatidylcholine (○) and phosphatidylethanolamine (Δ) recovered from the reaction in A.

Table 3. Effect of reagents on enzymic activity of soluble lysophospholipase (LPL), lysophosphatidylcholine-lysophosphatidylcholine acyltransferase (LAT), and microsomal acyl-CoA LAT¹

Reagents	% of activity		
	LPL	LAT	Acyl-CoA LAT
None	100	100	100
Methanol, 2%	96.6	98.3	87.5
Isopropanol, 2%	79.2	103.4	105.7
DFP, 1 mM	16.2	12.8	29.7
Ca ²⁺ , 20 mM	83.2	89.1	90.0
Iodoacetate, 1 mM	91.9	97.5	69.5

¹ All values are means of duplicate. DFP: diisopropyl phosphorofluoridate.

tivity was observed in the microsomal fraction of rat lung (16, 37).

The soluble enzymes of LPL and LAT reported in rat lung had the same optimum pH and general properties, suggesting one enzymic form possessing two catalytic reactions (2). However, the hydrolysis and acylation of lysoPC by the soluble fraction of rabbit lung had different optima pH (Fig. 3) in our studies, suggesting the LPL and LAT may be two different enzymes in rabbit lung. Nonetheless, both activities share a number of the same properties (Tables 2 and 3). Both enzymes were inhibited by serine-blocking reagent, DFP. They were also inhibited by palmitoyl-CoA, the substrate of the acylation of lysoPC in microsomal fraction. This inhibition may be because the detergent effect of acyl-CoA at high concentrations is greater than its

critical micellar concentration (3.6–6 μ M) (4). Although DEP did not affect acyl-CoA LAT of liver microsomes (21) or dog lung microsomes (12), the enzyme obtained from rabbit lung microsomes here was inhibited by this serine-blocking reagent (Table 3). The enzymic activity of LAT was also inhibited by the substrate, 1-palmitoyl-2-lysoPC, especially at high concentration (Fig. 5A). This inhibition may be the result of a phospholipase A₂ reaction occurring in the same reaction mixture. This suggestion is supported by the observation of a decrease of total concentrations of both PC and PE from the crude homogenate reaction mixture of LAT (Fig. 5B) (42). Phospholipase A₂ activity may have also occurred in the soluble fraction, accounting for the abnormal kinetic pattern (Fig. 5A) (42).

The substrate inhibition was also observed for the microsomal acyl-CoA LAT (Figs. 6A and 7A). Since the concentrations of PC and PE decreased with increasing 1-palmitoyl-2-lysoPC in the reaction (Fig. 6B). This inhibition was also a result of the contamination of phospholipase A₂ in the substrate as observed for LAT (42). On the other hand, the inhibition of the activity of acyl-CoA LAT by palmitoyl-CoA did not significantly decrease the concentrations of PC and PE (Fig. 7B) and may simply be a detergent effect on the activity of the acyltransferase itself.

Although some investigators (1–3, 12, 17, 18, 20, 36) have suggested that the PC-lysoPC cycle pathway may be important in the remodeling of the lung surfactant, the mechanism is not clear. Chevalier and Collett (8) observed that PC was initially synthesized in endoplasmic reticulum and then transferred to lamellar bodies through Golgi complex. Rooney *et al.* (29) recently reported that PC in the lamellar bodies was 61.5% dipalmitoyl and in the microsomes only 37.8%. Our results of the distribution of cycle pathway enzymes in the cell indicate that the cycle pathway may involve two distinct mechanisms, *i.e.*, one in the endoplasmic reticulum and one in the cytoplasm. If the cycle pathway is involved in regulation of the lung PC, it is yet to be determined whether: (1) the PC is remodeled in the endoplasmic reticulum and then transferred to the lamellar bodies or (2) the remodeled PC is formed in the cytoplasm via the transacylation of 2 molecules of lysoPC during the transfer from endoplasmic reticulum to the lamellar bodies. The results of Rooney *et al.* (29) and our observations of cytosolic LAT activity suggests that the acylation of lysoPC may resynthesize more dipalmitoyl-PC from 1-palmitoyl-2-lysoPC in the cytoplasm before the transfer of PC of other molecular species from endoplasmic reticulum to the lamellar bodies. Clearly, other mechanisms such as the transacylation of palmitoyl-CoA to the preformed PC in the lamellar bodies (11), or a fast turnover rate of dipalmitoyl-PC transfer from microsomes to the lamellar bodies by a specific transfer protein like Wirtz and Zilversmit (40, 41) observed for the exchange of phospholipids between liver mitochondria and microsomes cannot yet be excluded as an important step in the regulation of lung surfactant, PC synthesis, and turnover.

CONCLUSION

Biosynthesis of rabbit lung PC from the *de novo* CDP-choline pathway and its remodeling mechanism and degradation in the PC-lysoPC cycle pathway in the subcellular fractions have been studied. The lamellar body fraction lacked all enzymic activities of both pathways. The enzymes of CK, CyT, LAT, and LPL were found mainly in the soluble fraction, PCGT and acyl-CoA LAT in microsomal fraction. Results indicate that the lamellar bodies neither synthesize PC from CDP-choline pathway nor remodel PC from PC-lysoPC cycle pathway. The remodeling mechanism of the lung surfactant is possibly either the transacylation of 2 molecules of lysoPC (LAT reaction) in the cytoplasm or the acylation of lysoPC with acyl-CoA (acyl-CoA LAT reaction) in the endoplasmic reticulum.

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42. Recent observations show that the decrease of the concentrations of PC and PE in LAT and acyl-CoA LAT reactions resulted from the high concentration of 1-palmitoyl-2-lysoPC because of the contamination of phospholipase A₂ activity in the commercially supplied lysocompound.
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