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Species pattern of phosphatidylinositol from lung surfactant and a comparison of the species pattern of phosphatidylinositol and phosphatidylglycerol synthesized *de novo* in lung microsomal fractions

B. RÜSTOW,* Y. NAKAGAWA,† H. RABE,* K. WAKU† and D. KUNZE*

*Institute of Pathological and Clinical Biochemistry, Charite Hospital, Humboldt University, 1040 Berlin, German Democratic Republic, and †Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

1. Phosphatidylinositol (PI) is a minor component of lung surfactant which may be able to replace the functionally important phosphatidylglycerol (PG) [Beppu, Clements & Goerke (1983) J. Appl. Physiol. **55**, 496–502] without disturbing lung function. The dipalmitoyl species is one of the main species for both PI (14.4%) and PG (16.9%). Besides the $C_{16:0}$–$C_{16:0}$ species, the $C_{16:0}$–$C_{18:0}$, $C_{16:0}$–$C_{18:1}$, $C_{16:0}$–$C_{18:2}$ and $C_{18:0}$–$C_{18:1}$ species showed comparable proportions in the PG and PI fractions. These similarities of the species patterns and the acidic character of both phospholipids could explain why surfactant PG may be replaced by PI. 2. PI and PG were radiolabelled by incubation of microsomal fractions with [^{14}C]glycerol 3-phosphate (Gro3*P*). For 11 out of 14 molecular species of PI and PG we measured comparable proportions of radioactivity. The radioactivity of these 11 species accounted together for more than 80% of the total. The addition of inositol to the incubation system decreased the incorporation *in vitro* of Gro3*P* into PG and CDP-DG (diacylglycerol) of lung microsomes (microsomal fractions), but did not change the distribution of radioactivity among the molecular species of PG. These results supported the idea that both acidic surfactant phospholipids may be synthesized *de novo* from a common CDP-DG pool in lung microsomes.

INTRODUCTION

The lung secretes a surface-active material, consisting largely of phospholipids, which lines the alveoli (King & Clements, 1972). The major components reducing the surface tension and preventing the collapse of the alveoli is dipalmitoyl phosphatidylcholine (PC) (Montfoort *et al.*, 1971; King & Clements, 1972; Goerke, 1974).

PG seems to be another functionally important component of pulmonary surfactant, representing 7–12% of total lipid phosphorus (Rooney *et al.*, 1975; Sanders & Longmore, 1975; Hallman *et al.*, 1977). The great practical importance in clinical obstetrics and neonatalogy of this acidic phospholipid (Gluck, 1978; Kulovich *et al.*, 1979) has been concluded from the observation that PG was almost absent from the lung effluent of infants with respiratory-distress syndrome (Rooney *et al.*, 1975).

The exact role of PG in lung surfactant is not known. It has been reported that PG (i) decreases surface compressibility of surfactant (Hallman & Gluck, 1976), (ii) regulates surface activity of dipalmitoyl PC in artificial surfactant (King & MacBeth, 1981; Suzuki, 1982) and (iii) enhances the binding of dipalmitoyl PC with apoprotein (King & MacBeth, 1981). But there are also studies suggesting that PG may not be a critical component of surfactant function (Hallman & Epstein, 1980). It seems to be possible that surfactant PG may be largely replaced by PI without distributing normal lung function (Beppu *et al.*, 1983; Egberts *et al.*, 1985; Liau *et al.*, 1985). Normally the PI concentration is much lower than that of PG in mammalian lung surfactant, but in the surfactant of the rhesus monkey the concentration of the acidic phospholipids is reversed; PI is the major acidic phospholipid, whereas PG is only a minor component (Egberts *et al.*, 1987). In surfactant of chicken (Hallman & Gluck, 1976) and turtle (Lau & Keough, 1981), PG is virtually absent.

If PG and PI are, indeed, essential for surfactant function, it may be assumed that the acidic character of both phospholipids is an important factor. On the other hand, similarities of the species patterns might provide another explanation as to why surfactant PG may be replaced by PI. Therefore we compared the species pattern of PI isolated from surfactant with that of PG already published by ourselves (Schlame *et al.*, 1986).

The acidic surfactant phospholipids PG and PI show a different developmental pattern: the proportion of PG starts to increase near term, while at the same time the proportion of PI decreases (Hallman *et al.*, 1977). Two attractive ideas explaining this developmental event can now be discussed. (i) A decrease in serum inositol (Hallman & Epstein, 1980) or a striking decrease in the availability of inositol to specific lung cells (Hallman *et al.*, 1986) decreases PI synthesis with the consequence that the common precursor, CDP-DG, is available for PG synthesis. (ii) A high CMP level produced by increasing PC synthesis in foetal lung near term (Quirk *et al.*, 1980)

Abbreviations used: PI, phosphatidylinositol; PG, phosphatidylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; DG, diacylglycerol; Gro3*P*, glycerol 3-phosphate; $C_{x:y}$, fatty acid with *x* carbon atoms and *y* double bonds; DPG, diphosphatidylglycerol.

shifts the equilibrium of the CDP-DG:inositol phosphatidyltransferase toward CDP-DG, which then available for PG synthesis (Bleasdale & Johnston, 1982).

Both ideas presume that PG and PI are synthesized from a common CDP-DG pool. Because PI synthesis in lung tissue seems to be located in the microsomal fraction (Hallman & Gluck, 1975; Bleasdale *et al.*, 1979), the concept of a common CDP-DG pool presumes that surfactant PG should be synthesized also in microsomes (microsomal fractions) or the CDP-DG is rapidly equilibrated between the microsomal and other subcellular sites. On the other hand, the results of investigations about the intracellular localization of PG synthesis are contradictory. These are arguments supporting the assumption that PG synthesis may be located either in microsomes or in mitochondria.

We think that a comparison of the species patterns of PG and PI synthesized *de novo* in lung microsomes may be a useful approach to verify the concept of a common CDP-DG pool.

In the present paper we show that surfactant PI contains the dipalmitoyl species in the same range as shown for surfactant PG (Schlame *et al.*, 1986). In the species patterns of PI and PG synthesized *de novo* by [^{14}C]Gro3*P* acylation in lung microsomes, more than 80% of the radioactivity was similarly distributed in 11 out of 14 individual species of both phospholipids. The results support the idea that both PI and PG may be synthesized from a common CDP-DG pool in lung microsomes.

EXPERIMENTAL

Chemicals

ATP, CTP, CoA and Gro3*P* were obtained from Boehringer-Mannheim. *sn*-[U-^{14}C]Gro3*P* (sp. radioactivity 170 mCi/mmol) and [^{3}H]CTP (sp. radioactivity 25.6 Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Hepes and Florisil were from Serva, silica-gel HR was from Merck and CDP-dipalmitoylglycerol was from Sigma. All other chemicals were commercially obtained in analytical grade.

Methods

Isolation of lung microsomes and surfactant. The isolation of lung microsomes was performed as previously described by van Heusden *et al.* (1981). Surfactant was isolated as described by Schlame *et al.* (1986).

Lipid extraction and separation. Lipids were extracted as described by Bligh & Dyer (1959), with the exception that 0.1 M-HCl was used instead of water. Separation by two-dimensional t.l.c. was performed on silica-gel HR/Florisil (99:1, w/w) or on commercial silica-gel 60 plates (Merck) with chloroform/methanol/25% (w/v) NH_3 (13:7:1, by vol.) in the first direction and chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.) in the second direction. When the CDP-DG label had to be determined, the mixture for the second direction was chloroform/methanol/acetic acid/water (50:25:8:3, by vol.).

Composition of the incubation mixture used for the synthesis *de novo* of PG and PI. The incubation mixture contained 0.1 M-EDTA, 10 mM-cysteine, 0.167 mM-CoA, 150 mM-KCl, 3 mM-$MgCl_2$, 3.5 mM-ATP, 50 mM-Hepes, pH 7.8, 1 mM-[^{3}H]CTP (sp. radioactivity 6200 d.p.m./nmol), 0.75 mM-[^{14}C]Gro3*P* (sp. radioactivity 8–12000 d.p.m./nmol) and 1 mg of microsomal protein/ml in a final volume of 1 ml. For the determination of the species patterns of labelled PI and PG we used the same incubation mixture with 0.2 mM-inositol in a final volume of 5–10 ml.

Species analysis. Labelled PI and PG were extracted from silica gel, then converted into DG by phospholipase C (*Bacillus thuringiensis*) action, and DG was acetylated with acetic anhydride in pyrimidine. The DG acetates were fractioned into their individual molecular species by reverse-phase h.p.l.c. (model 655 instrument; Hitachi Co., Tokyo, Japan) using a 4.6 mm × 25 mm Zorbax ODS column (du Pont, Wilmington, DE, U.S.A.) eluted with acetonitrile/propan-2-ol/methyl t-butyl ether/water (72:18:8:2, by vol.) (Nakagawa & Horrocks, 1983). DG acetates produced from rat liver PC and dipalmitoyl PC were added to labelled DG acetates produced from labelled PI and PG before h.p.l.c. separation to get sufficient amounts for u.v. detection.

PI is a minor components of the phospholipids in microsomes and surfactant of rat lung. Therefore we used for the analysis of its species pattern a more sensitive h.p.l.c. method (Krüger *et al.*, 1984; Rüstow *et al.*, 1987). PI isolated from lung microsomes and surfactant was converted into DG, which was derivatized with α-naphthyl isocyanate and then separated, on a LiChrosorb RP-18 column by h.p.l.c. [Hewlett–Packard model 1084 B equipped with a fluorescence detector, Fluorichrome (Varian, Los Altos, CA, U.S.A.)], as described by Rüstow *et al.* (1987).

Other analytical methods. Protein was measured as described by Lowry *et al.* (1951), with bovine serum albumin as standard. We used a Philips PW 4700 liquid-scintillation counter with an automatic compensation of quenching for the measurement of radioactivity.

RESULTS AND DISCUSSION

The PI of non-pulmonary sources is characterized by a very high proportion of the $C_{18:0}$–$C_{20:4}$ species, and dipalmitoyl species seemed to be completely absent (Bishop & Strickland, 1970; Thompson & MacDonald, 1975, 1976; Hawthorne, 1982). In contrast, surfactant PI contained the $C_{18:0}$–$C_{20:4}$ species as a minor component (Table 1). The species patterns of surfactant PG (Schlame *et al.*, 1986) and PI are similar in five individual species that accounted together for 60% in both phospholipids. We assume that this similarity of the species patterns and the acidic character of both phospholipids may explain why PI is able to replace PG in lung surfactant.

Pulmonary surfactant is synthesized by the alveolar Type II cells (King, 1982; Goerke, 1974). Although there is no doubt about the cell type which synthesizes surfactant lipids, the intracellular site of PG synthesis is controversial. The most potent arguments for the localization of the PG synthesis in mitochondria are (i) co-distribution of a mitochondrial marker with PG synthesis (Mavis & Vang, 1981; Jobe *et al.*, 1981; Bleasdale *et al.*, 1985) and (ii) stimulation of DPG synthesis at the expense of surfactant PG (Liau *et al.*, 1984). Arguments for the localization in microsomes are (i) the high capacity of microsomes for PG synthesis

from CDP-DG (Hallman & Gluck, 1974, 1975) and (ii) inositol-induced decrease in PG synthesis in favour of PI production in Type II cells (Batenburg *et al.*, 1982) and a rough microsomal fraction of Type II cells (Battenburg *et al.*, 1985).

Table 1. Comparison of the species of PG and PI isolated from surfactant of adult rat lung

For PI the values are means ± S.E.M.; for PG, means and individual values are given.

Species Phospho-lipid ...	Content (%, w/w) PI ($n = 3$)	PG ($n = 2$)
$C_{16:0}$–$C_{16:0}$	14.4 ± 4.4	16.9 (11.6, 22.2)
$C_{16:0}$–$C_{18:0}$	2.4 ± 1.5	1.4 (0.9, 1.6)
$C_{16:0}$–$C_{18:1}$	27.3 ± 2.1	26.7 (24.6, 28.8)*
$C_{18:0}$–$C_{18:1}$	5.5 ± 1.8	3.9 (3.5, 4.3)
$C_{16:0}$–$C_{18:2}$	9.4 ± 2.1	9.8 (9.5, 10.1)
$C_{18:0}$–$C_{18:2}$	10.1 ± 3.1	2.0 (1.7, 2.3)
$C_{16:0}$–$C_{20:3}$	5.4 ± 1.7	1.2 (0.7, 1.7)
$C_{18:0}$–$C_{20:3}$	3.9 ± 1.1	1.1 (0.8, 1.4)
$C_{16:0}$–$C_{20:4}$ / $C_{18:1}$–$C_{22:6}$	3.8 ± 1.4	11.2 (8.1, 14.3)
$C_{18:0}$–$C_{20:4}$	3.9 ± 1.0	1.7 (1.6, 1.8)†
$C_{18:0}$–$C_{22:5}$	0.9 ± 0.6	1.6 (1.4, 1.8)
$C_{16:0}$–$C_{22:6}$	4.6 ± 0.5	10.6 (8.5, 12.7)
$C_{18:0}$–$C_{22:6}$ / $C_{18:1}$–$C_{18:1}$	3.2 ± 0.4	—
Not identified	5.8 ± 0.9	11.9 (7.5, 16.3)

* $C_{18:0}$–$C_{22:6}$ was recovered in this fraction.
† $C_{18:1}$–$C_{18:1}$ was recovered in this fraction.

On the other hand, the localization of the PI synthesis in the microsomal fraction of lung tissue is well established (Hallman & Gluck, 1975; Bleasdale *et al.*, 1979, 1985; Bleasdale & Johnston, 1982). Therefore the inhibitory effects of inositol on the PG synthesis of Type II cells (Batenburg *et al.*, 1982) and of a crude microsomal fraction of Type II cells (Batenburg *et al.*, 1985) support the idea that PI and PG may be synthesized in microsomes from a common CDP-DG pool.

In the '*de novo*' pathway, PA is the precursor of the acidic phospholipids, with CDP-DG as the intermediate. From investigations with liver microsomes it was assumed that neither CDP-DG formation from PA nor PI formation from CDP-DG showed selectivity for individual species of the substrates (Akino & Shimojo, 1970; Holub & Kuksis, 1972; Holub & Piekarski, 1976). Provided that such non-selectivity of the '*de novo*' pathway is also effective in lung microsomes, the species pattern of PI and PG synthesized *de novo* should be quite

Table 2. Incorporation of [^{14}C]Gro3*P* into CDP-DG, PG and PI of lung microsomes

Values are means ± S.D. ($n = 3$).

Addition	[^{14}C]Gro3*P* incorporation (nmol/10 min per mg microsomal protein) CDP-DG	PG	PI
None	0.54 ± 0.10	0.28 ± 0.06	0.05 ± 0.05
0.2 mM-Inositol	0.14 ± 0.07	0.16 ± 0.04	1.84 ± 0.30
1.0 mM-Inositol	0.08 ± 0.06	0.07 ± 0.05	2.01 ± 0.24

Table 3. Comparison of the species of PG and PI synthesized *de novo* with and without inositol via [^{14}C]Gro3*P* acylation in lung microsomes

Values are means ± S.E.M, except for the '+Inositol, 60 min' values, which are individual results from two experiments.

	Content (%, w/w)				
	−Inositol	+Inositol			
Time (min) ...	10	10		60	
n ...	3	3		2	
Species Lipid ...	PG	PG	PI	PG	PI
$C_{16:0}$–$C_{16:0}$	8.8 ± 1.6	9.7 ± 1.7	5.8 ± 1.0	8.3, 9.6	6.5, 5.6
$C_{16:0}$–$C_{18:0}$	1.4 ± 0.5	1.4 ± 1.0	0.3 ± 0.2	0.5, 0.3	0.2, 0.2
$C_{16:0}$–$C_{18:1}$ / $C_{18:0}$–$C_{18:2}$	12.8 ± 2.6	14.8 ± 0.8	13.8 ± 3.3	16.4, 13.4	15.6, 14.0
$C_{18:0}$–$C_{18:1}$	0.5 ± 0.3	0.9 ± 0.6	0.2 ± 0.1	0.1, 0.2	0.3, 0.1
$C_{16:0}$–$C_{18:2}$	21.0 ± 2.0	22.2 ± 3.1	17.7 ± 1.5	22.5, 20.6	16.0, 18.6
$C_{16:0}$–$C_{20:4}$	3.6 ± 0.1	3.3 ± 0.8	7.0 ± 0.1	5.0, 6.8	7.4, 7.4
$C_{18:0}$–$C_{20:4}$	2.3 ± 0.8	2.5 ± 0	1.7 ± 0.7	1.8, 1.4	2.0, 1.6
$C_{16:0}$–$C_{22:6}$	14.2 ± 2.5	13.6 ± 2.1	14.7 ± 1.0	12.0, 13.4	11.4, 13.4
$C_{18:0}$–$C_{22:6}$ / $C_{18:1}$–$C_{18:2}$	15.8 ± 2.9	13.6 ± 0.8	14.9 ± 2.5	14.3, 14.9	14.5, 14.4
$C_{18:1}$–$C_{18:1}$	3.9 ± 0.6	3.7 ± 0.1	3.7 ± 1.1	3.7, 3.8	4.1, 3.4
$C_{18:1}$–$C_{20:4}$	4.0 ± 1.0	4.1 ± 1.3	5.7 ± 0.9	4.1, 5.8	6.8, 6.2
$C_{18:2}$–$C_{20:4}$	3.6 ± 0.5	3.1 ± 0.3	6.5 ± 0.8	4.5, 5.3	6.1, 7.7
$C_{18:1}$–$C_{22:6}$	0.9 ± 0.3	1.1 ± 0.2	1.3 ± 0	0.8, 1.0	1.3, 1.2

similar. Probably dipalmitoyl PG and PI will be synthesized on the basis of the dipalmitoyl proportion of PA present (Kato *et al.*, 1984) or generated (Rüstow *et al.*, 1985) in rat lung microsomes.

Table 2 shows that addition of inositol to the incubation mixture decreased the formation of CDP-DG and PG. This result promotes the idea that microsomal PG and PI synthesis may be possible in lung tissue.

As shown in Table 3, the addition of inositol did not change the species pattern of PG synthesis *de novo*. In 11 out of 14 individual species the radioactivity of PG was similarly distributed as in the PI synthesized after addition of inositol. The radioactivity of these 11 individual species accounted together for more than 80% of the total in both phospholipids.

Investigations of non-pulmonary tissues has shown that the characteristic species pattern of PI has to be produced by remodelling of '*de novo*'-formed PI (Akino & Shimojo, 1970; Holub & Kuksis, 1972; Holub & Piekarski, 1976). Therefore the question arose as to whether '*de novo*' synthesis only, or both '*de novo*' synthesis and remodelling, is the reason for the measured distribution of radioactivity among the individual species of PI and PG. We extended the incubation time and compared the species patterns of PI and PG synthesized for 10 and 60 min (Table 3). This approach is based on the idea that subsequent remodelling would change the '*de novo*'-synthesized species pattern in the time course of incubation. Because the species patterns did not change during an incubation time from 10 to 60 min, a remodelling of '*de novo*' synthesized PI and PG seems to be excluded in our system. This idea presumed that remodelling processes are slow in relation to '*de novo*' synthesis.

The species pattern of surfactant PI was different from that of endogenous microsomal PI, which contained, like microsomal PI from non-pulmonary sources, mainly $C_{18:0}$–$C_{20:4}$ (23.2%) and $C_{16:0}C_{18:1}$ (11.1%), although $C_{16:0}$–$C_{16:0}$ (5.1%) represented a minor component. By contrast, the species patterns of surfactant PG and microsomal PG were similar (Schlame *et al.*, 1986). These data may be explained by the heterogenity of lung microsomes, which were derived from different cell types present in lung. Obviously lung cells, other than Type II pneumocytes, contributed significantly to microsomal PI. But it may be assumed that microsomes of Type II cells only contain significant amounts of PG and represent its synthesis site. Therefore, it might be assumed, from the PI formation at the expense of PG and CDP-DG in lung microsomes (Table 2), that the measured incorporation of Gro3*P* into PG and PI reflected mainly the capacity of Type II cell microsomes for the synthesis of both phospholipids from a common pool of the CDP-DG presursor. The extended similarity of the species patterns of PG and PI synthesized *de novo* (Table 3) supported this idea. This peculiarity of lung microsomes does not exclude the possibility that the mitochondria may be an additional site of synthesis of surfactant PG.

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