Normal surface properties of phosphatidylglycerol-deficient surfactant from dog after acute lung injury[1]

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Abstract Lung surfactant was isolated from bronchoalveolar lavage of dogs during the late phase of recovery (15 days) from acute alveolar injury induced by subcutaneous injection of N-nitroso-N-methylurethane. This surfactant was compared with surfactant from control dogs in terms of in vitro surface properties, phospholipid composition and protein content, and those of its subfractions. Phospholipid composition and protein content were similar in the two groups, except that phosphatidylglycerol (PG) was markedly reduced and phosphatidylinositol (PI) was increased in the experimental group. In both, isopycnic densities of their subfractions in continuous sucrose density gradient were identical. The time course of surfactant adsorption was similar in both groups. Minimum surface tension (γmin) was 4.1 ± 1.5 dynes/cm in the experimental dogs and 3.8 ± 1.3 dynes/cm in the controls. Surface compressibility (SC), stability index (SI), and dynamic respreadability (DR) of the surfactants from the two groups were nearly identical. When compared to an artificial surfactant composed of dipalmitoyl phosphatidylcholine (DPPC) and PG in 9:1 molar ratio, a mixture of DPPC-PI 9:1 prepared identically showed similar γmin, SC, SI, and DR, and a much higher surface adsorption rate. These results suggest that PG is not essential for normal in vitro surfactant function and that its role may be assumed by PI. — **Liau, D. F., C. R. Barrett, A. L. L. Bell, and S. F. Ryan.** Normal surface properties of phosphatidylglycerol-deficient surfactant from dog after acute lung injury. *J. Lipid Res.* 1985. **26:** 1338-1344.

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Phosphatidylglycerol (PG) is the most abundant acidic phospholipid in the lung surfactant (1-3). It comprises up to 11% of the surfactant phospholipids (1, 4). PG-deficient surfactant has been found in the lungs of prematurely born rabbits and of patients with adult respiratory distress syndrome (5-7), and its presence in surfactant has been used as an indicator of fetal lung maturity (8). PG has been reported to decrease surface compressibility of the surfactant from postnatal rabbit lung (5), to regulate surface activities of the dipalmitoyl phosphatidylcholine (DPPC) from artificial surfactant (9-12), and to enhance the binding of DPPC with apoprotein and the adsorption rate of this lipoprotein complex (11). However, the functional role of this phospholipid in surfactant has not yet been clearly defined.

Deficiency of PG in surfactant could be induced by feeding myoinositol to adult rabbits (13). This sugar affected only the levels of PG and phosphatidylinositol (PI) but not other components in the surfactant (14, 15). Beppu, Clements, and Goerke (14) and Hallman, Enhorning, and Possmayer (15) using this model recently reported that deficiency of PG in the surfactant did not affect its normal surface properties. The pressure-volume relationships and gas exchange of the lungs deficient in surfactant PG were normal (14). Hallman et al. (15) found that the ability of PG-deficient surfactant to improve lung stability when instilled into preterm rabbits did not differ from that of normal surfactant. These studies suggested that PG may not be a critical determinant of lung surfactant function and that its specific role in the surfactant may be assumed by PI.

Acute alveolar injury closely resembling that seen in the lungs of humans with adult respiratory distress syndrome can be induced in dogs by subcutaneous injection of N-nitroso-N-methylurethane (NNNMU) (16, 17). Progressive decreases in alveolar lavage disaturated phosphatidylcholine (DSPC) and PG and in lung compliance were found during the first 7 days after administration of

Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; DPPC, dipalmitoyl phosphatidylcholine; DSPC, disaturated phosphatidylcholine; DPG, diphosphatidylglycerol; TLC, thin-layer chromatography; TN buffer, 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl; SC, surface compressibility; SI, stability index; DR, dynamic respreadability; NNNMU, N-nitroso-N-methylurethane.

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NNNMU (18, 19). Thereafter, the quantities of DSPC in alveolar lavage and the lung mechanics recovered toward normal (17, 19). In contrast, the quantities of PG remained markedly decreased throughout this period (19). Surface properties of the surfactant isolated from this PG-deficient alveolar lavage were studied and compared with those from control dogs. Our results suggest that PG is not an essential component for normal surface activity of the surfactant.

MATERIALS AND METHODS

The experiments were carried out on adult male mongrel dogs weighing 14–28 kg. Only dogs that had normal lung mechanics during a control period were used for the study. The dogs were divided into two groups, control and experimental. The experimental dogs received a single subcutaneous injection of 7 mg of NNNMU/kg body weight in 5 ml of saline and were killed with pentobarbital during the late phase of recovery (15 days after NNNMU) (16). The control dogs received no injection of saline since 5 ml of it was considered to have negligible effect in the dogs. The lung was excised quickly, lavaged, and perfused with cold TN buffer (0.01 M Tris HCl, pH 7.4, 0.15 M NaCl).

Detailed procedures used for the alveolar lavage, perfusion, and the analyses of the quantities and composition of lipids from alveolar lavage have been reported from this laboratory (18–21). In brief, the lung was weighed and degassed in a vacuum, and the bronchus, pulmonary artery, and pulmonary veins were cannulated. It was then lavaged six times via the cannulated bronchus, each time with 10 ml/g predicted normal lung weight (PLW) of cold TN buffer. Predicted normal lung weight was calculated from preinjection body weight using the data of Frank (22). The pulmonary artery was then perfused at a pressure of 30 cm of water with 1500 ml of cold TN buffer. The lung was used for the analysis of tissue phospholipids and other studies. The alveolar lavages were pooled and centrifuged at 200 *g* at 4°C for 10 min to remove cells. The supernatant was further filtered through a 5-μm Millipore filter (filter type: SM; Millipore Co., Bedford, MA) to complete the removal of cells and was used for assays of lipid and protein. Two aliquots, 600 ml each, were lyophilized and extracted for lipid analysis (23). The remainder (3000–4000 ml) was used for the isolation of surfactant for measurements of surface activity and for lipid and protein analyses.

Surfactant was isolated by centrifuging the alveolar lavages at 27,000 *g* for 120 min at 4°C (Beckman L5-40 ultracentrifuge, SW 27 rotor). The pellet was suspended in TN buffer to yield a suspension containing 5 mg of phospholipids/ml. This was distributed into several portions and stored at −20°C until used. Lipids were extracted by the same method as used for the lavage.

Phospholipids were separated by thin-layer chromatography (TLC) on silica gel 60 using the following solvents: (A) chloroform–methanol–conc. ammonium hydroxide 85:30:6 (v/v/v) (20) and (B) chloroform–methanol–water 65:25:4 (v/v/v) (21). Phosphatidylcholine (PC) (solvent A) and phosphatidylglycerol (PG) (solvent B) from the lavage and disaturated phosphatidylcholine (DSPC) (solvent B) from both the lavage and surfactant were quantitated by fluorometry after spraying the TLC plates with Rhodamine 6G (20). Separation of the phospholipids (Table 2) from the surfactant was accomplished by two-dimensional TLC using solvent A for the first dimension and solvent B for the second dimension. Individual phospholipids were then isolated from the TLC plate and lipid phosphorus was determined by the modified method of Beveridge and Johnson (24). Isolation of DSPC was carried out by the method of Mason, Nellenbogen, and Clements (25) except that the separation of DSPC from the reaction mixture was carried out by TLC (solvent B) rather than by column chromatography. Quantification of phospholipids by fluorometry has been found to give results similar to those obtained by lipid phosphorus assay. Protein content of the lavage and the surfactant was determined by the method of Lowry et al. (26) with the addition of 1% sodium dodecyl sulfate (SDS) to the reagent. The addition of SDS was necessary to prevent the turbidity caused by lipids.

Subfractionation of the surfactant was carried out in a linear sucrose density gradient (27). The surfactant was suspended in 2 ml of TN buffer and layered over a 34-ml linear sucrose density gradient (0.1 M–1.0 M in TN buffer). The gradient was centrifuged on an SW 27 rotor at 90,000 *g* for 4 hr at 4°C. The fraction was aspirated, diluted with TN buffer, and recovered by centrifugation (27,000 *g*, 1 hr). The resulting pellet was suspended in TN buffer and its total phospholipids and protein were determined as above.

Artificial surfactant was prepared by mixing DPPC–PG, DPPC–PI, or DPPC–PS in a molar ratio of 9:1. The mixture was then dispersed in TN buffer to form multilamellar liposomes according to the method of Bangham, Hill, and Miller (28) with slight modification. Twenty mg of the mixture was dissolved in 0.4 ml of chloroform and dried under N_2 to a thin film in a 20-ml test tube containing 20 glass spheres (3 mm in diameter) instead of rotary evaporation used by Bangham et al. (28). The dry film was dispersed in 4 ml of TN buffer to form a dispersion by gentle shaking at room temperature for 3 min. The phospholipid concentration of the dispersion was 5 mg/ml. With this procedure, large multilamellar liposomes could be obtained (29).

Surface tensions of the natural and artificial surfactants

were measured in a modified Wilhelmy balance at 37°C. A Teflon trough (15 cm × 6 cm, maximum and minimum surface area of 65 and 13 cm) lined with Teflon tape and a movable Teflon barrier was used. The trough was covered with a transparent plastic cover. No surfactant material was applied until the TN buffer subphase (50 ml) exhibited a clean surface (70 dynes/cm). Natural surfactant in TN buffer was dried under N_2 and resuspended in 50 μl of isopropanol–water–chloroform 2:1:0.5 (v/v/v) (7, 30) and artificial surfactant was taken directly from the aqueous dispersion. The sample containing 60 μg of total phospholipids was added to the surface with a microsyringe and with the barrier in the fully expanded position. After 10 min the spread films were compressed from 65 to 13 cm at 1 cycle/100 sec. The surface tension versus surface area was recorded continuously by an X-Y recorder (Hewlett Packard, San Diego, CA) for up to 2 hr.

Surface adsorption was measured by adding samples of natural and artificial surfactants, both in TN buffer containing 100 μg of phospholipids to 50 ml of TN buffer (2 μg/ml) in a Teflon beaker (4.0 cm diameter), and stirring constantly for 5 min with a magnetic stirrer (240 rpm) (12). After cessation of stirring, lipids adsorbed on the surface during stirring were removed by suction with removal of 3 ml of buffer to give a clean surface (70 dynes/cm). A platinum plate was then immersed to measure the surface tension. The decrease of surface tension was recorded on a strip-chart recorder for 1 hr.

The following surface properties of the natural surfactant and the artificial surfactant were determined: *1*) the minimal surface tension (γmin) at the end of the second compressions; *2*) surface compressibility (SC), SC = (1/A) (dA/dγ), calculated from the slope of the γ-A curve on the second compressions at γ = 15 dynes/cm; *3*) stability index (SI), SI = 2 (γmax − γmin)/(γmax + γmin) at second compressions; *4*) dynamic respreadability (DR), determined from the collapse plateau ratio (cycles 2/1) (31); and *5*) surface adsorption rate (Adπ), expressed as increase in surface pressure (π) per min during the first 10 min of recording. Three different concentrations of surfactant phospholipids were measured for each preparation and each measurement was done in duplicate.

Difference between mean values in the control and experimental dogs were evaluated using a conventional unpaired *t*-test (32).

Dipalmitoyl-DL-α-phosphatidylcholine (DPPC, synthetic), unsaturated L-α-phosphatidyl-DL-glycerol (PG, egg yolk lecithin), unsaturated L-α-phosphatidylinositol (PI, bovine brain), and unsaturated L-α-phosphatidyl-L-serine (PS, bovine brain) were obtained from Sigma Chemical Co., St. Louis, MO. They were analyzed by one-dimensional TLC using either solvent A or B, and were found to give single spots. All compounds were used, therefore, without further purification.

TABLE 1. Lung weight and composition of alveolar lavage and surfactant from control and experimental dogs

Dog	N	Predicted Lung Weight (PLW)	Alveolar Lavage					Surfactant				
			PC	DSPC	PG	PI	Protein	PC	DSPC	PG	PI	Protein
		g	*mg/g PLW*									
Control	8	199 ± 6	1.56 ± 0.31	0.76 ± 0.20	0.13 ± 0.4	0.061 ± 0.02	1.77 ± 0.02	0.63 ± 0.11	0.29 ± 0.06	0.094 ± 0.022	0.019 ± 0.006	0.17 ± 0.04
Experimental	7	188 ± 21	1.37 ± 0.63	0.61 ± 0.21	0.04 ± 0.02	0.112 ± 0.03	1.89 ± 0.31	0.58 ± 0.10	0.28 ± 0.05	0.015 ± 0.003	0.049 ± 0.013	0.16 ± 0.05
P		NS	NS	NS	<0.001	<0.005	NS	NS	NS	<0.001	<0.001	NS

See Table 2 for abbreviations of phospholipids. Values are means ± SD. NS, not significantly different.

TABLE 2. Phospholipid composition and phospholipid/protein ratio of surfactant from control and experimental dogs

		Control (n = 7)	Experimental (n = 5)	P
Phosphatidylcholine	(PC)	77.7 ± 3.4	78.8 ± 2.7	NS
Disaturated PC	(DSPC)	35.9 ± 2.5	38.2 ± 3.6	NS
Phosphatidylglycerol	(PG)	11.7 ± 2.8	2.0 ± 1.2	<0.001
Phosphatidylethanolamine	(PE)	2.5 ± 0.9	3.7 ± 1.2	NS
Sphingomyelin	(SPH)	2.6 ± 1.4	3.6 ± 1.3	NS
Phosphatidylinositol	(PI)	2.4 ± 0.8	6.6 ± 1.9	<0.001
Phosphatidylserine	(PS)	nd	nd	
Lysophosphatidylcholine	(LPC)	4.2 ± 2.9	3.3 ± 1.9	NS
Phospholipid/protein		4.9 ± 1.2	4.6 ± 0.7	NS

Values are means ± SD and are given as percent of total phospholipids. NS, not significantly different; nd, not detectable. Phospholipid/protein ratio was expressed as mg of phospholipid/mg protein.

RESULTS

The predicted lung weights of the experimental dogs were not significantly different from those of controls (**Table 1**). The quantities of PC, DSPC, PG, and PI and of proteins were determined in both the lavage and surfactant (Table 1). Except for PG and PI, none of these quantities expressed as mg/g of predicted lung weight was significantly different between the two groups. PG in the lavage and in the surfactant from the experimental dogs was strikingly decreased and PI was increased.

The phospholipid composition and the ratios of phospholipid to protein of the surfactants are given in **Table 2**. The percentages of PC, DSPC, PE, LPC and the ratios of phospholipid to protein were similar in the two groups. PG was very much lower in the experimental dogs than in the control dogs ($P < 0.001$), and the reverse was true for PI ($P < 0.001$). PS was not detectable.

Two subfractions were found in the surfactant from each group. The isopycnic densities and the phospholipid to protein ratios of these fractions in the two groups were almost identical (**Table 3**).

Surface adsorption of the surfactants, expressed as surface pressure increase versus time, were similar between groups (**Fig. 1**). Representative surface tension versus area diagrams of the surfactants from both groups are demonstrated in **Fig. 2**. Surface tension decreased to below 5 dynes/cm after the surface area was compressed from 65 cm to 13 cm. The minimum surface tension, surface compressibility, stability index, and dynamic respreadability did not significantly differ between the groups (Table 3).

Surface properties of the artificial surfactants are shown in **Table 4**. The mixtures DPPC-PG, DPPC-PI, and DPPC-PS were highly surface-active. They decreased surface tension to 0 dyne/cm. Surface compressibility, stability index, and dynamic respreadability of these mixtures were nearly identical. They adsorbed nearly linearly for 20 min (figures not shown) and the adsorption rates of DPPC-PI and DPPC-PS were significantly higher than that of DPPC-PG ($P < 0.001$).

DISCUSSION

Subcutaneous injection of NNNMU in dogs induced acute alveolar injury closely resembling that described in the adult respiratory distress syndrome in humans (16–17). The earliest lesion was injury to both types of alveolar epithelial cells while the capillary endothelium remained intact (16). Epithelial necrosis was accompanied by interstitial and perivascular edema and by alveolar collapse. These changes coincided with decreased compliance and increased elastic recoil of the lung (17), a marked decrease in alveolar lavage DSPC (18), and abnormal surface properties of the isolated surfactant (33). During recovery,

TABLE 3. Surface properties of surfactant, and densities and phospholipid/protein ratios of surfactant subfractions from control and experimental dogs

	Control (n = 5)	Experimental (n = 5)	P
Surface properties			
γmin (dyne/cm)	3.4 ± 0.8	4.1 ± 1.6	NS
SC (cm/dyne)	0.075 ± 0.021	0.071 ± 0.018	NS
SI	1.74 ± 0.03	1.73 ± 0.05	NS
DR (cycle 2:1)	0.89 ± 0.03	0.90 ± 0.07	NS
Density (g/cm^3)			
Subfraction 1	1.05 ± 0.01	1.05 ± 0.02	NS
Subfraction 2	1.09 ± 0.01	1.09 ± 0.02	NS
Phospholipid/protein			
Subfraction 1	7.3 ± 1.7	6.2 ± 1.1	NS
Subfraction 2	3.6 ± 0.8	3.5 ± 1.4	NS

Subfractions were separated by continuous sucrose density gradient. Values are means ± SD. γmin, minimum surface tension; SC, surface compressibility; SI, stability index; DR, dynamic respreadability; NS, not significantly different. Phospholipid/protein ratio was expressed as mg of phospholipid/mg protein.

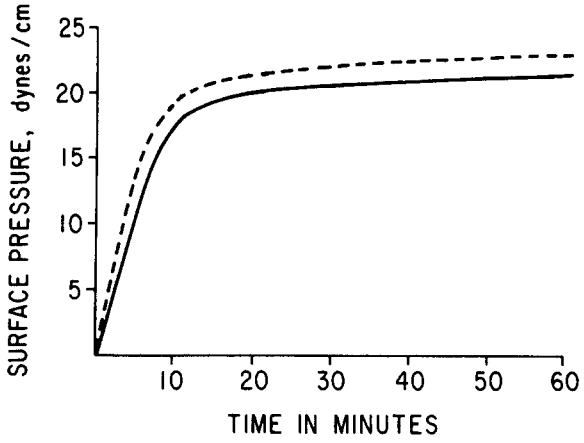

Fig. 1. Representative surface adsorption rates of surfactant from control (- - -) and experimental dogs (——). Each sample, containing 100 μg of phospholipids, was added to 50 ml of subphase solution. Surface pressure increase was measured continuously for 60 min.

massive regeneration of immature alveolar epithelial cells was followed by their differentiation to mature type II cells, recognizable by their cytoplasmic lamellar bodies. This differentiation coincided with improving lung mechanics and a return toward a normal amount of alveolar lavage DSPC (19). These findings suggest that, during this period, renewed production of normal surfactant occurred.

The present studies focus on characterization of the surfactants isolated from alveolar lavages of dogs during the late phase of recovery. Isolation of surfactant was carried out by commonly employed centrifugation procedures (7, 14, 27, 34). The yields of surfactant phospholipids accounted for 40% of total alveolar lavage phospholipids and were not significantly different from those of control dogs (Table 1). As shown in Table 1, the lungs during this period produced nearly normal amounts of PC, DSPC, and protein, determined either in the alveolar lavages or the isolated surfactants. Previous studies also showed that the fatty acyl group distribution of the lavage PC was almost identical in the two groups (35). However, the quantities of PG were persistently low and were accompanied by elevated levels of PI (Tables 1 and 2). Studies on the surfactant of the developing fetus reveal that PI initially increases parallel to DSPC, whereas PG appears later (5, 8). Competitive synthesis of PG and PI from a common precursor, CDP-diglyceride, was found in the lung and presumably takes place in type II cells (13). These findings suggest that induction of PI synthesis is accompanied by a decrease in PG synthesis and vice versa. Our previous study (19) on the quantities of PG and diphosphatidylglycerol (DPG) and the biosynthesis of DPG in this experimental model suggest that, during recovery, regenerating epithelial cells are the major site of the synthesis of PG and DPG. The decreased levels of PG in both alveolar lavage and lung tissue may be due to increased incorporation of PG into DPG, thus reducing the availability of PG for surfactant. Presumably, the DPG thus synthesized in the mitochondria is utilized for membrane construction in these new cells. It appears that PG deficiency is a characteristic of immature type II cells whether they are fetal or regenerating.

During the late phase of recovery, the in vitro surface properties, the densities of the subfractions, and the ratios of phospholipid to protein of the isolated surfactant, which is apparently deficient only in PG, were not different from those of control dogs (Figs. 1 and 2, Tables 2 and 3). Therefore, PG is apparently not a critical determinant for normal in vitro surfactant function. These findings agree with recent observations that PG-deficient surfactant isolated from inositol-fed adult rabbits possesses normal surface properties (14, 15). In order to determine whether the functional role of PG in surfactant can be assumed by PI, artificial surfactant containing either PG, PI, or another acidic phospholipid, phosphatidylserine (PS), in equal molar amounts were prepared. As shown in Table 4, surface properties (γmin, SC, SI, and DR) of both PI- and PS-containing artificial surfactants (DPPC-PI and DPPC-PS) were similar to those of PG-containing artificial surfactants (DPPC-PG). Surface adsorption rates of the two former artificial surfactants were much higher than those of PG-containing artificial surfactant. However, in comparison with natural surfactant (Fig. 1), their adsorption rates were low (0.65-0.75 dynes/cm per min vs. 1.8 dynes/cm per min). Metcalfe, Enhorning, and Possmayer (34) have demonstrated that artificial surfactant containing PI rather than PG was as effective as natural surfactant in prolonging survival of rabbit fetuses

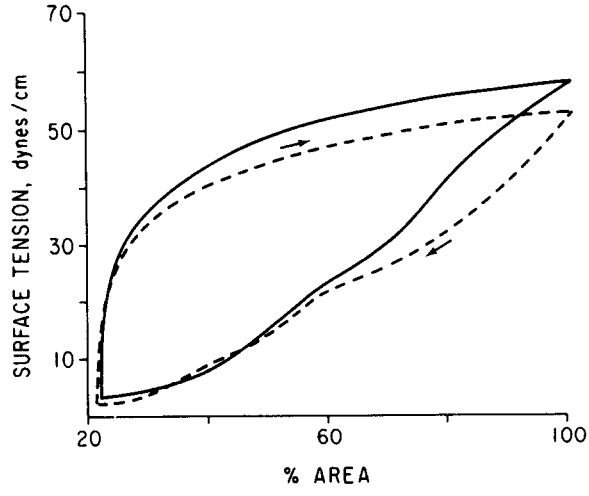

Fig. 2. Representative surface tension versus area diagrams of surfactant from control (- - -) and experimental dogs (——). Each sample, containing 60 μg of phospholipids, was applied to the surface in 50 μl of isopropanol-water-chloroform. Diagrams shown were recorded during the second cycle of compression and expansion of the surface.

TABLE 4. Surface properties of artificial surfactant

		DPPC-PG (9:1)	DPPC-PI (9:1)	DPPC-PS (9:1)	*P*
γmin	(dyne/cm)	0	0	0	NS
SC	(cm/dyne)	0.035 ± 0.003	0.028 ± 0.008	0.046 ± 0.004	NS
SI		1.60 ± 0.15	1.81 ± 0.11	1.81 ± 0.12	NS
DR	(cycle 2:1)	0.95 ± 0.04	0.96 ± 0.03	0.95 ± 0.02	NS
Adπ	(dyne/cm per min)	0.11 ± 0.03	0.75 ± 0.07	0.65 ± 0.05	<0.001

See Tables 2 and 3 for abbreviations of phospholipids and of surface properties, respectively. The mixture was dispersed in TN buffer by shaking in the presence of glass spheres and the dispersion was spread on the surface of the trough. Adπ, adsorption rate, was expressed as increment in surface pressure (dyne/cm per min) at 10 min. Values are means ± SD of four preparations. *P* values given are for the differences between DPPC-PI and DPPC-PG and between DPPC-PS and DPPC-PG. Surface properties of DPPC-PI and DPPC-PS are not significantly different. Adπ of DPPC-PI or DPPC-PS was higher than that of DPPC-PG ($P < 0.001$). NS, not significantly different.

delivered before term. In contrast, Ikegami, Silverman, and Adams (36) and Obladen, Klatt, and Bartholome (29) showed that a mixture of lipids containing PG was most efficient in restoring normal pressure-volume characteristics of surfactant-depleted animals when compared with mixtures containing other phospholipids.

Our results suggest that the presence of PG in the natural or the artificial surfactant is not essential for normal in vitro surfactant function and that its specific role may be assumed by other acidic phospholipids, e.g., PI or PS. However, the rapid turnover of PG in alveolar surfactant (37) and the speculations that PG might serve as a structural component of lamellar bodies (5) and as a precursor to DPG for membrane construction of lung cells (19) suggest additional roles for PG in lipid metabolism, or in the storage, secretion, or clearance of lung surfactant (14).

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