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PHOSPHATIDATE PHOSPHATASE

ACTIVITY AND PROPERTIES IN FETAL AND ADULT RAT LUNG

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Summary

The purpose of this work is to compare the properties of phosphatidate phosphatase (L-α-phosphatidate phosphohydrolase, EC 3.1.3.4) in fetal and adult rat lung and to establish the developmental profile of activity measured under optimal conditions. The maximal pH of 6.0—7.0 and the inhibition by fluoride, Ca^{2+} and detergents were similar for both adult and fetal. Phosphatidate phosphohydrolase activity was located in both mitochondria and microsomes. The localizations of marker enzymes indicated that the activity in these subfractions was not a result of cross contaminations. Very low activity was detected in the supernatant fraction and no Mg^{2+} requirement was demonstrable. The activity in the particulate fraction was about 50% of the adult from 18 day gestation until birth. Following birth, the activity rapidly increased to adult levels. Dipalmitoyl, dioleoyl and diacyl glycerol 3-phosphates are all utilized well as substrates. 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate was hydrolyzed faster under maximal conditions. The velocity-substrate curves tended to be sigmoidal, particularly when 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate was the substrate. Estimated apparent K_m values of 0.02—0.03 mM were obtained for fetal and adult preparations.

Introduction

Choline phosphoglycerides are synthesized in the lung primarily by the cytidine-dependent pathway [1—3]. The activity of this pathway increases with development coincident with the developmental rise in choline phosphoglyceride concentration [1,3—6]. The increase in the amount of choline phosphoglycerides is associated in time with the appearance of lamillar bodies in the

type II alveolar pneumocyte [7—10] and presumably reflects the maturation of the system for the production of 1,2-dipalmitoyl-*sn*-glycerol 3-phosphorylcholine, a necessary component of lung surfactant.

Previous investigations on the developmental regulation of the synthesis of choline phosphoglycerides in the lung concentrated on the reactions for the formation and utilization of CDPcholine [10—12]. However, since the final step in the synthesis of choline phosphoglycerides involves the transfer of phosphorycholine from CDPcholine to 1,2-diacyl-*sn*-glycerol, the efficient operation of the total pathway depends upon a continuous production of both 1,2-diacyl-*sn*-glycerol and CDPcholine. The amount of 1,2-diacyl-*sn*-glycerol available in the cell to react with CDPcholine depends upon both its rate of formation by the hydrolysis of phosphatidic acid and the rates of utilization in the formation of triglycerides and ethanolamine phosphoglycerides. Although modifications in the rates of synthesis of triglyceride and ethanolamine phosphoglyceride could effectively regulate the concentration of 1,2-diacyl-*sn*-glycerol, the regulation of the formation via the hydrolysis of phosphatidic acid by phosphatidate phosphatase appears more likely.

Phosphatidase phosphatase activity was first observed in the lung by Meban [13] who demonstrated by histochemical methods that the activity was localized in the type II granular pneumonocyte. Subsequently, phosphatidate phosphatase activity has been measured in adult and fetal rabbit lung [14], rabbit amniotic fluid [15], human amniotic fluid [16,17] and in lamillar bodies isolated from lung [18]. However, relatively little in-depth information is known about the comparative properties of the enzyme in fetal and adult lung.

The purpose of the present paper is to report our results on the detailed investigations of the activity and properties of phosphatidate phosphatase in lung from fetal and adult rats.

Experimental

Materials. Pregnant and non-pregnant (180—200 g) rats were obtained from Holtzman Company. The age of the fetuses was determined by considering the sperm positive date as day zero.

1,2-Diacyl-*sn*-glycerol 3-phosphate (from egg phosphatidylcholine) and 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate were from Sigma Chemical Co. 1,2-Dioleoyl-*sn*-glycerol 3-phosphate was purchased from Miles Laboratory. 1-Palmitoyl-*sn*-glycerol 3-phosphate was obtained from Serdary Research Laboratory. All were checked for purity by thin-layer chromatography on silica gel 60 plates (E-M Laboratories) in solvent systems A and B (see below). When the compounds showed greater than 5% impurities, they were purified by preparative thin-layer chromatography using solvent system B.

Sodium dodecyl sulfate, Tween-20, Lubrol WX and cetyl trimethyl ammonium bromide were obtained from Sigma Chemical Co. Sodium deoxycholate, and Triton X-100 were purchased from Fischer Scientific Co. Phospholipase D was purchased from Sigma.

Chromatography systems. Solvent systems for thin-layer chromatography were chloroform/methanol/acetic acid/water, 65 : 20 : 10 : 4 (Solvent A),

chloroform/methanol/4 M ammonium hydroxide, 75 : 37 : 7 (Solvent B); light petroleum/diethyl ether/acetic acid, 90 : 10 : 1 (Solvent C); diethyl ether/benzene/ethanol/acetic acid, 40 : 52 : 2 : 0.2 (Solvent D).

Preparation of 1,2-diacyl-sn-glycerol 3-phosphate. Lipid was extracted from rat lung or liver by the method of Folch [19]. Choline phosphoglycerides were isolated by DEAE-cellulose chromatography [20]. The isolated choline phosphoglycerides were hydrolyzed with phospholipase D [21] to form 1,2-diacyl-*sn*-glycerol 3-phosphate (phosphatidic acid) which was purified by preparative thin-layer chromatography using solvent system B. The area on the chromatogram corresponding to 1,2-diacyl-*sn*-glycerol 3-phosphate was scraped into tubes and recovered by extraction [22]. All lipid solutions were evaporated and stored under nitrogen at -40°C.

Preparation of radioactive 1,2-diacyl-sn-glycerol 3-phosphate. Radioactive 1,2-dioleoyl-*sn*-glycerol 3-phosphate was prepared enzymatically from *sn*-[U-^{14}C]glycerol 1-phosphate as described by Fallon [23]. The radioactive phosphatidic acid was purified by thin-layer chromatography on silica gel 60 plates with solvent system A. Radioactive 1,2-dioleoyl-*sn*-glycerol 3-phosphate had a specific activity of $1.26 \cdot 10^5$ cpm/μmol.

Preparation of subcellular fractions. Lung from adult rats (120—200 g) or from fetal rats was minced extensively with scissors and homogenized in a motor-driven Teflon glass homogenizer in 0.32 M sucrose/2.0 mM EDTA to obtain a 10% (w/v) homogenate. The homogenate was filtered through a 50 mesh stainless-steel wire filter. The filtered homogenate was centrifuged at $1000 \times g$ for 10 min. The sediment was washed twice with sucrose/EDTA (half the original volume of the homogenate). The combined $1000 \times g$ supernatant was centrifuged at $20\,000 \times g$ for 20 min. The crude mitochondrial pellet was resuspended in sucrose/ EDTA and collected again by centrifugation. The combined $20\,000 \times g$ supernatant was centrifuged at $100\,000 \times g$ for 60 min to obtain a pelleted microsome fraction and a $100\,000 \times g$ supernatant. The microsomes were suspended in 0.32 M sucrose (1.0 ml per g original lung).

Assay of phosphatidate phosphatase. The reaction mixture, unless stated otherwise, contained 100 mM Tris/maleate buffer pH 6.4, 0.35 mM phosphatidic acid and enzyme preparation in a volume of 1.0 ml. After the desired incubation time at 37°C, the reaction was stopped by the addition of 1.0 ml of 10% trichloroacetic acid. The amount of inorganic phosphate was measured in the supernatant. The amount of inorganic phosphate in zero time incubations was subtracted from all assay reactions. Initial control experiments indicated that no inorganic phosphate was released when the microsomes were incubated in the absence of phosphatidic acid nor was inorganic phosphate released when phosphatidic acid was incubated without microsomes.

Phosphatidic acid was added to the incubation mixtures as a sonicated suspension in the reaction buffer. The desired amount of phosphatidic acid was dissolved in hexane (approx. 0.1 ml per μmol phosphatidic acid). Tris/maleate buffer, pH 6.4, was added and the mixture sonicated for 1 min in a Heat Systems Model W185 sonifier at 50 W. The excess hexane was evaporated by blowing nitrogen through the solution. The use of hexane instead of diethyl ether produced phosphatidic acid preparations that were slightly more active as substrates in the reaction.

Analytical methods and marker enzyme assays. 5'-Nucleotidase and NADPH-cytochrome *c* reductase activities were determined as described by Mitchell and Hawthorne [24] and Sottocasa et al. [25], respectively. Succinate dehydrogenase activity was determined according to Pennington [26] using malonate in the blanks. The conditions for maximal activity for each enzyme were verified for each tissue fraction.

Inorganic phosphate was determined as described previously [4]. In the experiments using detergents, the resulting turbidity was cleared by the addition of sodium dodecyl sulfate [27] or by extracting once with 2.0 ml chloroform. Protein was estimated by the method of Lowry et al. [28].

Results and Discussion

General characteristics

The characteristics of phosphatidate phosphatase activity in homogenates and microsomes from adult and fetal lung were determined. Maximal activity was obtained at pH 6.0—7.0. The liberation of phosphorus was linear with time up to 20 min and proportional to protein up to 1.0 mg/ml in the assay. The addition of dithiothreitol, (1.0 mM), EDTA (1.0 and 10.0 mM), Mg^{2+} (1.0 and 10 mM), and iodoacetate (1.0 and 10.0 mM) to the assay mixture had no affect on the activity. 10 mM NaF produced a 70—80% inhibition. Calcium inhibited the activity 60% at 10 mM and 10—15% at 1.0 mM. Sodium deoxycholate produced no inhibition at concentrations of 3 mg/ml whereas sodium dodecyl sulfate, Lubrol WX, and Tween-20 at 3 mg/ml caused 80, 30 and 15% inhibition, respectively. Cetyl trimethyl ammonium bromide strongly inhibited the activity, 70—80% at 0.3 mg/ml. In all cases the effects were similar for both fetal and adult preparations. The detergent effects are very similar to those obtained with liver particulate phosphatidate phosphatase [29].

Radioactive 1,2-dioleoyl-*sn*-glycerol 3-phosphate was incubated under standard assay conditions with total particulate from fetal and adult lung. The total particulate is the material which is pelleted between 600 $\times g$ for 5 min and 100 000 $\times g$ for 60 min. After 20 min, the reaction mixtures were extracted with 2 : 1 chloroform/methanol by the method of Folch. The lipid extract was separated on thin-layer chromatography using both solvent systems C and D. Radioactive diglyceride was formed (140 and 80 nmol in 20 min for adult and fetal, respectively). No other radioactive products were detected.

Subcellular distribution

Phosphatidate phosphatase activity in both adult and fetal lung is located predominantly in particulate fractions (Table I). A small amount of activity is found in the soluble fraction in adult but no measurable activity is found in the soluble portions of fetal lung. The fetal lung apparently contains a higher proportion of activity in the mitochondria than the adult. A comparison of the distribution of selected marker enzymes (Table II) with the distribution of phosphatidate phosphatase indicates that the activity in both microsomes and mitochondria appears not to be a result of cross contamination. However, since the extent of contamination of these fractions by lysosomes and/or lamellar bodies is unknown, a definite conclusion cannot be made. The distribution

TABLE I

SUBCELLULAR DISTRIBUTION OF PHOSPHATIDATE PHOSPHATASE

The results are the average ± S.E. for four separate experiments. Fetal lung was from 21 day gestation rats. Phosphatidic acid prepared from rat liver phosphatidylcholine was used as substrate.

Fraction	Adult		Fetal	
	nmol/min per g lung	nmol/min per mg protein	nmol/min per g lung	nmol/min per mg protein
Homogenate	703 ± 51	4.1 ± 0.3	482 ± 41	4.9 ± 0.2
Nuclei and debris	316 ± 44	4.9 ± 0.5	212 ± 36	4.6 ± 0.7
Mitochondria	232 ± 32	16.7 ± 4	165 ± 21	19 ± 3
Microsome	300 ± 75	18.0 ± 4	107 ± 16	9.4 ± 2
Supernatant	35 ± 15	0.7 ± 0.3	0	0

pattern in lung is generally similar to that observed with other tissues where the activity is distributed among the subcellular fractions [29—34]. Evidence with other tissues indicates that the optimal substrate for the soluble phosphatidate phosphatase is particulate-bound phosphatidic acid [33—38]. We are not able to detect this type of activity in the lung supernatant. The use of membrane-bound substrate with lung supernatant does not increase the measurable activity (data not shown). This property of lung phosphatidate phosphatase is similar to that reported for heart where membrane-bound phosphatidate also was not a preferred substrate for soluble enzyme [39]. Reports with other tissues have suggested that the soluble enzyme requires Mg^{2+} for activity [40,41]. We dialyzed a portion of the adult lung supernatant against Tris/maleate buffer for 18 h. The activity in the dialyzed supernatant was determined in the presence and absence of Mg^{2+} (0.05—50 mM). No increase in activity was obtained at any of the Mg^{2+} concentrations. Supernatant was also assayed in the presence of 1.0 and 5.0 mM EGTA with 1.0 and 4.0 mM Mg^{2+}. Neither EGTA or EGTA plus Mg^{2+} produced any increase in enzyme activity.

Phosphatidate phosphatase activity during development

The activity in fetal lung was relatively constant at 70% of the adult from 18 day gestation (—4 day) until birth (Fig. 1). The activity increased rapidly

TABLE II

SUBCELLULAR DISTRIBUTION OF MARKER ENZYMES

The activity is presented as nmol/min per mg protein and is the average ± S.E. Mean for four experiments. Fetal lung was from 21 day gestation rats.

Fraction	5′-Nucleotidase		NADPH-cytochrome *c* reductase		Succinate dehydrogenase	
	Adult	Fetal	Adult	Fetal	Adult	Fetal
Homogenate	10 ± 2	10.6 ± 2	3.7 ± 0.4	9.5 ± 0.9	5.6 ± 0.8	13 ± 0.5
Nuclei and debris	9 ± 2	9 ± 0.9	3.3 ± 0.4	6.4 ± 7	3.1 ± 0.5	9.6 ± 1.5
Mitochondria	26.8 ± 6	34.2 ± 6	10.4 ± 2	16.2 ± 1.6	39.5 ± 1	61.5 ± 12
Microsomes	31.0 ± 11	22.3 ± 6	24.0 ± 7	16.2 ± 2.6	6.0 ± 1.7	5.6 ± 2
Supernatant	4.9 ± 2	1.3 ± 0.5	3.4 ± 0.6	2.6 ± 0.5	0	0

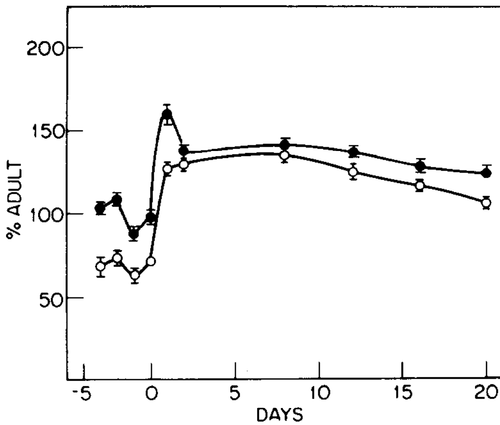

Fig. 1. Phosphatidate phosphatase activity during lung development. Each value is the average of 5 separate litters ± S.E.M. The activity is presented both per weight of lung, ○, and per mg total protein, ●. The adult values are 0.8 μmol/min per g lung and 8.3 ± 0.9 nmol/min per mg protein. The activity was measured in the total particulate fraction, i.e., that material which sedimented when a 10% homogenate was centrifuged at 100 000 × g 60 min.

after birth to reach 130% of the adult by 1 day and maintained this activity for 8—10 days before slowly dropping to adult values by 20 days. The activity in the lungs from groups of adult male, female and pregnant rats was not significantly different.

The developmental pattern in the rat differs from that reported for the rabbit [14]. The enzyme activity increases prior to birth in the rabbit. This apparent difference in the timing of the enzyme activity increase may be a reflection of the fact newborn rabbits are more developed at birth than are the newborn rats. The rapid increase after birth is consistent with the increased requirement for synthesis of choline phosphoglycerides for the formation of surfactant that is needed for lung function in the breathing animal. Presumably, the activity of phosphatidate phosphatase in fetal rat lung is sufficient to support the surge in biosynthetic activity prior to birth [1], when the continued requirement for choline phosphoglycerides is limited since most of the

TABLE III

SPECIFICITITY OF HYDROLYTIC REACTION FOR PHOSPHATIDIC ACID

The reaction was measured with adult lung microsomes under the conditions for phosphatidate phosphatase. The phosphatidic acid was from egg phosphatidylcholine.

Substrate	nmol/min per mg protein		
	0.10 mM	1.0 mM	1.8 mM
D-Glucose 6-phosphate	0.7	1.3	0.9
DL-α-Glycerol phosphate	0.3	0.9	1.2
β-Glycerol phosphate	0.1	1.1	0.8
p-Nitrophenyl phosphate	0.5	1.8	2.4
Phosphatidic acid		15.5	

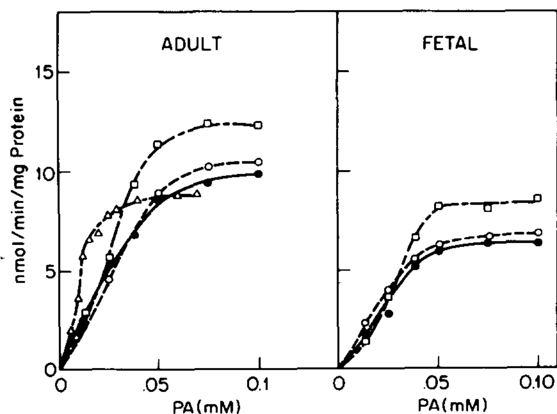

Fig. 2. Phosphatidic acid specificity for phosphatidate phosphatase activity. ●, 1,2-diacyl-*sn*-glycerol 3-phosphate prepared from egg phosphatidylcholine; ○, 1,2-dioleoyl-*sn*-glycerol 3-phosphate; □, 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate; △, monoacyl-*sn*-glycerol 3-phosphate. Microsome preparations were used as source of enzyme.

surfactant formed is for storage. Following birth an increased requirement for the synthesis of choline phosphoglycerides probably occurs because of the need for continuous formation of surfactant material for secretion. Thus, an increase in the capacity for choline phosphoglyceride synthesis would be met by increasing the activity of phosphatidate phosphatase.

Substrate specificity

The phosphatidate phosphatase reaction in lung microsomes is specific for phosphatidic acid (Table III). The rate of hydrolysis of glucose 6-phosphate, DL-α-glycerophosphate, β-glycerophosphate and *p*-nitrophenylphosphate is about 10% that of phosphatidic acid. Thus, under these conditions, the reaction is specific for phosphatidic acid. Little, if any, activity is due to deacylation of phosphatidic acid followed by hydrolysis of the glycerol 3-phosphate as has been reported to occur in liver [42]. The results described above with 1,2-diacyl-*sn*-[^{14}C]glycerol 3-phosphate also are consistent with this conclusion.

A comparison of the rate of hydrolysis of 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate, 1,2-dioleoyl-*sn*-glycerol 3-phosphate, and 1,2-diacyl-*sn*-glycerol 3-phosphate indicates that all are utilized well as substrates for phosphatidate phosphatase with both fetal and adult microsomes (Fig. 2). The 1,2-dipalmitoyl-*sn*-

TABLE IV

PHOSPHATIDIC SPECIFICITY FOR HYDROLYSIS BY ADULT LUNG MICROSOMES

The values presented show the standard deviation for triplicate assays. PA = phosphatidic acid.

Substrate	Velocity (nmol/min per mg protein)
0.1 mM diacyl PA	16.4 ± 0.20
0.05 mM diacyl PA + 0.05 mM dipalmitoyl PA	18.7 ± 0.18
0.1 mM dipalmitoyl PA	21.0 ± 0.20

glycerol 3-phosphate appears to give slightly faster rates of hydrolysis at saturating substrate concentrations. An additional series of measurements at 0.1 mM phosphatidic acid using a different adult microsomal preparation verified that the 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate consistently gives a higher reaction rate (Table IV).

Detailed information about substrate specificity for phosphatidate phosphatase from other tissues is relatively scarce. Early investigations with brain homogenates indicated that synthetically prepared phosphatidic acid was a poorer substrate than phosphatidic acid prepared from naturally occurring phosphatidylcholine [43—45]. Particularly noteworthy is the observation that 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate was inactive as a substrate in brain preparations [44]. McCaman et al. [46] found no significant difference in activity towards phosphatidic acids with different fatty acid compositions. Particulate enzyme from intestine generally showed highest activity with 1,2-dioleoyl-*sn*-glycerol 3-phosphate [47] and the activity towards disaturated phosphatidic acids was far lower. Thus, the greater activity towards 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate by lung appears to be somewhat unique and is consistent with the unique ability of lung to synthesis large amounts of 1,2-dipalmitoyl-*sn*-glycerol 3-phosphoryl choline. Substrate specificities obtained by adding phosphatidic acid exogenously to the assay must be interpreted with some caution due to differences in the physical state of the substrate. However, the dipalmitoyl species is the most insoluble and the most difficult to prepare in uniform suspensions.

The velocity-substrate curves shown in Fig. 2 do not follow Michaelis-Menten kinetics, but rather tend to be sigmoidal curves. Additional experiments with more determinations at lower substrate concentrations showed that a definite lag in the velocity vs. substrate curve occurs at low substrate concentrations. This result is particularly striking when 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate is used as substrate (Fig. 3). Biphasic substrate saturation curves have also been reported for phosphatidate phosphatase from *Bacillus subtilis* [48]. The failure of enzymes which utilize lipid substrates to follow Michaelis-Menten kinetics is not unusual and has been discussed at length by Gatt [49]. According to the classifications proposed by Gatt, the substrate curves observed for phosphatidate phosphatase indicate that under these conditions the enzyme utilizes micelles and that monomeric forms of phosphatidic acid are utilized at a much lower rate.

In an attempt to clarify this possibility several experiments were conducted in which the phosphatidic acid was prepared under different conditions. Sonication of the phosphatidic acid for 90 min gives a clear preparation. This preparation produces the same general velocity-substrate curve but a somewhat higher maximal velocity when compared to phosphatidic acid sonicated for 30 s. Phosphatidic acid from egg phosphatidylcholine was sonicated with equal amounts of phosphatidylcholine. The substrate-velocity curve was unchanged with this substrate preparation. Similarly, phosphatidic acid was sonicated in the presence of equal amounts of cholesterol. This preparation also gave the same substrate-velocity curve as phosphatidic acid alone.

Accurate calculations of K_m for phosphatidic acid are difficult because of the sigmoidal nature of the substrate-velocity curves. However, apparent K_m

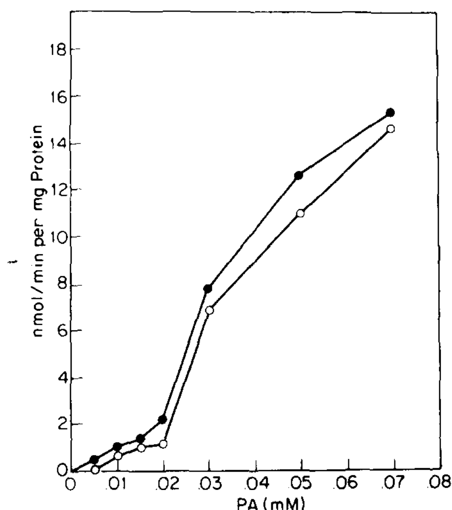

Fig. 3. Phosphatidate phosphatase activity versus 1,2-dipalmitoyl-*sn*-glycerol 3-phosphate concentration. •, dipalmitoyl phosphatidic acid prepared by 2 min sonication; ○, dipalmitoylphosphatidic acid prepared by 90 min sonication. Microsomes from adult lung were the source of enzyme. PA, phosphatidic acid.

values of 0.02—0.03 mM, estimated from the data shown in Fig. 2, indicate that the values are generally similar for fetal and adult preparations and for the various substrates used. These K_m values are generally 5—10 times lower than values reported for other tissues [35,36,41]. Two recently published studies of partially purified preparations from liver [40] and muscle [50] have reported K_m values of 0.05—0.06 mM, which approach the values found in lung.

Despite the evidence that the CDPcholine pathway operates primarily to form unsaturated pulmonary choline phosphoglycerides [51], our results indicate that the properties of phosphatidate phosphatase would allow this enzyme to function in the formation of 1,2-dipalmitoyl-*sn*-glycerol 3-phosphorylcholine. Furthermore, the results of studies on the enzyme activity during rabbit lung development as well as reports that phosphatidate phosphatase is present in human amniotic fluid have been interpreted to support a specific role for phosphatidate phosphatase in lung maturation [16—18]. The association of phosphatidate phosphatase activity with purified surface-active material [52] as well as the observation that glucocorticoids administration increases the activity [53] provide further support for the functional role of phosphatidate phosphatase in the regulation of the synthesis of choline phosphoglyceride in the lungs.

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