INDUCTION OF SURFACTANT PHOSPHATIDYLGLYCEROL IN THE LUNG OF FETAL AND NEWBORN RABBITS BY DIBUTYRYL ADENOSINE 3':5'-MONOPHOSPHATE

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SUMMARY: Dibutyryl adenosine 3':5'-monophosphate had an age-dependent effect on phosphatidylglycerol synthesis in the lung. In lung slices from preterm and term fetuses it specifically stimulated phosphatidylglycerol incorporation, but had no effect on immature or postnatal lung. At term the stimulation disappeared within one hour following the delivery, whereas in preterm animals the stimulation persisted. Also, dibutyryl adenosine 3':5'-monophosphate given to preterm animals at birth induced surfactant phosphatidylglycerol and increased the release of surfactant into alveolar spaces during the first neonatal hour.

INTRODUCTION

Besides dipalmitoyl lecithin the surfactant complex of the lung contains other components. Characteristically phosphatidylglycerol represents about 10 % or even more of phospholipids recovered from surfactant in the adults (1-3). The function of this unique molecule is still speculative. In the human newborn with respiratory distress syndrome phosphatidylglycerol is always lacking in lung effluent and sometimes it seems to be the only deficient surfactant phospholipid (4). This suggests that phosphatidylglycerol is important in stabilization of alveoli.

In the rabbit fetus from the age of 28 days large amounts of surfactant are present in alveolar lining as intracellular lamellar inclusion bodies. Many of those organelles release their contents into the alveoli first after the birth (5,6). The fetal surfactant contains little if any phosphatidylglycerol that first

Abbreviation: cyclic AMP, adenosine 3':5'-monophosphate

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appears at birth (7).

A recent evidence of Rooney et al. suggests that three days after cortisol administration to a 24-day-old fetal rabbit, lung phosphatidylglycerol synthesis was activated (8). According to the present evidence the perinatal regulation of surfactant phosphatidylglycerol does not involve only the biosynthesis of an enzyme protein, since cyclic AMP induces this phospholipid by a rapid mechanism apparently not requiring protein synthesis.

MATERIALS AND METHODS

The gestational age of New Zealand rabbits was known within eight hours. Pregnant does were killed by intravenous air, and hysterectomy was rapidly performed. When fetuses were used the animals were removed from their amniotic sacs and sacrificed before letting them breathe. The lungs were chilled to ice-cold Krebs-Ringer phosphate (9) and most of bronchial tissue was removed. Subsequently the slices weighing from 45 to 70 mg were prepared using Stadie-Riggs slicer (Thomas, Philadelphia, Pa.). The incubation with the radioactive isotopes took place at 37°C for 30 minutes in flat-bottomed flasks containing 0.7 ml of the medium. For further details see ref. 10.

The lipids were extracted and the phospholipids isolated using two-dimensional thin-layer chromatography (2). The first dimension was developed with chloroform-methanol acetic acid- H_2O (390-150-48-24 v/v) and the second with tetrahydrofuran-methyl-al-methanol- 2 N NH_4OH (400-285-78-42). To visualize phosphatidylglycerol spot in fetal animals 50 nmoles of carrier isolated from adult rabbit lungs was used in individual runs. The lipid spots were visualized with iodine, scraped and the radioactivity recorded using Wallac SC-20 liquid scintillation spectrometer.

Unsaturated lecithins were adducted with mercuric acid (10) and disaturated lecithins were subsequently isolated in two-dimensional thin-layer chromatography.

Proteins (11) and phospholipids (12) were quantified using standard methods.

Alveolar wash was performed essentially as described by Gluck et al. (13). The airways were lavaged through the tracheal cannula four times each time using 1.5 ml of ± 150 mM NaCl.

The results were expressed as the mean ± S.E.

RESULTS

Fig. 1 shows the rate of [^{14}C]-palmitate and [^{3}H]-glycerol incorporation into phosphatidylglycerol in lung slices from perinatal rabbits and the effect of dibutyryl cyclic AMP. Theophylline was routinely added to the incubation medium.

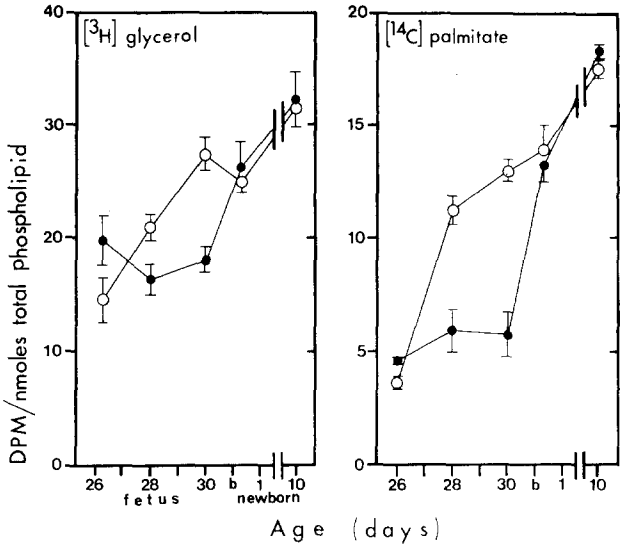

Fig. 1. Incorporation of glycerol and palmitate into phosphatidylglycerol in lung slices from perinatal rabbits. Effect of 0.25 mM dibutyryl cyclic AMP (—o—). For experimental details see METHODS and Table 1.

Omission of this addition did not change the rate of incorporation in any age groups. However, the effect of cyclic AMP was somewhat attenuated in the absence of theophylline (data not shown). The apparent rate of palmitate incorporation into phosphatidylglycerol increased more during the development than the corresponding rate of glycerol. This suggests an increase in the amount of saturated phosphatidylglycerol.

Between 28th and 30th fetal day dibutyryl cyclic AMP significantly stimulated phosphatidylglycerol incorporation whereas in previable fetuses and postnatal animals it had no effect.

Table 1 shows the effects of cyclic AMP on phosphatidylglycerol incorporation into lung slices, when the fetuses were first removed from the uterus and then kept in room air at 30°C for one hour. Consequently in 28-day-old animals the stimulation was even higher than in the fetuses. In striking contrast the

Table 1. Incorporation of [^{3}H] glycerol and [^{14}C] palmitate into phosphatidylglycerol in lung slices of the newborn one hour following the delivery.

	Dibutyryl cyclic AMP	DPM/nmoles total phospholipid	
		^{3}H	^{14}C
Newborn, 28 fetal days	-	16.4±1.4 (10)	6.4±0.2
	+	27.1±1.9*	11.5±0.7*
Newborn, 30 fetal days	-	24.8±2.0 (6)	12.8±0.6
	+	24.9±1.6	14.0±1.1

The incubation took place in Krebs-Ringer bicarbonate (9) containing 0.061 mM [1-^{14}C] palmitate (29 mCi/mmoles), 0.005 mM [1(3)-^{3}H] glycerol (1400 mCi/mmoles), 0.8 mM theophylline and when indicated 0.25 mM dibutyryl cyclic AMP.
*Significant difference ($p < 0.001$) as compared to that without cyclic AMP.

term animals after one hours air-breathing did not display any stimulation at all by the drug.

Table 2 presents the effects of dibutyryl cyclic AMP on palmitate and glycerol incorporation into lecithin and phosphatidylinositol. Palmitate incorporation significantly increased between 26th and 30th fetal days suggesting the activation of the synthesis of the saturated surfactant phospholipids. In addition, cyclic AMP seemed to decrease phosphatidylinositol incorporation in 28- and 30-day-old fetuses.

28-day-old premature animals were chosen to further study the effect of dibutyryl cyclic AMP on surfactant. This seemed interesting since the cyclic nucleotide stimulated phosphatidyl-

Table 2. Incorporation of [^{3}H] glycerol and [^{14}C] palmitate into lecithin and phosphatidylinositol in lung slices from rabbits of different ages

	Dibutyryl cyclic AMP	DPM/nmoles total phospholipid			
		Lecithin		Phosphatidyl-inositol	
		^{3}H	^{14}C	^{3}H	^{14}C
Fetus, 26 days	-	164±12 (6)	320±10	78±3	25±1
	+	181±10	343±17	68±5	24±1
Fetus, 28 days	-	136±9 (10)	676±34	99±8	59±4
	+	152±8	631±29	80±4*	49±4*
Fetus, 30 days	-	155±7 (6)	481±20	76±2	52±2
	+	165±9	466±17	71±3	46±1*
Newborn, 10 days	-	89±2 (4)	426±31	62±3	40±3
	+	81±6	480±27	56±2	33±3

For experimental details see METHODS and Table 1.
*$p < 0.1$ as compared to that without cyclic AMP.

glycerol incorporation both in the fetus and in the newborn, suggesting that the triggering of the increase in phosphatidylglycerol synthesis was deficient. Therefore dibutyryl cyclic AMP or saline alone was intraperitoneally given to the newborn at birth. During the first neonatal hour the controls often had retractions and cyanosis in room air, whereas those who received the drug were acyanotic with no apparent respiratory distress.

Table 3 shows the assays of the total phospholipids in the alveolar wash and the residual lung in 28-day-old rabbits one hour after their removal from the uterus. The amount of phospholipids recovered during airway lavages was higher after dibutyryl

Table 3. Total phospholipids in alveolar wash and in the residual lung in 28-day-old rabbits one hour after the delivery. Effect of intraperitoneal cyclic AMP at birth*

	Control	Cyclic AMP
Alveolar wash phospholipids		
nmoles/animal	395±31 (8)	639±40** (9)
nmoles/mg protein	1699±154	1975±160
Residual lung phospholipids		
nmoles/animal	5705±439	5618±430
nmoles/mg protein	175±6	164±8
Total lung phospholipids		
nmoles/animal	6100±441	6257±424
nmoles/mg protein	186±6	181±7
Alveolar wash/ Total lung		
nmoles/nmoles x 100	6.5±0.4	10.2±0.6**

*10 mg of dibutyryl cyclic AMP in 0.1 ml of 75 mM NaCl or saline alone was intraperitoneally given to the newborn within two minutes after their removal from the uterus.

**Significant difference ($p < 0.001$) as compared to the control.

cyclic AMP than that of the controls. In controls 6.5 % of total phospholipid was recovered in alveolar washes, whereas after cyclic AMP the corresponding percentage was 10.2. The low sphingomyelin content (Table 4) and high content of disaturated lecithin (control: 49 % of total lecithin, dibutyryl cyclic AMP:54 %) indicates that the material recovered during the lavages was largely surfactant. Therefore, the drug seems to increase the release of surfactant into alveoli (cf. ref. 14).

Table 4 shows the percentage distribution of individual

Table 4. The percentage phospholipid composition in alveolar wash and in residual lung after the alveolar lavage in 28-day-old rabbits one hour after the delivery. Effect of intraperitoneal dibutyryl cyclic AMP at birth.

	% of total phospholipids			
	Alveolar wash		Residual lung	
	Control	Cyclic AMP	Control	Cyclic AMP
Lecithin	73.8±2.3	72.4±2.7	55.1±2.2	56.3±2.6
Phosphatidylglycerol	0.4±0.3	3.4±0.3*	0.7±0.1	1.1±0.1**
Phosphatidylinositol	12.6±0.4	11.7±0.7	4.0±0.1	4.2±0.3
Phosphatidyl-ethanolamine + Phosphatidylserine	7.8±1.0	6.5±0.6	25.2±1.3	24.0±0.7
Sphingomyelin	1.9±0.1	2.1±0.2	7.1±0.2	7.2±0.3
	96.5±2.1	96.1±2.9	92.1±2.6	92.8±3.0

For experimental details see Table 3.
*Significant difference ($p < 0.0001$) as compared to the control.
**Probably significant difference ($p < 0.05$) as compared to the control.

phospholipids in the lung. In alveolar lavage phosphatidylglycerol became detectable within one hour following dibutyryl cyclic AMP, whereas in controls this phospholipid remained virtually absent (control: 2±2 nmoles/animal, dibutyryl cyclic AMP: 22±2). Surfactant phosphatidylglycerol appeared even if 0.5 mg of puromycin was given 5 minutes prior to the cyclic nucleotide (17±1 nmoles/animal). The residual lung after the alveolar wash always contained some phosphatidylglycerol and the administration of cyclic AMP only slightly increased this phospholipid (control:

40±4 nmoles/animal, dibutyryl cyclic AMP: 62±6). The percentage distribution of the other phospholipids in alveolar wash and the residual lung did not reveal significant difference.

DISCUSSION

In the present study the induction of phosphatidylglycerol in the lung by dibutyryl cyclic AMP was demonstrated. Its promptness and failure of puromycin inhibition suggest that the effect was not due to stimulation of protein synthesis. Other aspects of the mechanism of the induction are open and remain to be elucidated.

Traces of phosphatidylglycerol found in mitochondria serve as precursor of cardiolipin (15). In contrast surfactant phosphatidylglycerol is qualitatively the second phospholipid after lecithin in adults and seems to be an endproduct rather than a precursor (16). According to the present results only phosphatidylglycerol recovered from alveolar lavage was induced by cyclic AMP at birth. In contrast, phosphatidylglycerol in the residual lung was only slightly increased. This could be due to selective induction of phosphatidylglycerol associated to lamellar bodies. Moreover, cyclic AMP did not increase the incorporation into phosphatidylglycerol in lung slices from the immature lung that hardly contain any surfactant, supporting the view that the stimulation specifically involves surfactant phosphatidylglycerol.

Glucocorticoids given on the 24th day to the fetus increase three days later the activity of glycerophosphate phosphatidyltransferase in lung homogenate (8), the rate-limiting enzyme of phosphatidylglycerol synthesis (17,16). This finding is not necessarily contradictory to the present one since the induction could be a sequential process: The enzymes may be synthesized first. As the second step cyclic AMP could activate the pathway

by as yet unknown mechanism and induce surfactant phosphatidylglycerol. Therefore, glucocorticoids may have a permissive role in the cyclic AMP mediated effect.

Prompt disappearance of cyclic AMP stimulation following the neonatal induction of phosphatidylglycerol (Table 1) suggests that cyclic nucleotides are physiologically involved in perinatal regulation of the surfactant component. According to this assumption hormone(s) sofar unidentified regulate cyclic nucleotide levels in the alveolar epithelial lining.

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REFERENCES

1. Rooney, S. A., Canavan, P. M., Motoyama, E. K. (1974) Biochim. Biophys. Acta, 360, 56-67.
2. Hallman, M., and Gluck, L. (1974) Biochem. Biophys. Res. Commun., 60, 1-7.
3. Sanders, R. L., and Longmore, W. J. (1975) Biochemistry, 14, 835-840.
4. Hallman, M., Feldman, B. H., and Gluck, L. (1977) Pediat. Res., 11
5. Kikkawa, J., Motoyama, E. K., and Gluck, L. (1968) Am. J. Pathol., 52, 177-209.
6. Gluck, L., Sribney, M., and Kulovich, M. V. (1967) Pediat. Res., 1, 247-265.
7. Hallman, M., and Gluck, L. (1976) J. Lipid Res., 17, 51-56.
8. Rooney, S. A., Gross, I., Gassenheimer, L. N., and Motoyama, E. K. (1975) Biochim. Biophys. Acta, 398, 433-441.
9. Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1964) Manometric Techniques, pp. 132-133, Burgess Publishing Company, Minnesota.
10. Hallman, M., and Raivio, K. (1974) Pediat. Res., 8, 874-879.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem., 193, 265-275.
12. Bartlett, G. R. (1959) J. Biol. Chem., 234, 466-471.
13. Gluck, L., Motoyama, E. K., Smits, H., and Kulovich, M. V. (1967) Pediat. Res., 1, 237-246.
14. Stahlman, M. T., Gray, M. E., Liew, S., and Chytil, F. (1974) Pediat. Res., 8, 470.
15. Hostetler, K. Y., Van Den Bosch, H., and Van Deenen, L. L. M. (1971) Biochim. Biophys. Acta, 239, 113-119.
16. Hallman, M., and Gluck, L. (1975) Biochim. Biophys. Acta, 409, 172-191.
17. Kiyasu, J. Y., Pieringer, R. A., Paulus, H., and Kennedy, E. P. (1963) J. Biol. Chem., 238, 2293-2298.