University of Manitoba
Faculty of Graduate Studies

Ca^{2+}-PS-dependent Protein Kinase C Activity in Fetal, Neonate and Adult Rabbit Lung and the Release of Surfactant-Related Material from Isolated Fetal Rabbit Type II Alveolar Cells.

Submitted in partial fulfillment of the degree of Master of Science,

University of Manitoba, September, 1996

Submitted by:

Emile Samuels.

5274671

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

*****

COPYRIGHT PERMISSION PAGE

Ca^{2+}-PS- DEPENDENT PROTEIN KINASE C ACTIVITY IN FETAL, NEONATE AND ADULT RABBIT LUNG AND THE RELEASE OF SURFACTANT-RELATED MATERIAL FROM ISOLATED FETAL RABBIT TYPE II ALVEOLAR CELLS

BY

EMILE SAMUELS

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank Dr. J.E. Scott , my advisor, for all his help and immeasurable patience.

Also invaluable to this project were the assistance and guidance of Ms. Elke Grief and Dr. S-Y. Yang.

I would like to thank the Manitoba Lung Association for their financial support.

Finally, I would like to thank my family for their support, understanding and encouragement throughout this project.

TABLE OF CONTENTS

ABSTRACT 8

INTRODUCTION 10

2.1 SUBDIVISIONS 10
2.1a Conducting Region 11
2.1b Respiratory Region 13

2.2 EPITHELIUM 14
2.2a Macrophages 15
2.2b Fibroblasts 15
2.2c Type I Pneumocytes 16
2.2d Type II Pneumocytes 17

2.3 SURFACTANT 18
2.3a Background 18
2.3b Composition 22
2.3c Synthesis 24
2.3d Function 29

2.4 INITIATION AND REGULATION OF SURFACTANT METABOLISM 30
2.4a Synthesis 31
2.4b Secretion 34
2.4c Recycling 38

2.5 PROTEIN KINASE C AND SURFACTANT 39

MATERIALS AND METHODS 42

3.1 MATERIALS 42

3.2 METHODS 42
3.2a Subcellular Fractionation Procedures 42
3.2b Protein Quantification 44
3.2c Phospholipid Quantification 44
3.2d Analysis of Lamellar Body Fraction 45
3.2e Enzyme Assays 47
3.2f Inhibition Assays 48
3.2g Delipidation of Fractions 48
3.2h Isolation of Pre-Type II Alveolar Cells 49
3.2i Addback Assays 50
3.2j Synthesis of Surfactant-Related Phospholipid 51
3.2k Release of Surfactant-Related Phospholipid 53
3.2l Test of TPA Cytotoxicity 53
3.2m Statistics 54

RESULTS **55**

4.1 WHOLE LUNG 55
4.1a Protein and Phospholipid 55
4.1b Analysis of Lamellar Body Phospholipids 59
4.1c Protein Kinase C Activity 59
4.1d Specific and Total PKC Activity 60
4.1d.i Specific Activity 68
4.1d.ii Total Activity 71
4.1e Inhibition Assays 71
4.1f PKC Activity in Delipidated Fractions 76

4.2 ISOLATED CELLS 78
4.2a PKC Activity in Lamellar Body Fraction 78
4.2b Distribution of Radioactivity in Fetal Type II Alveolar Cells After TPA Exposure 78
4.2c Release of Radioactive Precursors in Response to TPA Stimulation 79
4.2d Remaining Radioactivity after TPA induced Release 90

DISCUSSION **93**

5.1a Phospholipid and Protein levels in the Developing Rabbit Lung 95
5.1b Phospholipid Composition in Lamellar Body Fraction 96
5.1c PKC Activity in Rabbit Lung 96
5.1d Subcellular Distribution of PKC Activity in Rabbit Lung 97
5.1e PKC activity in fetal and neonate lung 98
5.1e.i Specific Activity 98
5.1e.ii Total Activity 99
5.1f Inhibition of PKC Activity 100
5.1g PKC Activity in Delipidated Fractions 101
5.1h PKC Activity and Lamellar Bodies 101
5.1i PKC and Synthesis of Surfactant-related Phospholipid 102
5.1j PKC and Release of Surfactant-related Phospholipid 103

5.2 CONCLUSIONS 104

5.3 FUTURE DIRECTIONS 105

REFERENCES **107**

LIST of TABLES and FIGURES

FIGURE 1 : LIPID BIOSYNTHESIS 28
TABLE 1 : ADDBACK PREPARATIONS 52
TABLE 2 : PROTEIN AND PHOSPHOLIPID CONTENT OF SELECTED RABBIT LUNG SUBCELLULAR FRACTIONS 56
FIGURE 2 : PROTEIN AND PHOSPHOLIPID CONTENT FROM ADULT RABBIT LUNG 58FRACTIONS 58
TABLE 3 : PHOSPHOLIPID PROFILES FROM RABBIT LUNG LAMELLAR BODY FRACTION FROM 3 AGE GROUPS. 61
FIGURE 3 : CA^{2+}-PS-DEPENDENT AND TOTAL PROTEIN KINASE C ACTIVITY IN ADULT RABBIT LUNG LAMELLAR BODIES AND MICROSOMES 63
FIGURE 4 : A TIME COURSE FOR CA^{2+}-PS-DEPENDENT AND TOTAL PROTEIN KINASE C ACTIVITY IN MICROSOMAL FRACTION OF ADULT AND GESTATIONAL DAY 24 RABBIT LUNGS 65
FIGURE 5 : SPECIFIC AND TOTAL ACTIVITY OF CA^{2+}-PS-DEPENDENT PROTEIN KINASE C ACTIVITY IN SUBCELLULAR FRACTIONS ISOLATED FROM ADULT RABBIT LUNG 67
FIGURE 6 : SPECIFIC ACTIVITY OF CA^{2+}-PS-DEPENDENT PROTEIN KINASE IN HOMOGENATE AND SUBCELLULAR FRACTIONS OF FETAL NEWBORN AND ADULT RABBIT LUNGS. 70
FIGURE 7 : TOTAL ACTIVITY OF CA^{2+}-PS-DEPENDENT PROTEIN KINASE IN HOMOGENATE AND SUBCELLULAR FRACTIONS OF FETAL NEWBORN AND ADULT RABBIT LUNGS. 73
FIGURE 8 : EFFECT OF THE PKC INHIBITORS ACRIDINE ORANGE, 9-AMINOACRIDINE , AND SANGIVAMYCIN ON ENZYME ACTIVITY IN LAMELLAR BODY AND MICROSOMAL FRACTIONS. 75
TABLE 4 : ACTIVITY OF RABBIT LUNG FRACTIONS AFTER DELIPIDATION. 77
FIGURE 9 : CA^{2+}-PS-DEPENDENT PROTEIN KINASE C ACTIVITY IN LAMELLAR BODY FRACTION AND MICROSOMAL FRACTIONS ISOLATED FROM FETAL TYPE II ALVEOLAR CELLS. 81
FIGURE 10 : RESULTS OF THE ADDBACK ASSAYS ON SAMPLES EXPOSED TO SFMEM AND TPA. 83
FIGURE 11 : DISTRIBUTION OF RADIOACTIVITY AFTER CONCURRENT INCUBATION OF FETAL ALVEOLAR TYPE II CELLS WITH TPA AND RADIOACTIVE PRECURSORS. 85
FIGURE 12 : DISTRIBUTION OF RADIOACTIVITY IN MATERIAL RELEASED BY PRELABELED FETAL ALVEOLAR TYPE II CELLS AFTER EXPOSURE TO TPA. 87
FIGURE 13 : ELECTRON MICROGRAPH OF MATERIAL COLLECTED FROM CULTURE MEDIA OF FETAL ALVEOLAR TYPE II CELLS EXPOSED TO TPA. 89
FIGURE 14 : DISTRIBUTION OF RADIOACTIVITY IN PRELABELLED FETAL ALVEOLAR TYPE II CELLS AFTER EXPOSURE TO TPA 92

ABSTRACT

The fetal lung secretes significant quantities of surfactant during late gestation in preparation for respiration which must begin immediately after birth. Although initiation of surfactant synthesis/secretion may be accelerated, the underlying mechanisms of the process itself remain to be resolved. An important pathway in adult lung has implicated the Ca^{2+}-PS-dependent enzyme protein kinase C (PKC) in its regulation. The present study was undertaken to characterize the activity of Ca^{2+}-PS-dependent PKC in adult lung and to determine if PKC was involved in the processes of initiation of synthesis and secretion of surfactant-related compounds in fetal and neonate lung. Protein and phospholipid levels, and activity profiles of Ca^{2+}-PS-dependent PKC were determined from subcellular fractions of fetal, neonate and adult rabbit lung. The enzyme's activity in the lamellar body fraction from whole lung and isolated type II cells was examined in greater detail. Additionally, the effect of PKC activation on uptake and release of phosphotidylcholine precursors [^{32}P] and [^{3}H]choline by isolated fetal type II pneumocytes was observed using the PKC activator tetradecanoylphorbol acetate (TPA). Protein levels displayed a biphasic pattern, peaking on gestational days 27 and 30. Phospholipid levels were found to increase significantly on day 27. Phospholipid:protein ratios in the putative lamellar body fraction also displayed a biphasic pattern with peaks on gestational day 27 and postnatal day 1. Ca^{2+}-PS-dependent PKC specific activity was found to follow the same biphasic pattern in the subcellular fractions.

Although enzyme activity localized in the lamellar body fractions isolated from type II cell cultures was found to be due to contamination, TPA stimulation of these cells appeared to cause translocation of Ca^{2+}-PS-dependent PKC to this fraction. TPA stimulation of these cells also caused significantly greater uptake of the precursors [^{32}P] and [^{3}H]choline during concurrent incubation. In cells prelabelled with isotope, stimulation with TPA caused higher levels of isotope to be released into the culture medium. Upon subcellular examination of the cells, the putative lamellar body fraction was found to be affected. These results indicate that PKC is active in the developing lung during the period of initiation of the surfactant cycle, and may be involved in the regulation of that cycle.

INTRODUCTION

Respiratory System - General Overview of Structure and Arrangement

The respiratory system may be divided into three functional components associated with: a) ventilation, b) conduction, and c) gas exchange. The ventilating component consists of the thoracic cage, intercostal muscles, diaphragm and elastic tissues of the lung. The conducting portion consists of the nose, pharynx, larynx, trachea, bronchi, and the proximal bronchioles. Gas exchange takes place in the distal regions of the lung, comprised of respiratory bronchioles, alveolar ducts and sacs. Since the primary focus of the study is at the respiratory level of the lung, only these regions will be considered in further detail.

2.1 SUBDIVISIONS

The lung is an extremely complex organ which is reflected in the large number of cell types which have been identified. Some forty different specialized cells have been observed (Sorokin, 1970). The brief description which follows outlines the general structure of the lung. However, the main focus of the present studies will be function at the level of the alveolus. This latter area will be reviewed in detail.

2.1a Conducting Region

Inspired air enters the nose and passes through the nasopharynx and oropharynx and into the larynx. These regions of the conducting system initiate the processes of adjusting the humidity and temperature of inspired air to match that of the body. The nasal cavity contains several small projections in its lumen which promote a turbulent airflow. This allows uniform contact of air with the epithelial mucosal coat, warming and humidifying the air. Posteriorly the pharynx is structurally continuous with the nasal cavity, and in its laryngeal regions is the point of separation of respiratory and alimentary tracts.

Intervening between the pharynx and trachea is the larynx, a hollow tube, partially enclosed by cartilage plates bound together by muscle and ligaments. The interior surface is covered by a thick lamina propria and epithelium. Housed within the submucosa are a number of mixed glands responsible for secreting and maintaining a mucous layer over the epithelium.

The trachea extends from the base of the larynx and passes inferiorly along the ventral surface of the neck. The tracheal adventitia is composed of loose connective tissue with numerous blood vessels and nerves. This layer merges with surrounding tissue externally while internally it blends into submucosa or perichondrium as it approaches the lumen. Reinforcing this airway are 16 to 20 hyaline U-shaped cartilages. Embedded in the fibrous connective tissue that forms the wall, each cartilage contains a gap on the dorsal side which is bridged by a band of smooth muscle. The cartilage perichondrium

give rise to radially oriented collagen fibres. These fibres terminate at the elastic membrane which separates lamina propria from submucosa. These fibres therefore anchor the lamina propria to the cartilage skeleton. As in lamina propria of the previous segments, this layer contains numerous glands. These glands are predominantly mucous in nature and pass their secretions onto the epithelial surface via ducts that pierce the lamina propria.

The trachea bifurcates in the mediastinum giving rise to paired airways, the primary bronchi of each lung. These are smaller than the trachea but retain a similar structure. Entering the lungs at the hilum with blood vessels and nerves, they form the bronchial tree, each generation of airway dividing dichotomously to form smaller airways. Initially these airways follow the same structural plan as the trachea, but as the lumen diameter decreases, gradual changes take place within their walls. The cartilage component decreases forming complete rings around larger bronchi, and irregular plates in the smaller bronchi. The amount of smooth muscle increases to form a layer of muscularis consisting of two bands arranged helically around the airway. The presence of glands, blood vessels and nerves allows the submucosal layer to retain most of its size. However lamina propria thins out until the epithelium sits atop an encircling elastic membrane. Elastic tissue, an important part of the ventilating system, becomes more conspicuous as bronchial lumen decreases. The airways to this point are still termed bronchi, but as lumen diameter approaches 1 mm, they are termed bronchioles, and the wall undergoes further changes.

2.1b Respiratory Region

As bronchioles branch and form progressively smaller airways, the muscularis of the wall becomes a discontinuous layer. Within the terminal bronchioles, the last generation of conducting airways, the lumen is encircled respectively by an epithelium, lamina propria, scattered smooth muscle cells and adventitia. These bronchioles divide dichotomously producing respiratory bronchioles. While essentially similar in structure to terminal bronchioles, respiratory bronchiolar epithelium is interrupted by sporadic alveoli. As air progresses further down the branching airways, the number of alveoli opening directly into the airway increases. The final airway gives rise to paired alveolar ducts surrounded by grapelike clusters of alveoli.

Alveolar structure is supported on a frame of collagenous and elastic fibres. A rich blood supply is provided by capillaries interwoven in this framework and covered externally by the alveolar epithelium. Smooth muscle cells may persist as single fibres encircling the entrance to an alveolus. The alveolar wall allows diffusion to take place between inspired air and the rich capillary bed present in the wall. This diffusion is dependent on the minimal connective tissue layer separating a very thin alveolar epithelium from the capillary endothelium. In fact in most regions this potential space between endothelial and epithelial cells is obliterated as their basal laminae fuse forming the blood-air barrier. The entire structure is supported by a mesh of collagenous and elastic fibres. Capillaries

are woven through this framework covered by epithelium and as noted a muscularis is absent having terminated at the level of the alveolar ducts.

2.2 EPITHELIUM

Differences in function of the respiratory system from nasal cavity to alveoli are reflected in the epithelial lining. A defining characteristic of the conducting airways is its respiratory epithelium, principally ciliated pseudostratified epithelium. This epithlium is columnar in the upper airways, and gradually shortens to low cuboidal in distal bronchi. Islands of stratified squamous cells may be found in areas of the tract exposed to irritating stimuli.

Interspersed within this layer are several cell types. Basal cells are associated with the basal membrane but do not reach the luminal surface. Accounting for a large proportion of nonciliated cells are mucous or goblet cells which appear similar to those of the gastrointestinal tract. Their secretions are combined with that of submucosal glands to produce a mucous layer covering the epithelium. As the caliber of the airway decreases and submucosa disappears, a corresponding reduction in gland tissue occurs.

In addition to conditioning inspired air before delivery to the respiratory section of the lung, the mucous coat forms part of an important defense mechanism. Inspired dust particles are trapped in mucous as the air is humidified. This layer is pushed towards the glottis by the coordinated sweeping motion of the surface cilia.

Ciliated cells persist as the dominant cell type in the bronchiolar epithelium, though they are now low cuboidal in form. Mucous cells however, have been replaced by non-ciliated Clara cells. These cells which are characteristic of the smaller airways, produce secretions suspected to include mucolytic and proteolytic enzymes, lipids and polysaccharides (Widdicombe and Pack, 1982). It has been suggested they contribute to the pulmonary surfactant in some manner (Ueda et. al., 1985), although the extent of thier contribution is still under debate.

Four major cell types are present within the respiratory regions of the lung. They are: macrophages, fibroblasts and type I and type II pneumocytes.

2.2a Macrophages

Alveolar macrophages provide a primary line of defence at the level of the alveolus against microorganisms and particulate material which have not been trapped by the mucous layer of the upper airways. Macrophages are able to move freely over the alveolar surface and engulf foreign matter. There is also ample evidence that macrophages have an important role in clearing spent surfactant from alveoli (Sueishi et al., 1977; Desai et al., 1978; Coalson et al., 1987).

2.2b Fibroblasts

Residing in the connective tissue compartment of the interstitium, these spindle-shaped cells are similar to fibroblasts observed in other tissues. While a functional role in the adult lung has not been defined, during fetal lung

development, fibroblasts appear to play an essential role in regulating differentiation of type II cells through production of a mediating protein, fibroblast-pneumocyte factor (FPF) (Post et al., 1984a). FPF has been shown to increase the rate of lung maturation (Smith, 1979) and phospholipid synthesis in fetal type II cells (Post et al., 1984a). In addition a mechanical induction through transluminal foot processes has been shown to occur (Snyder and Magliato, 1991). It has also been suggested that fibroblast-epithelial cell interaction is important for regulating replication and development in type II cells (Scott and Das, 1994).

2.2c Type I Pneumocytes

Although they account for only ~11% of total lung cell population, type I pneumocytes cover ~96% of alveolar surface area (Crapo et al., 1982). Their primary function is to provide an intact gas permeable surface. Small amounts of connective tissue may intervene between type I pneumocytes and interstitial endothelial cells, in many places basal laminae of endothelial and type I epithelial cells may be fused. In addition to their close proximity to each other, both cell types have very attenuated cytoplasm. In fact prior to the age of electron microscopy, a good deal of speculation occurred in regard to the presence or absence of a continuous cellular layer in the alveolus. These characteristics of the type I pneumocyte greatly reduce the diffusion distance between air in the alveolar space and blood in the capillary bed.

2.2d Type II Pneumocytes

Interspersed among type I cells lining alveoli are the type II alveolar cells. Type II cells are cuboidal in shape and therefore tend to push the basal membrane into the interstitial space and may concurrently protrude to variable degrees into the luminal space. Type II pneumocytes account for 15% of lung cell population (Crapo et al., 1982) and are generally considered multifunctional cells.

While surfactant production is clearly a major function of the type II pneumocytes, there is substantial evidence that these cells participate in a number of functions including epithelial regeneration, fluid homeostasis and defense. Injury of the alveolar epithelium tends to affect type I cells to a greater degree due to their attenuated cytoplasm and large surface area. However it is the type II pneumocytes that respond to damaged type I cells by dividing (Adamson and Bowden, 1974), and subsequent daughter cells transform to adopt the characteristics of type I cells (Woodcock-Mitchell et al., 1986). It has also been suggested that type II cells act as the precursors of type I cells during normal growth in fetal and immature lungs (Kauffman et al., 1974). A fluid transport function has also be attributed to the type II cells at least in tissue culture (Mason et al., 1982).

As mentioned above, of major functional interest with regard to type II cells is the production of pulmonary surfactant. A characteristic of type II cells are the lamellar bodies, the intracellular storage form of surfactant. A possible

relationship between these organelles and the alveolar surface film was first made by Macklin in 1954 (Macklin, 1954). Subsequent studies confirmed their relationship using electron microscopic autoradiography to trace radioactively labeled precursors to various surfactant components through type II cells. (Buckingham et al., 1966; Chevalier and Collet, 1972).

Secretion of surfactant takes place by exocytosis of lamellar bodies into the alveolar space. Inhibition of cytoskeletal function in lung slices with agents such as vinblastine and colchicine (Marino and Rooney, 1980) reduces surfactant secretion suggesting involvement of the cytoskeleton in the secretory process.

Radiographic studies using disaturated phosphatidylcholine, a major constituent of surfactant (see section on Surfactant Composition, 2.3b), suggest that type II cells also aid in surfactant clearance (Geiger et al., 1976) and recycling of certain phospholipids and protein components back to the alveolus (Hallman et al., 1981).

The functions of type II cells concerning the surfactant cycle will be dealt with in greater detail in later sections.

2.3 SURFACTANT

2.3a Background

The first estimations of mechanical forces involved in lung function were done in 1929 by von Neergaard (von Neergaard, 1929). By comparing pressure-

volume relationships of air and liquid-filled lungs, von Neergaard recognized the paramount importance of surface tension forces in maintaining lung expansion. Estimating that tissue elasticity only accounted for a third of the total force, he correctly concluded that the remainder of the pressure was due to the force of surface tension. In order to explain higher surface forces in a system with a constant surface tension, he assumed alveoli to be saucer-like in shape. Upon inflation the alveoli bulge out but never becomes completely hemispherical. The reason for this theoretical limit is that in a system with constant surface tension, the pressure required to inflate alveoli increases until a hemispherical shape is reached, then decreases rapidly after a hemispherical shape is attained. Increased surface forces were now attributable to the increasing alveolar radii. A rough analogy may be drawn to inflating a balloon. Initially it is difficult, but once the balloon is inflated beyond a critical radius of curvature, it expands readily. von Neergaard considered only a model with a constant surface tension never taking into consideration the concept of a variable surface tension.

Von Neegaard's work remained unnoticed for the next twenty-five years and was in fact duplicated in 1954 by E.P. Radford (Radford, 1954) in an attempt to measure alveolar surface area. Radford postulated that the difference in surface energy between blood and lung extracts could be accounted for by a surface that was in a semi-solid phase or lined by a highly surface active material. He dismissed both ideas as unlikely.

The discovery in 1959 that Neonatal Respiratory Distress Syndrome (NRDS) was related to a deficiency in surfactant (Avery and Mead, 1959) greatly increased the interest in mechanisms of surfactant synthesis and secretion. It is now understood that surfactant prevents alveolar collapse at end expiration as well as decreasing the force required for initial lung expansion by imparting to the alveolar surface the ability to vary the surface tension with changing alveolar volumes.

Studying the problem of pulmonary edema, Pattle reported that bubbles from lung extracts persisted for a long time. This indicated that surface tension of the alveolar lining layer approached zero (Pattle, 1955). However this conclusion was in conflict with existing concepts. Pressure differences between air- and liquid-filled lungs suggested a much higher surface tension (Mead, 1961).

Attempting to reconcile these conflicting reports, the significance of a paper by Macklin (Macklin, 1954) concerning the presence of a mucopolysaccharide lining within the alveolus was recognized by Clements. This investigator designed an instrument capable of measuring surface tension of lung extracts at different degrees of inflation. Values of ~10 dynes/cm were obtained for lungs at minimum surface area as compared to ~46 dynes/cm when inflated, suggesting that surface tension varied as a function of the degree of inflation (Clements, 1958).

Variable surface tension within alveoli allowed a change in the concept of alveolar structure. Increasing surface forces could now be attributed to an increase in tension rather than alveolar shape. The Laplace equation is given by $p=2\gamma/r$, where p represents the air pressure inside a bubble of radius r and surface tension γ. This equation indicates that smaller alveoli require greater air pressure to prevent collapse. Since alveoli are interconnected, this pressure difference would deflate smaller alveoli as air flows from high to low pressure areas. Increasing surface tension with alveolar size equalized these forces. The shape of alveoli could now be thought of as a bubble, with each having an effect on adjacent alveoli.

To confirm that the lining layer was responsible for the variation observed in the surface tension, a nonionic detergent, Tween 20 was used to replace the endogenous alveolar lining with one which displayed constant surface tension. Pressure-volume relationships were determined which showed similar inflation/deflation curves (Clements et al., 1961; Radford, 1962). This indicated that replacement of the surface layer by Tween 20 eliminated the variation in surface tension associated with the endogenous alveolar lining material. The functional importance of this surface film renewed interest in pulmonary surfactant. Independent laboratories engaged in identification of the major surface active component reached a similar conclusion (Buckingham, 1961; Klaus et al., 1961; Pattle and Thomas, 1961). Although the methods used to

isolate surfactant were different, a common conclusion arose that a lipoprotein material constituting a surface active lining existed at the level of the alveolus.

2.3b Composition

After the presence of pulmonary surfactant was established, research into the composition, synthesis and function of this material followed quickly. It was observed that two pools of surfactant could be isolated from the adult lung. The first is present as intracellular organelles within type II pneumocytes, lamellar bodies. The second pool, isolated by bronchoalveolar lavage (BAL), is the extracellular material lining the respiratory portion of the lung. It was Macklin that first suggested a possible link between these organelles and the extracellular material lining alveoli (Macklin, 1954). A later study observed the coincidental appearance of lamellar bodies and surfactant during lung development in fetal mice (Buckingham and Avery, 1962). Later studies confirmed the precursor-product relationship of lamellar bodies and surfactant by following radioactively labelled phospholipid precursors through the cell and onto the alveolar surface (Chevalier and Collet, 1972). Further support was provided by comparison of lamellar body and extracellular surfactant lipid profiles (Haagsman and van Golde, 1991).

Lipids represent ~90% of the total surfactant and are primarily responsible for the surfactant surface activity. The remainder is composed of surfactant-associated proteins, which are generally of a low molecular mass, hydrophilic or hydrophobic in nature.

The lipid fraction is divisible further into separate compounds. The major phospholipid in surfactant is phosphatidylcholine which accounts for ~85% of the total. Of this appoximately 50% occurs as disaturated phosphatidylcholine (DSPC), of which greater than 95% is dipalmitoylphosphatidylcholine (DPPC). It is generally recognized that the capacity of surfactant to alter surface tension is due to the presence of DSPC. Unsaturated phosphatidylcholine (USPC), represents ~15% of total lipids. In addition, acidic phospholipids, phosphatidylglycerol (PG) and phosphatidylinositol (PI), important constituents of surfactant, account for ~10% and ~5% of lipids respectively. The amount of PG in surfactant is unusually high. Generally it accounts for only a small amount of total glycerophospholipid in animal cell membranes and acts predominantly as a precursor to cardiolipin synthesis (Bourbon, 1991). In fact PG's unusual prevalence in mature surfactant has formed the basis of a diagnostic test of lung maturity in the fetus (Bent et al., 1982). Other phospholipids present in surfactant include phosphatidylethanolamine (PE~5%), phosphatidylserine (PS, 3%), sphingomyelin (SM, 3%), and lysophosphatidylcholine (LPC, >1%). Neutral lipids represent 15% of surfactant lipids. These are cholesterol (Ch, ~10%) and triacylglycerols and assorted free fatty acids (Akino, 1992).

In addition to the phospholipids, surfactant proteins which were first identified in 1973 by King (King et al., 1973), have recently been shown to play important roles in surfactant function. At present, four surfactant proteins have been identified, two are hydrophilic, SP-A and SP-D, while the other two are

proteolipids, SP-B and SP-C and extremely lipophilic. Relative amounts and functions of each have yet to be determined, although SP-A is clearly the most abundant (Whitsett and Weaver, 1991).

2.3c Synthesis

A considerable body of evidence now indicates that a majority of the protein and lipid components of surfactant are synthesized within the type II pneumocyte. The number of different cell types identified in lung combined with the paucity of certain of these, has presented a hurdle in understanding surfactant synthetic pathways and has indeed defined the problem with identifying surfactant regulatory mechanisms. The development of methods to isolate alveolar type II cells from the lung has allowed major advances in understanding these pathways. It is generally clear that the steps involved in synthesis of individual phospholipid components operate in a fashion which is similar to that of other cells: differences exist only in relative activity at specific steps. An overview of the biosynthesis of the major phospholipids in surfactant is given below and shown in Figure 1.

The first step in lipid synthesis is formation of phosphatidic acid (PA). This molecule may be synthesized via two interconnected pathways which produce the intermediate 1-acylglycerol-3-phosphate (1-AG-3-P). One pathway starts with circulating glucose, which appears to be the major starting point in *de novo* synthesis of glycerophospholipids (Batenburg et al., 1978). This is taken in by the cell and converted to dihydroxyacetone-phosphate (DHAP). Acylation and

reduction steps produce 1-AG-3-P. The other pathway starts with free glycerol, which is phosphorylated to form glycerol-3-phosphate (G-3-P). This molecule then undergoes an acylation step to produce 1-AG-3-P. PA is then produced by a secondary acylation of 1-AG-3-P which proceeds much faster than the first (Haagsman and van Golde, 1991), producing phosphatidic acid. It has been estimated that 60% or more of the PA synthesized in isolated type II cells is via the DHAP pathway (Mason, 1978). This figure however has been questioned by Batenburg, who gave a value closer to 50% (Batenburg, 1992). The importance of PA lies in its being a precursor common to PC as well as acidic phospholipids PI and PG. It is therefore a point of divergence of the respective pathways (Bourbon, 1991; Haagsman and van Golde, 1991; Batenburg, 1992).

Synthesis of PC molecules requires hydrolysis of PA to form diacylglycerol (DAG). Cytidylyldiphosphocholine (CDP-choline) formed by the sequential action of choline kinase and cholinephosphate cytidylyltransferase (CP-CyT) on choline is incorporated into DAG by cholinephosphotransferase (CP-T). This incorporation marks the division between the hydrophilic and lipophilic synthetic intermediates. Good evidence from pulse-chase and pool size studies indicate that in type II cells CP-CyT catalyzes the rate-limiting step in DSPC synthesis (Possmayer et al., 1981; Post et al., 1982; Post et al., 1984b).

Generally enzymes responsible for PC synthesis do not appear to exhibit acyl group specificity (Crecelius and Longmore, 1983; Post et al, 1983). It has

also been suggested that enzymes involved in PC synthesis, from choline kinase to cholinephosphotransferase, are organised into an ordered array, effectively channeling the product of one reaction to the next enzyme (George et. al., 1989). The ability of CP-CyT to associate with actin suggest that the cytoskeleton plays a role in this organisation.

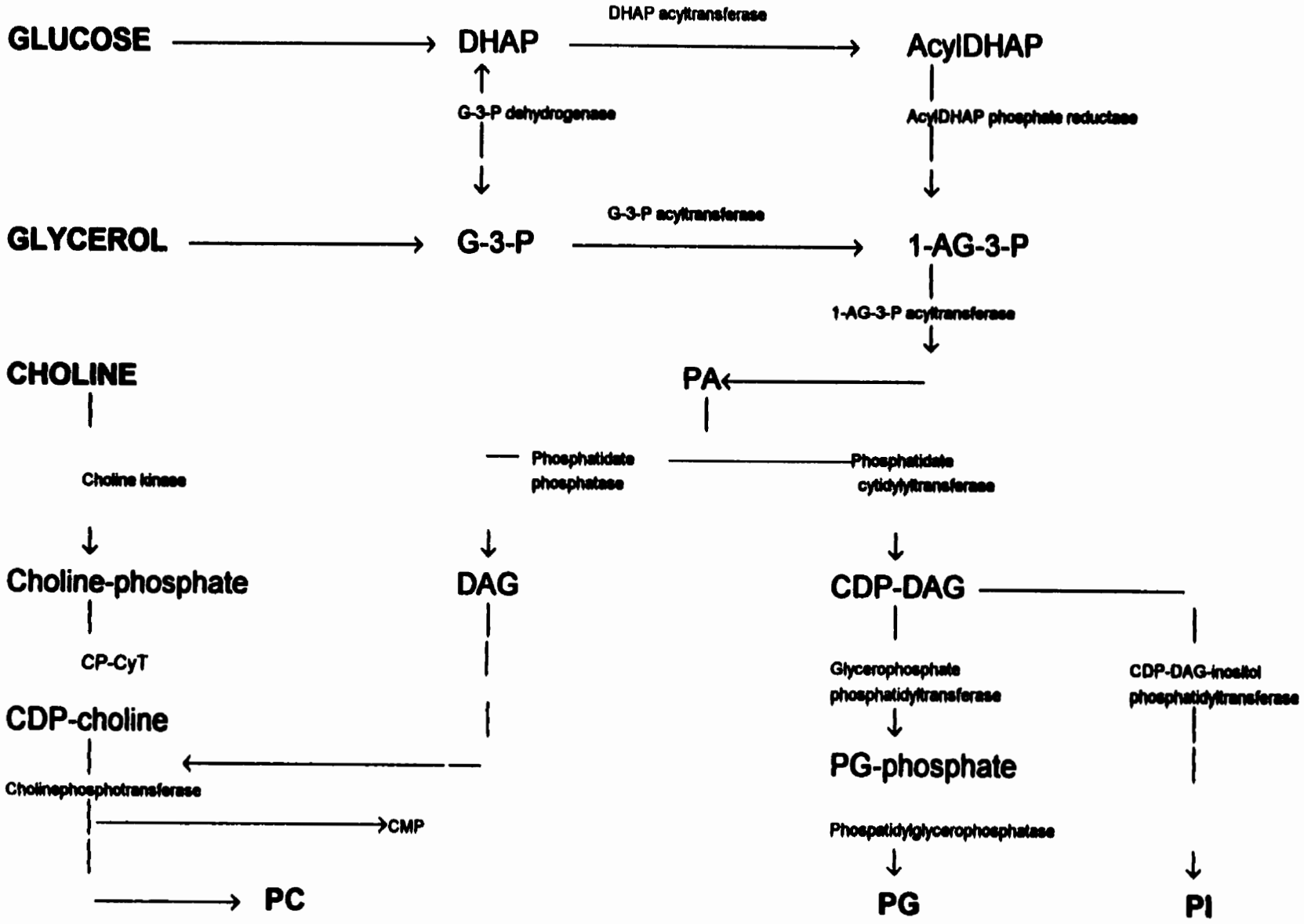
Unsaturated PC is also converted to saturated forms by a deacylation-reacylation reaction or a transacylation reaction, the former appearing to be of greater importance in lung tissue (Batenburg et al., 1979; Crecelius and Longmore, 1984). It has been estimated that 55% of DPPC synthesized passes through this remodeling pathway (Den Breejen et al., 1989), the remainder is converted directly from saturated DAG. PA may also be converted, in the presence of cytidine triphosphate into CDP-diacylglycerol, a precursor common to acidic phospholipids PI and PG. This molecule is converted directly to PI, or phosphatidylglycerophosphate, the latter is dephosphorylated to PG.

Synthesis of PE follows a route similar to PC synthesis. Although the same diacylglycerol pool is utilized, very little saturated PE is found (Akino, 1992). Synthesis of PS involves a catalyzed head group exchange of PC or PE with free serine. The overwhelming majority of surfactant cholesterol appears to be imported from the pool of circulating lipoproteins (Hass and Longmore, 1980).

Although specific to type II cells and possibly bronchiolar cells, synthesis of surfactant-associated proteins appears to be similar to protein synthesis in

Figure 1: Biosynthesis of the lipids phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol. The abbreviations used in the figure are:

DHAP	Dihydroxyacetone-phosphate
AcylDHAP	Acyldihydroxyacetone-phosphate
G-3-P	Glycerol-3-phosphate
1-AG-3-P	1-acylglycerol-3-phosphate
DAG	Diacylglycerol
CDP-DAG	Cytidine 5'-diphosphate diacylglycerol
CDP-choline	Cytidine 5'-diphosphate choline
CP-CyT	Cholinephosphate-cytidylyltransferase
CMP	Choline monophosphate
PG-phosphate	Phosphatidylglycerol-phosphate
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol


GLUCOSE
DHAP
DHAP acyltransferase
AcylDHAP
G-3-P dehydrogenase
AcylDHAP phosphate reductase
GLYCEROL
G-3-P
G-3-P acyltransferase
1-AG-3-P
1-AG-3-P acyltransferase
CHOLINE
PA
Phosphatidate phosphatase
Phosphatidate cytidylyltransferase
Choline kinase
Choline-phosphate
DAG
CDP-DAG
CP-CyT
CDP-choline
Glycerophosphate phosphatidyltransferase
CDP-DAG-inositol phosphatidyltransferase
PG-phosphate
Cholinephosphotransferase
CMP
Phospatidylglycerophosphatase
PC
PG
PI

other cells. Translation of the genomic sequence is followed by modifications, the amount and type of which varies among the four known proteins. Interest in the surfactant-associated proteins has increased dramatically in the last two years as evidence has appeared suggesting that they are important in many aspects of surfactant function (Cockshutt and Possmayer, 1992). Review of the associated literature is beyond the scope of this project.

2.3d Function

The function most often associated with surfactant is the ability to stabilize alveoli at low volumes. It is generally accepted that the major constituent of surfactant, DSPC, is primarily responsible for the ability of surfactant to vary surface tension (Clements, 1977). Because of its amphiphilic nature, DSPC orients as a monolayer at the air-water interface with the nonpolar acyl chains directed into the alveoli and the polar head group, in this case choline, towards the aqueous hypophase lining the alveolus. As alveolar surface area is decreased during expiration, DSPC molecules forming the monolayer move closer to each other. Repulsive forces between adjacent molecules increase, resisting further decreases in surface area (Hills, 1988). In order to function, such a lipid monolayer must be below its gel-liquid transition temperature. Above this temperature, DSPC molecules lose their ordered state. This transition temperature for DSPC is 41°C (Hawco et al. 1981). The presence of other lipid species in surfactant appears also to be important to the overall function. In particular, the acidic phospholipids, PI and PG, are important in

promoting DSPC adsorption as a monomolecular layer covering the alveolar surface (Hallman and Gluck, 1976; Hallman et al., 1977)

A role for surfactant in alveolar fluid homeostasis has been postulated by Pattle (Pattle, 1955). Subsequent studies in which surfactant was removed from alveoli have shown an increase in liquid flow into the alveolar space from the capillary bed (Bachofen et al., 1979). A suggested mechanism of action involves compression of the liquid phase between surfactant and the alveolar wall equalizing that of the capillary bed pressure forcing liquid in the opposite direction (Walters, 1992).

Defense also appears to be an important part of surfactant function. Several mechanisms of action have been proposed. Simply covering the alveolar surfaces prevents evaporation which protects cells from dehydration. An interaction has also been proposed between surfactant and alveolar macrophages which seems to provide protection from bacteria. Macrophage activity against bacteria coated with surfactant compared to uncoated bacteria was significantly increased (O'Neill et al., 1984). Surfactant defense also potentially extends to inhibition of pathogen proliferation (Coonrod et al., 1984).

2.4 INITIATION and REGULATION of SURFACTANT METABOLISM

Initiation of surfactant synthesis and secretion during late gestation is critical for survival of the newborn. Lipid and protein biosynthetic products are stored intracellularly as lamellar bodies. Extrusion of these organelles into the

alveolar space begins prior to birth when major structural changes occur. It has been suggested that exocytosis of lamellar bodies causes distortion of the organelle, which begins a process of unraveling lamellar material producing tubular myelin which is generally regarded as an intermediate between lamellar bodies and the surfactant monolayer (Chander and Fisher, 1990). Surfactant from the monolayer may be degraded or recycled. The total pool of surfactant DSPC within the lung has been estimated to be about twice that required to coat all alveolar surfaces (Wright and Clements, 1987). In addition to this, surfactant replacement occurs at ~10% (Young et al., 1981) of the total per hour in adult animals, but up to 90% per hour in newborns (Jacobs et al., 1983). This suggests that the processes of surfactant synthesis, secretion, and clearance are tightly regulated as well as being coordinated with each other.

2.4a Synthesis

In spite of years of research, mechanisms controlling surfactant and DSPC synthesis in particular, remain speculative. Major work has focused on the morphological and biochemical changes which occur in lung tissue during development particularly during initiation of surfactant synthesis. A substantial amount of evidence indicates that glucocorticoids (Liggins, 1969; Taeusch et al., 1972; Rooney et al., 1979) or thyroid hormones (Rooney et al., 1978; Liggins et al., 1988) are primary agents responsible for lung maturation and initiation of surfactant and DSPC synthesis. These hormones act in concert with other

factors to initiate surfactant synthesis and possibly secretion into the alveolar space.

Although type II cells contain appropriate receptors, the action of glucocorticoids on type II cell phospholipid synthesis appears to be mediated predominantly by interstitial fibroblasts (Post et al., 1984a). These latter cells produce and secrete a low molecular weight protein, fibroblast-pneumocyte-factor (FPF), in response to these hormones. This interaction is confirmed by studies in which FPF is eliminated by addition of monoclonal antibodies or using pure type II cell cultures (Smith, 1979; Post et al. 1984c). Furthermore autoradiographic studies show increased glucocorticoid receptor binding in fibroblasts just prior to initiation of surfactant synthesis (Beer et al., 1984; Caniggia et al., 1991). Intracellularly FPF stimulates DSPC (Post and Smith, 1984; Post et al., 1984a) synthesis in fetal lung cells by activating enzymes in phospholipid biosynthesis pathways. Although several appear to play a role in regulation, CP-CyT which catalyzes the rate-limiting step in DSPC synthesis (Post et al., 1983)(see section on Surfactant Synthesis, 2.3c.), has received the most attention.

Another mechanism through which glucocorticoids may function to regulate CP-CyT is by inducing an increase in the amount of fatty acid synthase available (Xu et. al., 1989). The ability of phospholipids, PG in particular (Feldman et al., 1978; Gilfillan et al. 1985), and fatty acids to activate CP-CyT has been known for several years. Recently Batenburg and Elfring (Batenburg

and Elfring, 1992) demonstrated that exposure of fetal type II cells to fibroblast-conditioned cortisol-containing medium increased the fatty acid synthase activity. Furthermore, both cortisol and fibroblast-conditioned media were required to obtain maximal activity. This suggests a mesenchymal-epithelial interaction is essential to up regulate this enzyme.

Activation of CP-CyT within the cell and it's site of action remain a topic of debate. While CP-CyT was initially thought to be activated by translocation from cytosol to membranes of the endoplasmic reticulum upon stimulation (Weinhold et al., 1984), later studies failed to show reduced cytosolic activity expected with increased microsomal activity (Aeberhard et al., 1986; Chander and Fisher, 1988). Furthermore recent evidence suggests that this enzyme is not influenced by activation of cAMP-dependent protein kinase but activity does decrease under phosphorylating conditions (Zimmermann et al., 1994; Radika and Possmayer, 1985). A comprehensive overview of the mechanisms involved in the regulation of CP-CyT are yet to be described.

Recent evidence has suggested glucocorticoid-induced acceleration of lung maturation may also involve surfactant associated proteins. While regulation of surfactant protein synthesis appears to be independent of glucocorticoids, the 5' flanking region of the SP-A gene has been found to contain glucocorticoid responsive elements (Boggaram et al. 1988) capable of regulating transcription (Boggaram et al. 1989). Low concentrations of hormone

appear to increase mRNA levels while higher concentrations inhibit the mRNA accumulation (Mendelson and Boggaram, 1991).

Administration of thyroxine (T_4), triiodothyronine (T_3) or thyrotropin releasing hormone (TRH) has been shown to accelerate lung maturation and increase surfactant production (Wu et al., 1973; Rooney et al., 1978; Hitchcock, 1979). Thyroid hormones which differ in action from glucocorticoids, may act directly in type II pneumocytes (Smith and Hitchcock, 1983), possibly by potentiating the effect of FPF (Smith and Sabry, 1983). This effect does not appear to be extended to surfactant proteins (Ballard et al., 1986).

2.4b Secretion

It is generally accepted that surfactant secretion, the process of exocytosis of lamellar bodies into the alveolar space, is initiated prior to birth. Indeed, this process forms the basis for prenatal assessment of lung maturity through amniocentesis. Nevertheless, major regulatory factors involved in secretion remain unclear. Furthermore separation of factors regulating only secretion is difficult as the entire surfactant cycle appears to be tightly regulated and evidence of reciprocal regulation has appeared (Scott, 1992; Kresch, 1994). Studies have utilised a number of models in examining surfactant secretion including *in vivo* and *in vitro* systems such as organ culture, perfused lung slices as well as both adult and fetal type II cell cultures. These studies have identified numerous mechanisms that affect secretion as measured by DSPC release. These include distention (Oyarzun and Clements, 1978), microtubule disruption

with colchicine and vinblastine (Delahunty and Johnson, 1976), cholinergic (Goldenberg et al., 1969), adrenergic (Brown and Longmore, 1981) and purinergic (Gilfillan and Rooney, 1988) activation. Recent studies suggest that changes in the physical state of extracellular DSPC (Suwabe et al., 1992; Scott, 1992) as well as the presence of SP-A (Rice et al., 1987) in the alveolar space also affect secretion of surfactant.

Involvement of the cytoskeleton in surfactant secretion has been demonstrated through the use of the microtubular transport inhibitors colchicine (Delahunty and Johnson, 1976) and cytochalasin (Tsilibary and Williams, 1983). In these cases secretion rates in lung slices were reduced. Actin filaments also appear likely to function in concert with microtubules and intermediate filaments to transport lamellar bodies to the apical surface. However using cultured type II cells, the opposite effect whereby secretion was unaffected or increased in response to the same agents was observed by Dobbs and Mason (Dobbs and Mason, 1979). While this may be due to changes in cytoskeletal structure in cultured cells which grow in an attenuated fashion on culture substrate resulting in reduction in cell thickness compared to the *in vivo* situation (Mason, 1978), a clear explanation has not appeared. Additionally, the disruption of cytoskeletal elements separating lamellar bodies from the cell membrane allow the two to fuse and exocytosis to occur. This may also account for the discrepancy in these findings (Wright and Dobbs, 1991).

Lung distention has been shown to increase the amount of phospholipid that can be recovered from the lungs by bronchoalveolar lavage (Oyarzun and Clements, 1978; Nicholas et al., 1982). In this case the mechanism producing secretion has not been defined. However studies using cultured cells indicate two possible routes. The first was observed in fetal cells where cyclic stretching of cells, imitating fetal breathing movement-induced distention increased cellular cAMP levels (Scott et al., 1993). The second mechanism may be calcium (Ca^{2+}) mediated. Ca^{2+} levels increased in adult cells after a single stretch (Wirtz and Dobbs, 1990).

Increasing cellular Ca^{2+} by treating cells with the ionophore A23187 also stimulates secretion (Sano et al., 1985; Pian et al., 1988). Verapamil, a Ca^{2+}-channel blocker, was shown to inhibit Ca^{2+} uptake to the endoplasmic reticulum. This increases the intracellular Ca^{2+}, while increasing secretion, cAMP formation and phosphoinositide hydrolysis; these effects correlated with elevated secretion of DSPC (Warburton et al., 1989a).

Receptor-mediated secretion has also been extensively studied. Adrenergic agonists which increase cellular cAMP levels have been found to increase secretion (Dobbs and Mason, 1979; Brown and Longmore, 1981). Cholera toxin which bypasses the receptor to cause persistent activation of adenylate cyclase by ADP-ribosylation of its G protein, also stimulates secretion (Mescher et al., 1983). Presumably the cAMP produced activates cAMP-dependent protein kinase (PKA) which phosphorylates an effector molecule(s)

which in some manner is tied to the secretory response. In contrast to adrenergic stimulation, cholinergic agonists have clearly been shown not to affect secretion of surfactant related material in isolated type II cells (Hung, 1980; Brown and Longmore, 1981).

Adenosine compounds, in particular ATP, are potent agonists of secretion. Acting via purinoceptors (Gilfillian and Rooney, 1987; Gilfillian and Rooney, 1988) they appear to activate at least two pathways in stimulating surfactant secretion. P_1 agonists increase cellular cAMP and presumably activate a cAMP-dependent kinase (Griese et al. 1993). P_2 purinergic receptors cause hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2), producing diacylglycerol (DAG) and inositol trisphosphate (IP_3) (Warburton et al., 1989b; Rice et al., 1990 Tio et. al., 1991).

IP_3 mobilizes Ca^{2+} from intracellular stores. This Ca^{2+} may be involved with activation of a protein kinase C (PKC) together with DAG resulting from PIP_2 hydrolysis (Bell, 1986). Experimentally this enzyme may also be activated directly by 12-O-tetradecanoyl-13-phorbol acetate (Kikkawa et al., 1983; Kraft and Anderson, 1983). This agent has proven to be the most potent stimulator of type II cell surfactant-related secretion to date (Wright and Dobbs, 1991). These observations strongly suggest that PKC activation plays an important role in the secretory process of type II cells.

The presence of surfactant in alveoli has also been shown to modulate secretion of surfactant-related material. This effect appears to be mediated by

PC and surfactant protein A. The presence of DPPC in culture media at physiological temperatures decreased secretion of surfactant-related material (Scott, 1992; Suwabe et al., 1992). In addition the magnitude of the effect on secretion was dependent on characteristics in relation to fatty acid acyl groups of the extracellular phospholipid (Scott, 1992; Suwabe et al., 1992). Surfactant protein A (SP-A) also inhibited secretion after stimulation by a variety of agonists (Dobbs et al., 1987; Rice et al., 1987). The mechanism of DPPC and SP-A inhibition is unknown, but the general pattern which affects PKA-, PKC- and Ca^{2+}-dependent pathways suggests it acts at a step distal to second-messenger production which may be common to all (Chander and Fisher, 1990; Suwabe et al., 1992).

2.4c Recycling

In addition to *de novo* synthesis of surfactant components, evidence has recently been presented that type II pneumocytes from animals of various ages reutilize surfactant taken up from the alveolar space or the culture medium (Hallman et al., 1977; Chander et al., 1983; Jacobs et al., 1983; Scott, 1992). The contribution of recycling to the intracellular surfactant pool has been estimated to be as high as 90% in newborn rabbits (Jacobs et al., 1983) but appears to decline to 40% in adults (Jacobs et al., 1985), although the latter figure has been questioned (Magoon et al., 1983). Feedback mechanisms appear to be important in lipid uptake by type II cells. Extracellular SP-A affects rates of uptake by both type II cells and alveolar macrophages. Once

incorporated into type II cells, lipid components may be degraded or recombined directly with other surfactant-related precursors. Factors which control these processes are only beginning to be defined (Wright and Dobbs, 1991).

2.5 PROTEIN KINASE C and SURFACTANT

The enzyme protein kinase C was discovered in 1977 (Takai et al., 1977; Inoue et al., 1977) and has since been implicated in signal transduction pathways and functions of many cell types (Kikkawa and Nishizuka, 1986). In type II cells evidence indicates that ATP binds to P_2 purinergic receptors and activates the phosphoinositide cascade. Phospholipase C-mediated hydrolysis of membrane phosphoinositides produces two intracellular second messengers, DAG and the inositoltrisphosphate (IP_3) (Tio et. al., 1991;Griese et al. 1993).

Evidence indicates that IP_3 generation is associated with Ca^{2+} mobilisation from intracellular stores (Berridge and Irvine, 1984). The released Ca^{2+} may function with DAG to activate cytosolic PKC. Association of Ca^{2+}, DAG and PKC in the cytosolic compartment causes translocation to cell membranes where it associates with phosphatidylserine to complete activation of the PKC complex (Huang, 1989).

Enzyme activation may also be achieved experimentally using the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) or related compounds (Castegna et al., 1982; Kikkawa et al., 1983; Kraft and Anderson, 1983). This approach has proven extremely useful in many studies examining the role of

PKC activation in cell function. However TPA activation is not physiological and evidence indicates activation may be permanent, accounting for at least in part, the tumour-promoting activity of TPA (Bazzi and Nelsestuen, 1989).

Isolated adult type II pneumocytes have been found to secrete DSPC in response to exposure to TPA (Dobbs and Mason, 1978; Mescher et al., 1983; Sano et al., 1985). In fact TPA and adenosine trisphosphate (ATP) are probably the most potent agonists promoting surfactant-related phospholipid secretion (Mason, 1992). In fetal type II cells addition of TPA may also increase incorporation of [^{3}H]-choline into DSPC (Scott, 1994). These results suggest an important role for PKC in secretion and in fetal type II cells, perhaps synthesis of surfactant related components.

Differentiation of type II cells and their ability to synthesize and secrete surfactant into the alveolar space begins prior to birth. It has been previously shown that alveolar type II cells first appear in the rabbit fetus after gestational day 24 (Kikkawa et al., 1968; Kikkawa et al., 1971). Studies by Williams (Williams, 1977) and Faridy and Thliveris (Faridy and Thliveris, 1987), have shown clearly that lamellar bodies and surfactant components are secreted by the fetal lung prior to birth. Specifically lamellar bodies are detectable in alveoli by the 27th gestational day.. This suggests that whatever the secretory mechanisms regulating release, they are at the very least in place at this time of gestation. If PKC activation is a prerequisite to surfactant synthesis and/or

secretion regulation, then it may be active in fetal cells during the period when surfactant synthesis/secretion is initiated.

Although a role for PKC activation has been hypothesized in surfactant release from adult type II pneumocytes, the activity of this enzyme has not been previously studied in either fetal or newborn lung. The present study was conducted to characterize the activity of PKC at different stages of fetal lung development and compare this to the activity profile in adult lung. In addition, the effects of TPA-induced PKC activation in isolated fetal type II cells was also determined.

MATERIALS and METHODS

3.1 MATERIALS

Timed pregnant New Zealand White rabbits were obtained from the Blue Farm Rabbitry, St. Pierre, Manitoba. The time of mating was known to within 1 hour and this time was designated as day 0. Chemicals were obtained from Baxter-Canlab (Mississauga, Ont.) or Sigma Chemical Co. (St. Louis, Mo.). Tissue culture materials were from Gibco (Mississauga, Ont.). Protein Kinase C assay kit was obtained from Amersham (Oakville, Ont.). Radioisotopes ([^{32}P]-orthophosphoric acid [specific activity 1,100Ci/mmol], [g -^{32}P]-adenosine-5-trisphosphate [specific activity 3,000 Ci/mmol], [methyl-^{3}H] choline chloride [specific activity 90 Ci/mmol] were obtained from New England Nuclear. Centrifugation steps were carried out using the Joaun CR3000, and International Equipment Company (IEC) centrifuges IEC B-20 and B-60A. The latter two required IEC rotors A-237, A-321, and SB-283.

3.2 Methods

3.2a Subcellular Fractionation Procedures

Fractionation procedures were carried out as described previously (Oulton et al., 1986) using lung tissue from rabbits of 24, 27 and 30 gestational days as well as postnatal day 1 and adult. Tissue was also taken from 18 day fetuses but not fractionated due to the small amount of tissue available per litter. Pregnant New Zealand White rabbits were anaesthetized by intravenous

injection of Euthanyl (sodium pentobarbitol, 240 mg/mL) into the lateral ear vein and fetuses were delivered by hysterotomy and decapitated. Lungs were dissected from the thorax and weighed. They were placed in ice cold Hank's Balanced Salt Solution (HBSS) (Gibco), minced with scissors and homogenised in 9 volumes (mLs:gm of tissue) of buffer A (0.145 M NaCl, 0.01 M Tris/HCl, 0.001 M EDTA, 0.005 M EGTA, 10 ug/ul Leupeptin, 5 ug/ul phenylmethylsulfonyl fluoride, 0.005 M B-mercaptoethanol) with a Polytron homogeniser. Cellular debris and unbroken cells were pelleted by centrifuging the homogenate at 140xg for 10 minutes. This fraction was discarded as its composition has not been characterized and probably represents a mixture of unbroken cells, debris, and connective tissue (Oulton et al., 1986). The nuclear fraction was collected as a pellet using centrifugation at 1,500xg for 15 minutes. The supernate was spun at 10,000xg for 30 minutes to obtain a crude mitochondrial fraction. Lamellar body and purified mitochondrial fractions were isolated by centrifuging a portion of the resuspended crude mitochondrial designated Mito[1], fraction over a discontinuous sucrose gradient (5 mL of 0.25 M sucrose over 5 mL 0.68 M sucrose) in a swinging bucket rotor (SB-283) at 68,000xg for 60 minutes as described by Oulton et. al. (Oulton et. al., 1986). The lamellar body fraction collected at the interface of the sucrose solutions, while a second mitochondrial fraction, designated Mito2, pelleted beneath the gradient. The supernate from the 10,000xg centrifugation was spun at 100,000xg for 90 minutes. This produced a microsomal fraction (pellet) and a cytosolic fraction (supernate). All

pelleted fractions were resuspended in homogenizing buffer. Protein kinase C activity was assessed immediately following subfractionation. The remainder of the fractions were frozen at -70°C prior to analysis.

3.2b Protein Quantification

Protein assays were done using the Bio-Rad protein assay kit available from Bio-Rad Laboratories (Mississauga, Ont.), which is based on the method of Bradford (Bradford, 1976). This kit produces a color change upon the formation of a complex between the Coomassie blue dye and sample protein. The intensity of the color may then be compared spectrophotometrically to that formed by known amounts of bovine serum albumin at 595 nm.

3.2c Phospholipid Quantification

Phospholipid was quantified in the organic solvent soluble fraction by determining phosphorus concentrations in the samples using a method based on that of Bartlett (Bartlett, 1959). All tubes used in this assay were previously washed in a phosphate-free detergent. Phosphorus was released by charring an aliquot of the sample in a test tube with 1 mL of 70% perchloric acid for 15 minutes on a Kjeldahl rack. Free phosphorus was reacted with 2 mL of 1.25% ammonium molybdate in 0.5 N sulphuric acid followed by 1 mL of 10% ascorbic acid at 60°C. Samples were quantified against standards of known phosphorus concentrations by comparison of their spectrophotometric absorbances at 820 nm. Phosphorus concentrations were converted to phospholipid by multiplying by a factor of 25, the approximate ratio of their respective molecular weights.

3.2d Analysis of Lamellar Body Fraction

In order to determine the relative phospholipid components of the lamellar body fraction and to determine developmental changes, phospholipids were separated and quantified. Phospholipid component of lamellar body fractions of day 27 gestation, day 1 postnatal, and adult rabbits was isolated from the samples following the Bligh and Dyer protocol (Bligh and Dyer, 1959). One volume of the fraction was mixed with six volumes of chloroform:methanol (1:2). After vortexing, two volumes of chloroform were added and the mixture was vortexed again. The phases were separated by the addition of 2.6 volumes of 1% potassium chloride and centrifugation at 140xg for 5 minutes. The top aqueous fraction was removed and discarded. The bottom organic fraction was dried under nitrogen and resuspended in 1 mL of chloroform:methanol (20:1). Total phospholipid content was determined by assaying the phosphorus content of a 50 µL aliquot. A 600 µL aliquot of this fraction was then set aside for DSPC isolation and quantification, the remainder was used for determination of phospholipid profiles.

To characterize the phospholipids, a 250 µL aliquot was applied to thin layer chromatography plates (LK5D, 19 channel, Whatman). Standards (phosphatidylglycerol, phosphatidylethanolamine, phosphatidyl-L-serine, phosphatidylinositol, phosphatidylcholine, sphingomyelin and lysophosphatidylcholine) were plated in adjacent channels. The plates were

developed in a solvent system of chloroform:ethanol:water:triethylamine, 30:34:8:35 as described by Touchstone et al. (Touchstone et al., 1980). The solvent front was allowed to run to the top of the plate, The plates were removed, air dried and run in the same direction a second time in the same solvent system. Phospholipids were visualized by staining with Molybdenum Blue (Vaskovsky and Kostetsky, 1968). Relative amounts of each phospholipid were determined by scraping the appropriate spot off and quantifying the phosphorus as described above. Phospholipids in this system separated adequately, save for PS and PI which could not be resolved as separate spots.

Phospholipid profiles were also determined from lamellar body fraction of isolated type II cells cultured from day 24 gestation fetuses. Although these are not lamellar bodie proper, they contain a phospholipid material and exhibit lamellar-like structure (Scott, 1992). Prior to fractionation, isolated fetal type II cells were first incubated with ^{3}H-choline and ^{32}P-inorganic phosphorus for 24 hours. Phospholipid separation was done as described above with the exception that the quantification step was achieved by counting radioactivity in the phospholipid fractions on a scintillation counter (model LS 5800, Beckman). Quench was determined using H# (Beckman Instant, Palo Alto, Ca.).

Determination of the DSPC levels in the fractions was done by a modification of the column chromatography technique developed by Mason (Mason et al., 1976). The 600µL portion of the organic fraction was dried under air, and resuspended in 1mL of CCl_4 containing 3.14mg of OsO_4, vortexed and

allowed to incubate at room temperature for 30 minutes. The fraction was dried under air and resuspended with 5mL of chlorofrom:methanol (20:1) and vortexed. This was applied to activated neutral alumina columns (4cms x 1cm). Each column was eluted with 10mL of chloroform:methanol (20:1) to remove neutral lipids. Disaturated phospholipids were eluted with 5mL of chloroform:methanol:ammonium hydroxide (70:30:2). The eluant was collected in vials and allowed to evaporate to dryness. Eluant from whole lung was quantified by phospholipid assay while those from the cultured cells were measured by scintillation counting.

3.2e Enzyme Assays

Protein kinase C was assayed using a modification of the technique developed by Hannun et al. (Hannun et al., 1985). 100 μg of fraction protein was incubated with 1 mM Ca^{2+}, 8 mole% phosphatidylserine, 2.5 mM dithiothreitol, 15 mM Mg^{2+}, 50 mM [g-^{32}P]ATP (total activity 0.25 mCi), and 75 mM synthetic substrate peptide in 50 μL of 50 mM Tris buffer at pH 7.5 at a temperature of 25°C for 3 minutes. Reaction was initiated by addition of 25 μL of 12 mM TPA in the same buffer and terminated by addition of 100 μL of ice cold dilute orthophosphoric acid. The reaction mixture was flushed onto a peptide-binding paper, and washed twice with 5% acetic acid. The papers were placed into vials with 10 mL of Ready Protein+ Scintillation Cocktail (Beckman), and counted in a scintillation counter (model LS 5800, Beckman). ^{32}P counts with an efficiency approaching 100% under these conditions. Quench was corrected by H# which

is based on the spectrum of ^{137}Cs. Calcium-phosphatidylserine-independent activity was determined using the above assay without calcium, TPA or phoshatidylserine. In all cases calcium-phosphatidylserine-dependent activity was determined by subtracting the calcium-phosphatidylserine independent activity from the total activity.

3.2f Inhibition Assays

The effect of known PKC inhibitors was measured in lamellar body and microsomal fractions. Protocol was identical to that used above. Acridine orange, 9-aminoacridine, which have been shown to localize to lamellar bodies (Fabisiak et al., 1987) and sangivamycin were used to inhibit PKC activity at concentrations of 0.0 - 2.0 mM (acridine orange and 9-aminoacridine) and 0.0 - 0.2 mM (sangivamycin).

3.2g Delipidation of Fractions

Subcellular fractions, lamellar bodies in particular, contain very high phospholipid concentrations (Oulton et al., 1993). This may interfere with PKC activity, which is phospholipid dependent. High levels of phospholipid may also interfere with protein quantification ((Friedenauer and Berlet, 1989; Kirazov et al., 1993). Portions of samples were delipidated prior to protein quantification and enzyme assay. The procedure used was that of Fiscus and Schneider (Fiscus and Schneider, 1966). Briefly, 2 mL samples of each fraction were mixed with 20 mL of acetone at 4°C. Precipitate was collected by centrifuging at 10,000xg for 10 min. The pellet was washed with 5 mL of butanol and collected

again by centrifugation. The pellet was dried at 0°C under a stream of nitrogen. The pellet was resuspended in a small volume of homogenization buffer prior to protein and enzyme assay.

3.2h Isolation of Pre-Type II Alveolar Cells

A more thorough examination of the processes of surfactant synthesis and secretion is possible if surrounding influences are kept at a minimum. The use of tissue culture techniques allows the isolation of nearly pure type II cell populations from lung in which some 40 different cell types occur (Sorokin, 1970). Fetal pre-type II cells were isolated on the 24th day of gestation by trypsinization and differential adhesion as described previously (Scott et al., 1983). These cells have been characterized for the ability to produce surfactant-related disaturated phosphatidylcholine (Scott et al., 1986) and lamellar bodies (Scott, 1992).

Pregnant New Zealand White rabbits were anaesthetized and fetuses were removed by hysterotomy and decapitated. Lungs were dissected from the thorax and placed in ice-cold HBSS. After removal of the heart and major airways, tissue was cut into cubes of ~1 mm with sharp scissors and chopped on a Sorval Tissue Chopper. Cells were enzymatically dissociated into a monocellular dispersion by stirring with 100 mL trypsin solution (0.5 g/L), and 80 mg DNAse I (525 U/mg) for 40 minutes. The mixture was filtered through 3 layers of 150 μm Nitex gauze, and protease activity was stopped by overloading with 100 mL of 10% carbon stripped Fetal Calf Serum (sFCS) in Minimum

Essential Medium (MEM). Cells were pelleted at 140xg for 8 minutes, resuspended in 50 mL of MEM with 10% sFCS (v/v) and plated in 75 cm^2 flasks for 1 hour to remove fibroblasts. This was repeated a second time for a 1/2 hour duration. Unattached cells were collected by centrifugation at 250xg for 8 minutes, resuspended in 20 mL MEM with 10% sFCS (v/v) and plated in 25 cm^2 flasks at a density of not less than $1x10^5$ per cm^2. Cells were 95% pure type II as ascertained by osmium tetroxide or trichrome staining (Mason et al., 1976) (Scott, 1994). All cells were used within 3-4 days of isolation.

3.2i Addback Assays

To determine if the observed lamellar body fraction PKC activity was endogenous or the result of contamination, an addback technique was employed (McMurray, 1974). Isolated pre-type II cells were exposed to fibroblast conditioned medium (FCM) for 24 hours to induce differentiation (Smith, 1979; Scott, 1992). After this period cells were transferred to fresh media with or without 10^{-4}M TPA for 2 hours, as shown in Table 1. Medium was removed and the cell monolayer washed with ice-cold HBSS. Cells were scraped into homogenisation buffer A, homogenised and subfractionated as described previously (see section 3.2a). Following subfractionation of the cells, the amount of nonremovable microsomal contamination in the lamellar body fraction was determined by assaying for the marker enzyme cytochrome reductase C. This gave a baseline percentage of microsomal content in lamellar body fraction. Increasing amounts (1.5 µL increments) of microsomal fraction were added to a

fixed amount of lamellar body fraction. Protein kinase C activity was assayed as previously indicated in these fractions. Specific activity was plotted versus microsomal content and the best-fit lines were fit by computer and extrapolated to 0 microsomal content. Enzyme activity is considered endogenous to the LB fraction if the extrapolated line crosses the y-axis before the x-axis, i.e. activity is present at zero contamination.

3.2j Synthesis of Surfactant-Related Phospholipid

The effect of TPA-induced PKC activation on radiolabelled precursor incorporation into surfactant-related phospholipid was determined. Briefly, isolated type II cells were incubated with TPA (10^{-7}-10^{-4} M) simultaneously with the precursors [^{3}H]-choline and [^{32}P]-inorganic phosphorus in Minimum Essential Medium (MEM) for 4 hours. After the incubation, medium was removed and discarded. Cells were washed with HBSS, scraped from the culture vessels into HBSS, homogenised. The cell homogenate was mixed with adult lung homogenate which acted as a carrier. Subcellular fractions, including lamellar bodies, were isolated as described previously (Oulton et al., 1986).

Table 1. Addback experiments were conducted on the following lamellar body preparations isolated from fetal type II cells.The cells were grown in either serum free Minimum Essential Media (sfMEM) or sfMEM containing fibroblast Conditioned Media (Cm in sfMEM, 20%, v/v). Cells were subsequently transferred to fresh media with or without phorbol ester (concentration 10 μM).

	sfMEM	CM in sfMEM	Phorbol Ester
Preparation 1	X		
Preparation 2		X	
Preparation 3	X		X
Preparation 4		X	X

3.2k Release of Surfactant-Related Phospholipid

To determine if PKC activation is associated with release of surfactant-related material, isolated pre-type II cells were allowed to incorporate the precursors [^{3}H]-choline and [^{32}P]-inorganic phosphorus for 24 hours. Prelabelling media was decanted and replaced with TPA in MEM (10^{-4} M) for 2 hours. Media was collected, filtered to remove cells and saved. Cells were scraped from the culture flasks, homogenised and subcellular fractions including lamellar bodies were isolated from cells as described for the lung tissue.

Subfractionation of the media was carried out as done previously with tissue, to determine if material released in response to TPA was similar to lung lamellar bodies. Medium collected from control or TPA-exposed cells was centrifuged at 100,000xg for 1 hour. The pellet was mixed with adult rabbit lung homogenate as carrier and isolated by sequential centrifugation as described above (Oulton et al., 1993). The [^{3}H] and [^{32}P] radioactivity were measured in 50 µL aliquots of each subfraction and extrapolated to total fraction volume. Additionally, the cells were also subfractionated in the same manner in an attempt to determine the compartment of origin of the released radioactivity. Phospholipid profiles were also determined in the released material by thin layer chromatography as described previously (section 3.2d)

3.2l Test of TPA Cytotoxicity

In order to address the question of whether TPA could have a toxic effect, the cells were incubated for 1, 2, 4, 8 and 24 hours with 10^{-4} M TPA. The media

was collected and filtered to remove dead cells. Lactate dehydrogenase activity in the media was determined using a kit (LDL-20) available from Sigma Chemical Co. (St. Louis, Mo.). This assay measures the activity by monitoring spectrophotometrically color changes produced at 340 nm as the enzyme converts substrate lactate into pyruvate.

3.2m Statistics

Analyses of differences between groups were conducted by *post hoc* application of Duncan's Multiple Range Test after determining that such comparisons were valid by analysis of variance.

RESULTS

4.1 WHOLE LUNG

4.1a Protein and Phospholipid

Protein and phospholipid content of microsomal, lamellar body and cytosolic subcellular fractions of fetal, newborn and adult lungs are shown in Table 1. Protein levels in all three fractions increased to a maximum on gestational day 30. The levels of phospholipid content of the microsomal and cytosolic compartments displayed a biphasic pattern prior to birth. The lamellar body fraction phospholipid content peaked on gestational day 27. The ratio of phospholipid to protein was highest in the lamellar body fraction at all timepoints. This ratio displayed a biphasic pattern peaking on day 27 and again on day 1 postnatal. This ratio was also highest in the lamellar body fraction of post-natal day 1 rabbits.

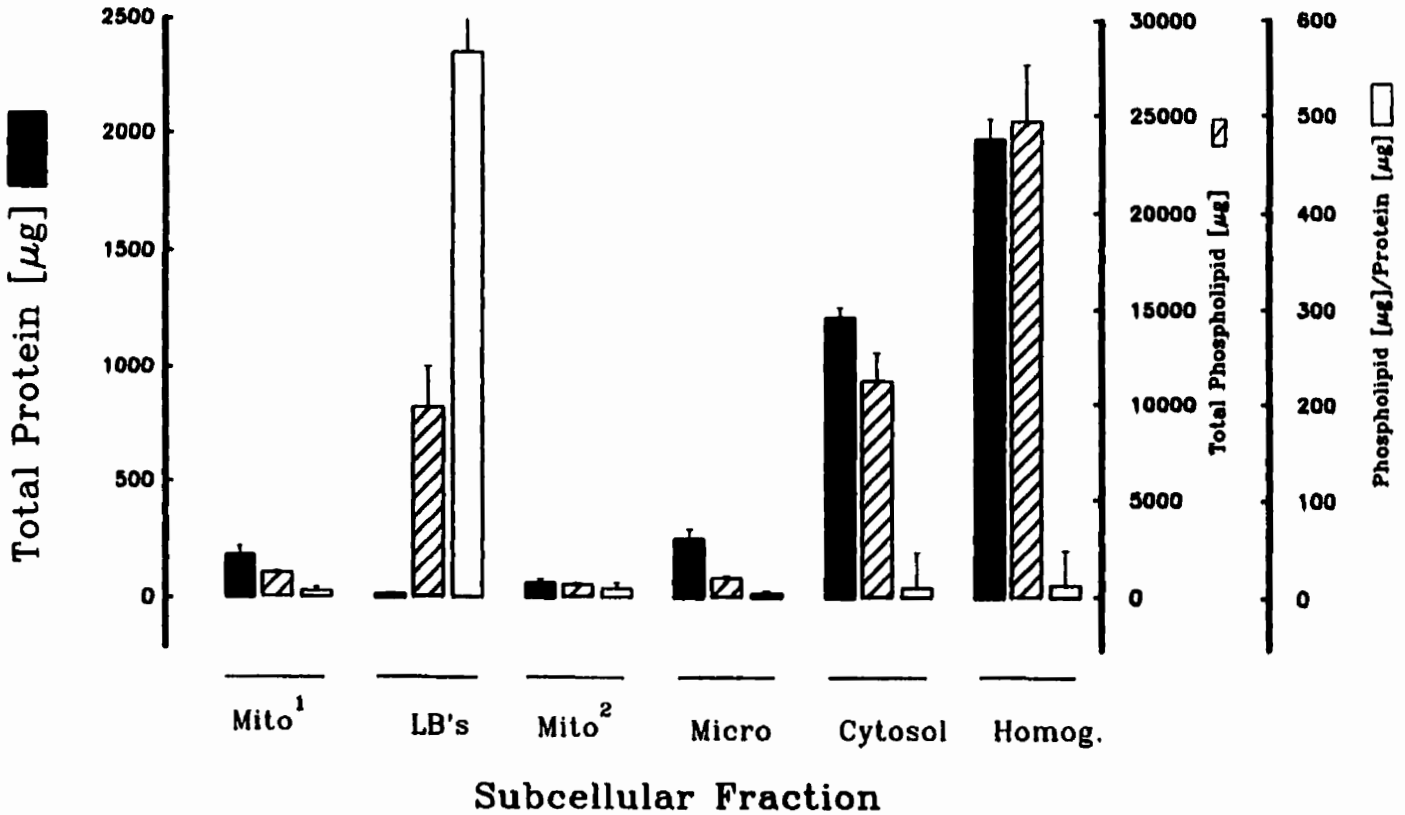
A graphical representation of total phospholipid and protein in tha adult rabbit lung, and the resulting ratio of the two are shown in Figure 2. The lamellar body fraction of adult lung is observed to contain a much greater levels of phospholipid than any other fraction.

Table 2. Protein and phospholipid content in the microsomal, cytosolic and lamellar body fractions isolated from whole lung homogenate by centrifugation, as described in Materials and Methods. In order to facilitate comparison, values for both protein (Prot) and phospholipid (PL) content are expressed as mg/ gm lung tissue. Phospholipid to Protein ratio (PL/Prot) is unitless.All values represent the mean ± standard error of the mean of a minimum of three replicates.

	Microsomes			Cytosol			Lamellar body		
	Prot	PL	PL/Prot	Prot	PL	PL/Prot	Prot	PL	PL/Prot
Gestational day 24	16.25 ± 1.926	11.40 ± 1.743	0.702 ± 0.202	62.34 ± 2.146	32.16 ± 1.864	0.516 ± 0.105	0.241 ± 0.056	1.91 ± 0.384	7.92 ± 2.619
Gest day 27	20.16 ± 1.523	2.59 ± 0.141	0.128 ± 0.0925	87.76 ± 4.848	11.52 ± 0.861	0.131 ± 0.032	0.621 ± 0.136	10.06 ± 1.154	16.20 ± 2.913
Gest day 30	62.26 ± 5.127	12.63 ± 1.004	0.203 ± 0.037	118.56 ± 6.089	25.57 ± 2.691	0.216 ± 0.065	1.010 ± 0.259	8.68 ± 1.041	8.59 ± 2.005
Post day 1	13.55 ± 0.953	11.55 ± 0.853	0.852 ± 0.169	102.01 ± 5.846	21.89 ± 1.457	0.215 ± 0.062	0.291 ± 0.097	6.81 ± 0.438	23.38 ± 4.515
Adult	15.67 ± 1.242	18.89 ± 1.427	1.21 ± 0.296	91.87 ± 7.294	32.32 ± 2.864	0.352 ± 0.039	1.51 ± 0.176	23.45 ± 2.233	15.53 ± 2.042

Figure 2: Protein, phospholipid and protein:phospholipid ratio of the subcellular fractions from adult rabbit lung. Subcellular fractions of adult lungs were prepared as described in Materials and Methods. Protein and phospholipid content were measured in aliquots of each subcellular fraction as described in Materials and Methods. Each bar represents the mean ± standard error of the mean of three replicates from five samples.

Fig 2. Protein and phospholipid content of fractions from adult rabbit lung



4.1b Analysis of Lamellar Body Phospholipids

Lamellar bodies isolated from rabbit lungs of 27th gestational day, postnatal day 1, and adult animals were analysed for their phospholipid composition and the results are shown in Table 3. Phosphatidylcholine was the major constituent, accounting for between 80-85% of the total lipid content in all three samples of lamellar body material. Analysis of a separate aliquot indicated that the disaturated form of phosphatidylcholine accounted for approximately 50% of the total lipid in the fraction. The relative percentage of phosphatidylglycerol increased from day 27 of gestation to adult levels. Phosphatidylinositol and phosphatidylserine accounted for 8% of total phospholipid in day gestational day 27, increasing to 10% in postnatal day 1, followed by a small decline to 7% in the adult lung lamellar bodies decreased from approximately 1% during gestation to a postnatal level of 0.15%.

4.1c Protein Kinase C Activity

Figure 3 depicts total PKC activity detected, with and without the addition of the cofactors Ca^{2+} and PS, in lamellar body and microsomal fractions in adult rabbit lung. The addition of these cofactors increased microsomal PKC activity by approximately 50%, but did not have a significant effect in lamellar body fractions. All assays displayed nonenzymatic phosphorylation at a level of 6%.

Figure 4 shows a time course for total and Ca^{2+}-PS-dependent phosphorylation in the microsomal fraction of 24th gestational day and adult rabbit lungs. Total activity increased up to 5 minutes of incubation in all samples

before reaching a plateau. This time response was reflected in the proportion of PKC activity dependent on Ca^{2+} and PS, which also peaked at this time.

4.1d Specific and Total PKC Activity

Subcellular fractionation of lung produced five fractions: a nuclear fraction, as well as mitochondrial, lamellar body, microsomal and cytosolic fractions. Lungs from animals of 18 and 20 gestational days were not subfractionated due to their small size. Ca^{2+}-PS-protein kinase C activity was detected in all fractions isolated. A representative graph of the total versus specific activity in adult lung fractions is shown in Figure 5. The greatest proportion of activity is observed in the cytosolic compartment (approximately 85%). A significant proportion is localised in the lamellar body fraction (3% in the case of adult lung). the remainder was distributed among the particulate fractions.

Specific activity detected in the lamellar body and cytosolic fractions was found to be of similar magnitude. Specific activity in both these fractions was substantially higher than that detected in the particulate fractions.

Table 3. Phospholipid distribution of common glycerophospholipids in the lamellar body fraction from Day 27 gestation, Day 1 postnatal and Adult lungs. The amount of disaturated phosphatidylcholine for the three age groups was also determined from a separate aliquot. Values are given as a percentage of total lipid and represent the mean ± SEM of a minimum of three determinations. (*PG: phosphatidylglycerol, PE: phosphatidylethanol, PI: phosphatidylinositol, PS: Phosphatidylserine, PC: phosphatidylcholine, SM: sphingomyelin, LPC: lysophosphatidylcholine*)

	PG	PE	PI, PS	PC	SM	LPC	DSPC
Day 27 Gestation	2.478 ± 0.137	1.256 ± 0.080	8.336 ± 0.756	86.684 ± 1.344	1.110 ± 0.1423	0.136 ± 0.034	50.997 ± 1.895
Day 1 Postnatal	4.352 ± 0.219	2.394 ± 0.720	10.464 ± 0.727	81.544 ± 1.704	0.269 ± 0.129	0.976 ± 0.216	55.183 ± 4.235
Adult	6.597 ± 0.304	0.754 ± 0.064	7.171 ± 0.232	84.717 ± 1.156	0.310 ± 0.140	0.452 ± 0.186	49.634 ± 0.729

Figure 3: Ca^{2+}-PS-independent and total protein kinase C activity in adult rabbit lung lamellar bodies and microsomes. The assay method is as described in Materials and Methods. The plus and minus signs below the bars denote the presence or absence of calcium and phosphatidylserine in the reaction. The lone bar to the left of the graph depicts the amount of background phosphorylation that occurs in the absence of the added subcellular fractions to the reaction mixture. The asterisk indicates a significant difference at the $p<0.05$ level. Each bar represents the mean ± standard error of the mean of three replicates from at least five lung samples.

Fig 3. Total and Ca^{2+}–PS–independent protein kinase C activity in adult rabbit lung lamellar bodies and microsomes

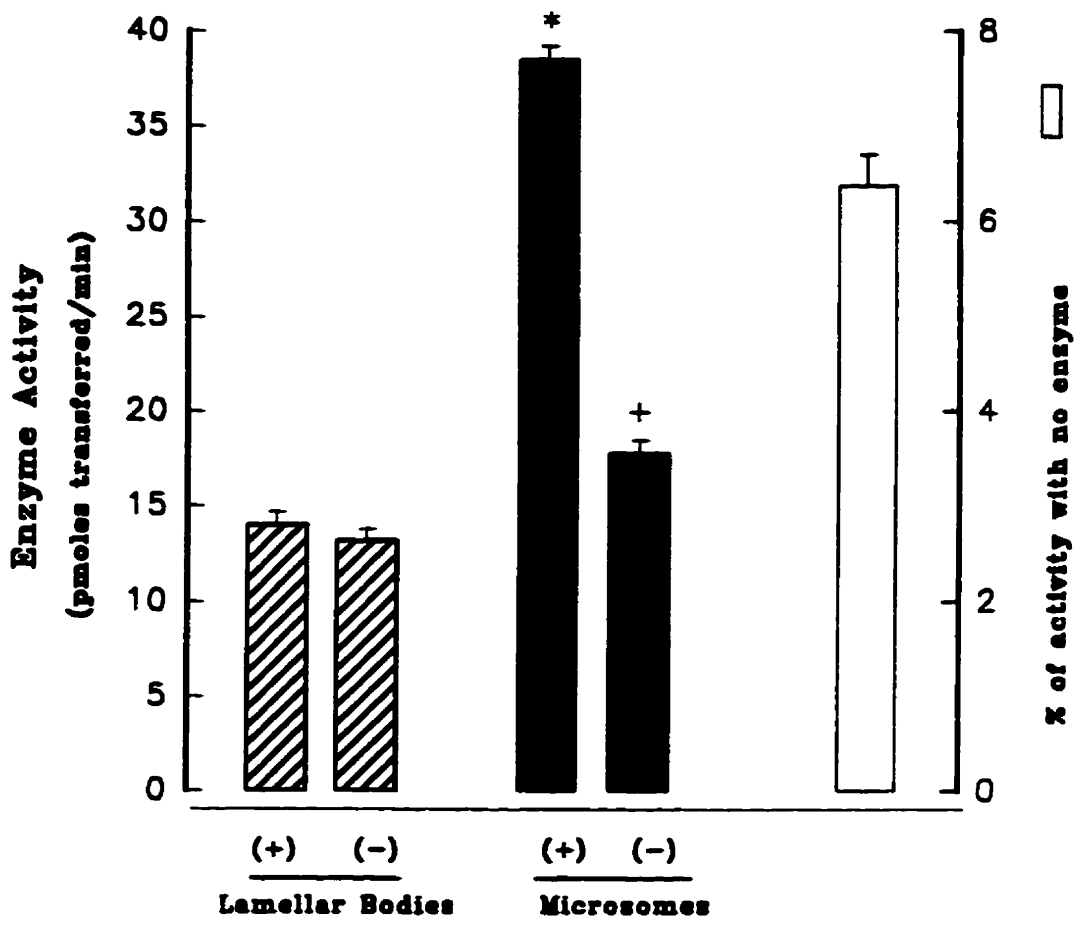


Figure 4: A time course for Ca^{2+}-PS-dependent and total Protein Kinase C activity in microsomal fraction of adult and gestational day 24 rabbit lungs. The assay was carried out as described in Materials and Methods over 15 minutes with identical amounts of protein. Each point represents the mean ± standard error of the mean of three replicates from at four three lung samples.

Fig. 4. Time course for Ca −PS−dependent and Total PKC activity in adult and fetal rabbit lungmicrosomal subcellular fractions.

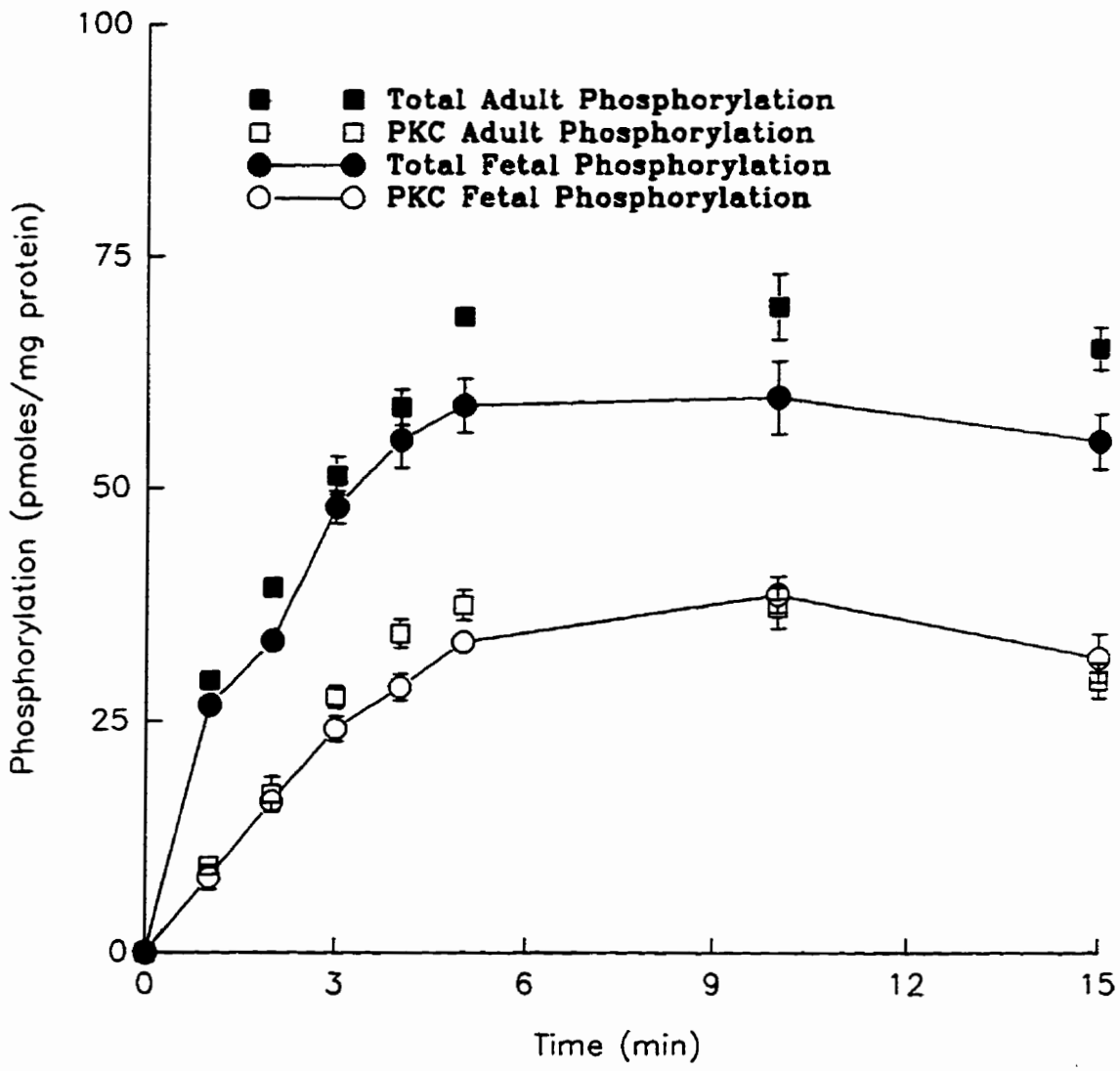
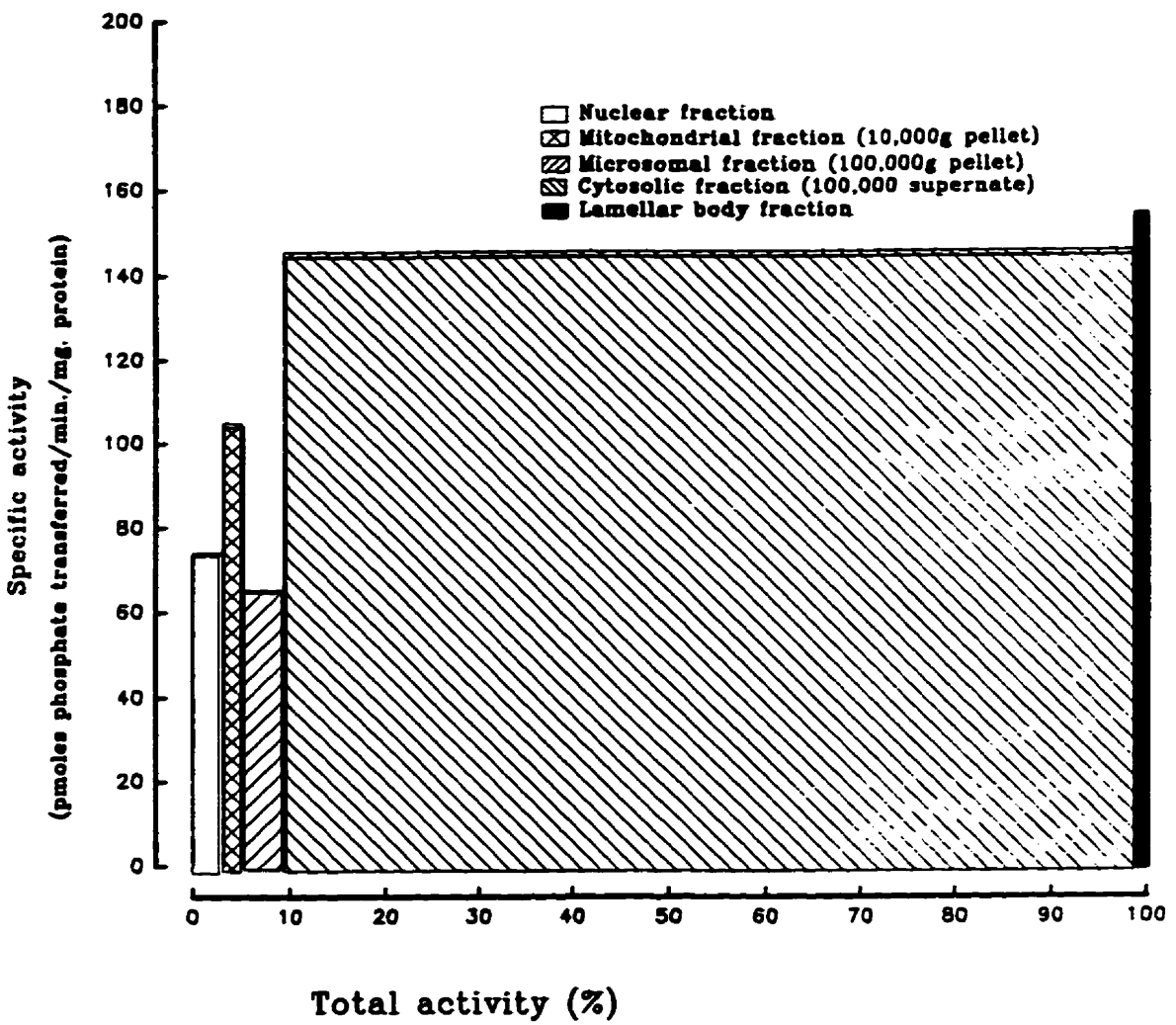


Figure 5: Specific and total activity (expressed as a %) of Ca^{2+}-PS-dependent protein kinase C activity in subcellular fractions isolated from adult rabbit lung. Isolation and enzyme assay of the subcellular fractions were as described in Materials and Methods. Each bar represents the mean ± standard error of the mean of six samples.

Fig 5. Ca^{2+}–PS–dependent protein kinase C activity in adult rabbit lung subcellular fractions

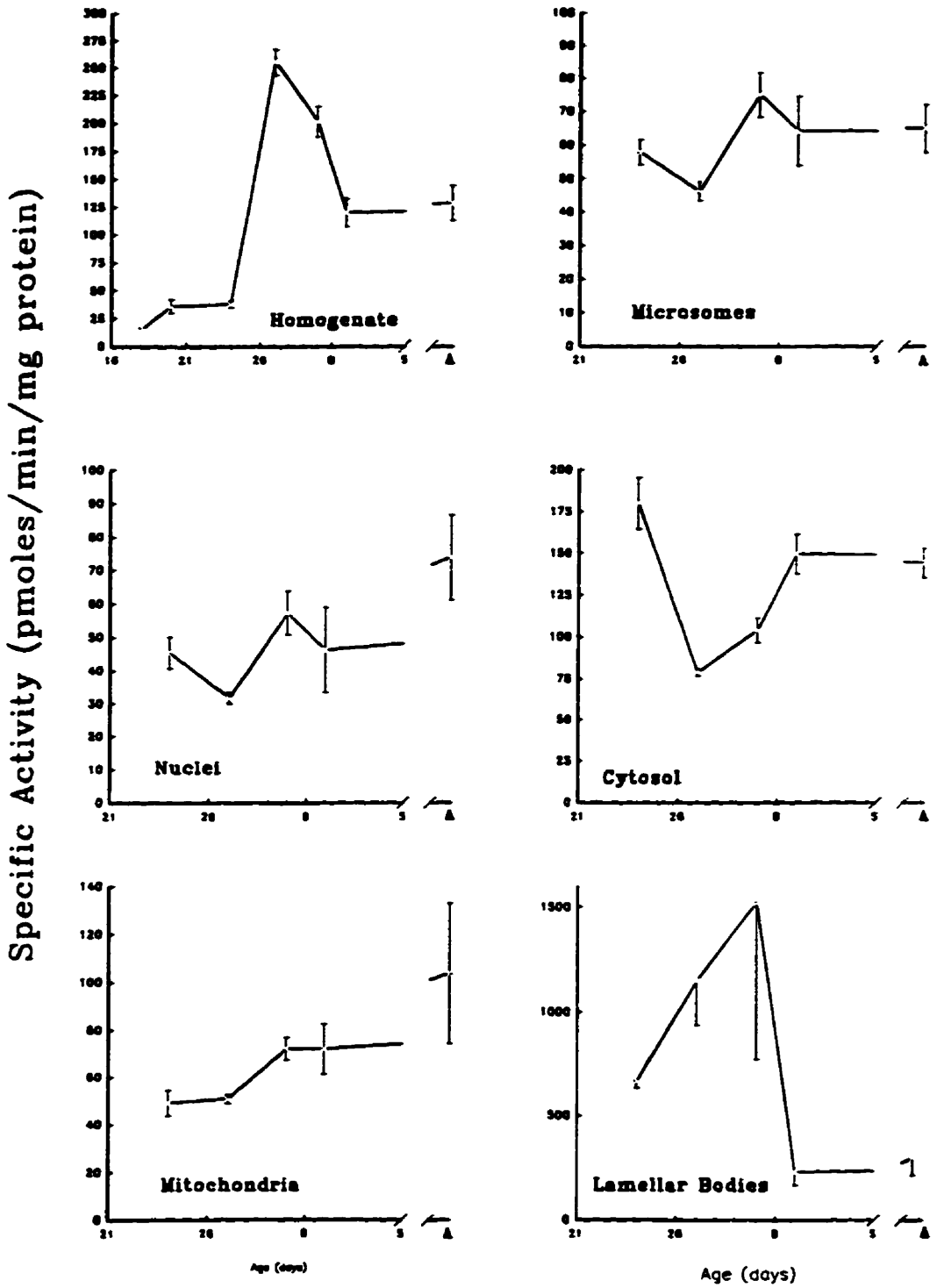


4.1d.i Specific Activity

Changes in specific activity of Ca^{2+}-PS-dependent PKC with age is shown in Figure 6. Homogenate specific activity was low prior to and including gestational day 24, but increased dramatically (approaching 10 fold) to peak on day 27 gestation. This activity is decreased to roughly 50% of peak activity by postnatal day 1, which approximates specific activity in the adult. The nuclear and microsomal activity followed similar patterns decreasing between gestational days 24 and 27, increasing slightly just prior to birth. The mitochondrial fraction displayed a continuous increase throughout the range. The wide fluctuations of the homogenate was not reflected in any of these three fractions. Cytosolic specific activity decreased by approximately 50% between gestational days 24 and 27, but recovers almost 90% of the specific activity by postnatal day 1. Activity in the lamellar body subfraction displayed great variability with age as well as within each individual age group. The general trend of the activity increased by greater than 100% between days 24 and 30. Specific activity decreased to approximate adult levels by postnatal day 1, 10% of the peak activity.

Figure 6: Specific activity of Ca^{2+}-PS-dependent protein kinase in homogenate and subcellular fractions of fetal newborn and adult rabbit lungs. Coordinates of the horizontal axis indicates gestational age (21-30), birth (B), postnatal age (1-5) and adult (A). Each point represents the mean ± standard error of the mean of Ca^{2+}-PS-dependent protein kinase C activity assayed in triplicate in isolated fractions from a minimum of three animals (adults) or three litters (fetuses and newborns) at each time.

Fig. 6. Ca^{2+}-PS-dependent PKC activity in developing, neonatal and adult rabbit lung subcellular fractions.



4.1d.ii Total Activity

Figure 7 depicts the changes in total Ca^{2+}-PS-dependent PKC in the same fractions shown in Figure 6. Homogenate total enzyme activity was low on the gestational days 18 and 20, but increased considerably after this, peaking on day 27. The variation of total enzyme activity with age in nuclear, mitochondrial, microsomal and cytosolic activity were of a similar form, characterized by a biphasic pattern with high activity on gestational days 24 and 27, followed by a sharp decrease immediately prior to birth, increasing again on postnatal day 1. In contrast the lamellar body fraction peaked on day 27, before a rapid decrease to adult levels by postnatal day 1.

4.1e Inhibition Assays

Figure 8 shows the response of microsomal and lamellar body PKC activity when assayed in the presence of varying concentrations of inhibitors. Sangivamycin was the most potent of the three inhibitors used with an IC_{50} (inhibitor concentration producing 50% reduction in normal activity) of 0.025mM in the microsomal fraction. Inhibition of microsomal PKC activity by acridine orange and 9-aminoacridine gave IC_{50} of 0.2 and 0.6mM respectively. The lamellar body fraction was resistant to inhibition, giving erratic responses to increasing inhibitor concentrations.

Figure 7: Total activity of Ca^{2+}-PS-dependent protein kinase in homogenate and subcellular fractions of fetal newborn and adult rabbit lungs. Coordinates of the horizontal axis indicates gestational age (21-30), birth (B), postnatal age (1-5) and adult (A). Each point represents the mean ± standard error of the mean of Ca^{2+}-PS-dependent protein kinase C activity assayed in triplicate in isolated fractions from a minimum of three animals (adults) or three litters (fetuses and newborns) at each time.

Fig. 7. Ca -PS-dependent PKC activity in developing, neonatal and adult rabbit lung Subcellular fractions.

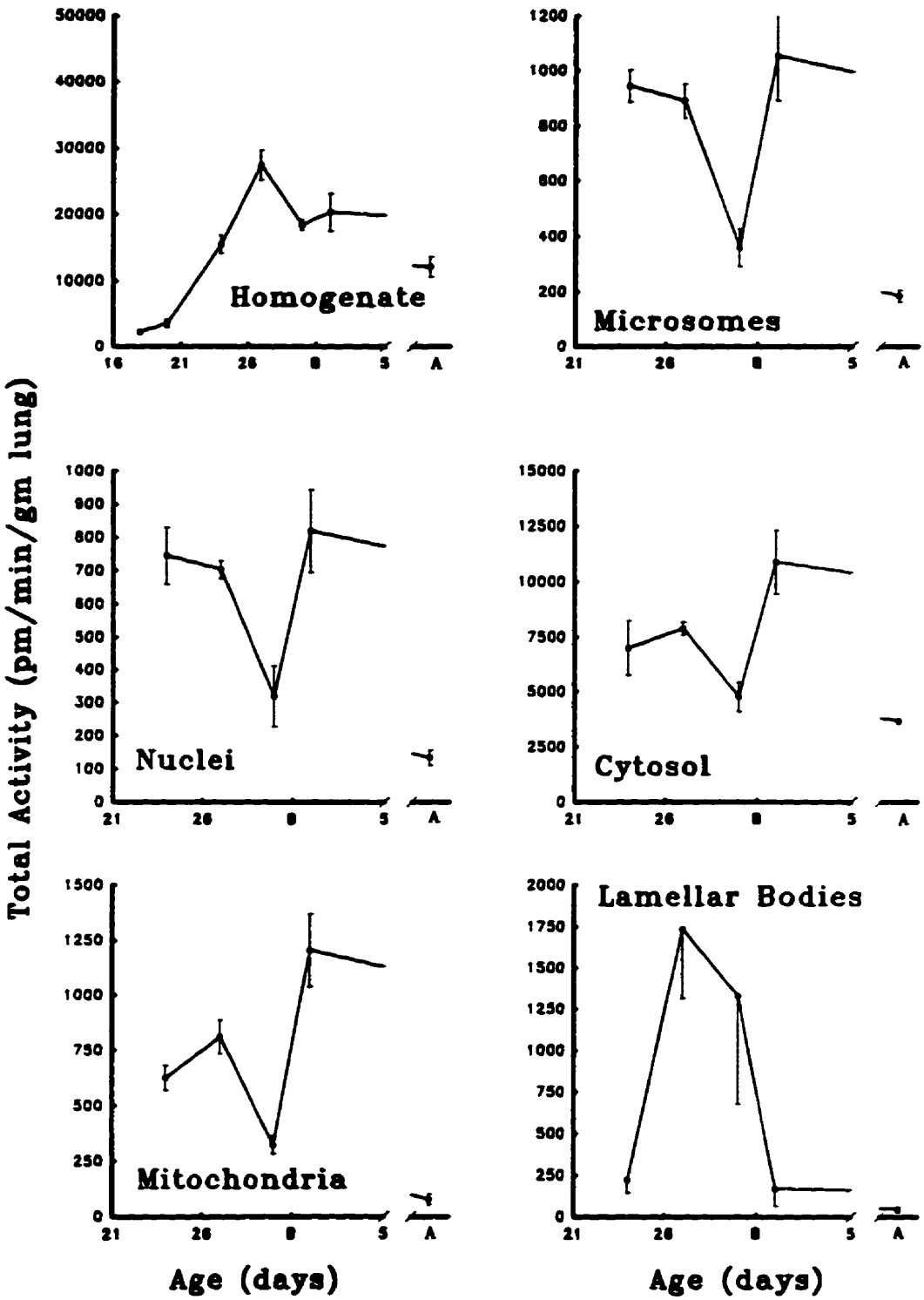
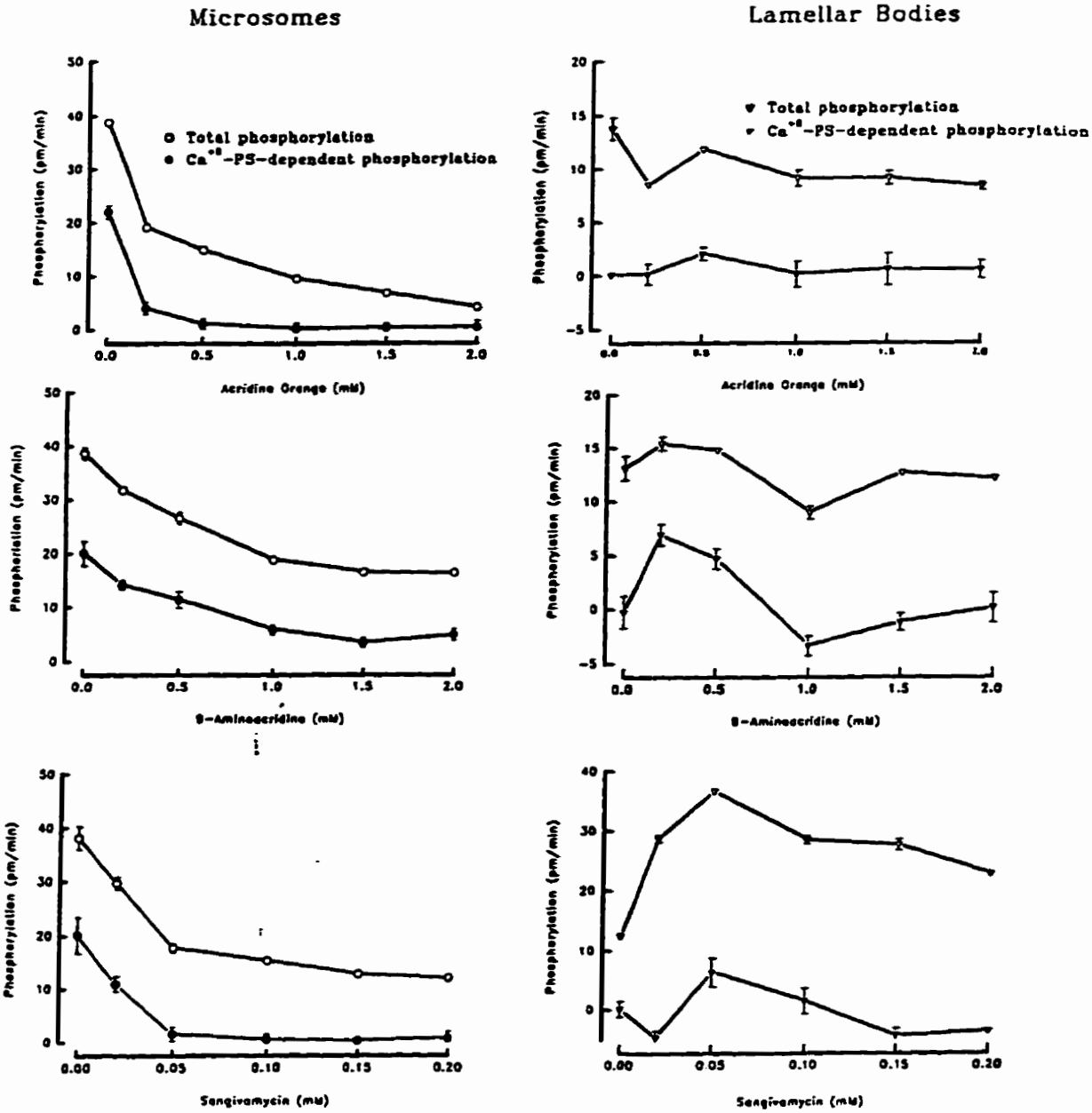


Figure 8: Effect of the PKC inhibitors Acridine Orange, 9-Aminoacridine and sangivamycin on enzyme activity in lamellar body and microsomal fractions. Identical amounts of protein from adult lamellar body and microsomal fractions were incubated with various concentrations of the three above inhibitors, and PKC activity assayed. Results are expressed as pmoles of [^{32}P] transferred per minute. Each point represents the mean ± standard error of the mean of at least four replicates.

Effect of Ca^{+2}–PS–dependent PKC inhibitors on enzyme activity



4.1f PKC Activity in Delipidated Fractions

Table 4 shows the Ca^{2+}-PS-dependent PKC activity in isolated fractions from day 27 gestation, day 1 postnatal and the adult lung that were subjected to a delipidation procedure. Most fractions displayed substantial reductions in specific activity after delipidation. In the lungs of day 27 gestational day fetuses, the crude mitochondrial and lamellar body fractions retained a greater level of activity. Delipidation removed the majority of the Ca^{2+}-PS-dependent PKC activity in the homogenate and microsomal fraction.

Fractions isolated from postnatal day 1 animals displayed an opposite reaction to the delipidation. The homogenate and the microsomal fraction retained a greater proportion of activity after delipidation.

Table 4 Effect of sample delipidation on Ca^{2+}-PS-dependent PKC activity in subcellular fractions of fetal, neonate and adult rabbit lung. Results are expressed as specific activty (pmoles phosphate transferred/minute/mg of protein) and represents the mean ± standard error of the mean of a minimum of three deteminations.

Fraction	Day 27 Gestation		Day 1 Postnatal		Adult	
	Whole	Delipidated	Whole	Delipidated	Whole	Delipidated
Homogenate	313.4 ± 11.2	26.9 ± 3.1	100.6 ± 11.5	82.7 ± 6.7	162.9 ± 14.5	71.5 ± 8.6
Mitochondria	54.0 ± 3.6	46.2 ± 3.9	101.6 ± 3.8	27.4 ± 3.2	85.6 ± 3.2	46.1 ± 2.8
Lamellar Body	941.6 ± 64.8	236.4 ± 17.8	131.2 ± 6.0	38.7 ± 3.0	297.4 ± 51.1	42.6 ± 5.5
Microsomal	76.8 ± 6.9	13.2 ± 1.6	66.0 ± 6.9	23.7 ± 1.5	92.5 ± 11.2	67.6 ± 2.4

4.2 ISOLATED CELLS

4.2a PKC Activity in Lamellar Body Fraction

Ca^{2+}-PS-dependent PKC activity in lamellar body and microsomal fractions of control and TPA-exposed cells is shown in Figure 9. Lamellar body PKC activity was not significantly affected by exposure to TPA. However, activity in the microsomal fraction did increase in response to incubation with TPA. Figure 10 illustrates the results observed in the addback assays conducted on the different preparations of lamellar body fractions isolated from cultured cells, and treated as shown in Table 1 (see Materials and Methods, addback assay.). The preparations not exposed to TPA produced a curve that when extrapolated, crossed the y-axis below zero activity at zero microsomal content. The curve generated from the subcellular fractions collected from cells exposed to TPA gave a positive y-intercept when extrapolated to zero microsomal contamination.

4.2b Distribution of Radioactivity in Fetal Type II Alveolar Cells After TPA Exposure

Radioactivity in subcellular fractions isolated from fetal rabbit lung cells of day 24 gestation fetuses exposed to [^{3}H] choline with or without TPA are shown in Figure 11. Inorganic [^{32}P] showed an identical profile and is not shown for simplicity. Mitochondrial, microsomal and lamellar body subfractions from cells exposed to TPA displayed a significantly greater ($p<0.05$) in radioactive precursor incorporation compared to the same fractions from unstimulated cells. Total membrane and cytosolic incorporation also showed differences, although

the TPA induced decline in cytosolic incorporation did not reach significant levels.

4.2c Release of Radioactive Precursors in Response to TPA Stimulation

Figure 12 shows relative amounts of radioactivity released by the cells after subfractionation of the media by the previously described centrifugation steps. Fractions isolated do not necessarily indicate the presence of a true fraction, i.e. a nuclear or mitochondrial, but rather a fraction of material that pelleted under conditions normally used to isolate that fraction from a cell homogenate. Cells allowed to incorporate the radioactive precursors prior to TPA stimulation were found to release significantly greater levels of radioactivity into the culture medium than cells not treated with TPA. The subfraction corresponding to lamellar bodies from TPA-exposed cells contained a ten fold increase of inorganic ^{32}P and ^{3}H-choline. Significantly higher amounts of radioactivity were also seen in the mitochondrial and microsomal fractions. Figure 13 shows ultrastructural characteristics of material collected from the culture medium of fetal type II cells exposed to 10^{-4} TPA. Diffuse liposomal or micellar-like structures were detected. In addition, membranous whorls resembling lamellar body-like material but in a somewhat distended state were present. No evidence was was found indicating the presence of damaged cells or subcellular organellar components.

Figure 9: Ca^{2+}-PS-dependent protein kinase C activity in lamellar body fraction and microsomal fractions isolated from fetal type II alveolar cells. Cells were induced to differentiate by exposure to conditioned medium (CM) for 24 hours prior to incubation with TPA. Cells were transferred to fresh medium with or without 10^{-4} TPA for 4 hours. Subfractions were isolated as described as in Materials and Methods. Each bar represents the mean ± standard error of the mean for a minimum of six determinations. +, significantly different ($p<0.05$) from the cell samples not exposed to conditioned medium. *, significantly different ($p<0.05$) from the cell samples not exposed to TPA.

Fig. 9. Effect of 4β phorbol ester of Ca^{2+}-PS-dependent protein kinase C activity to the microsomal or lamellar body compartments in isolated fetal type II alveolar cells.

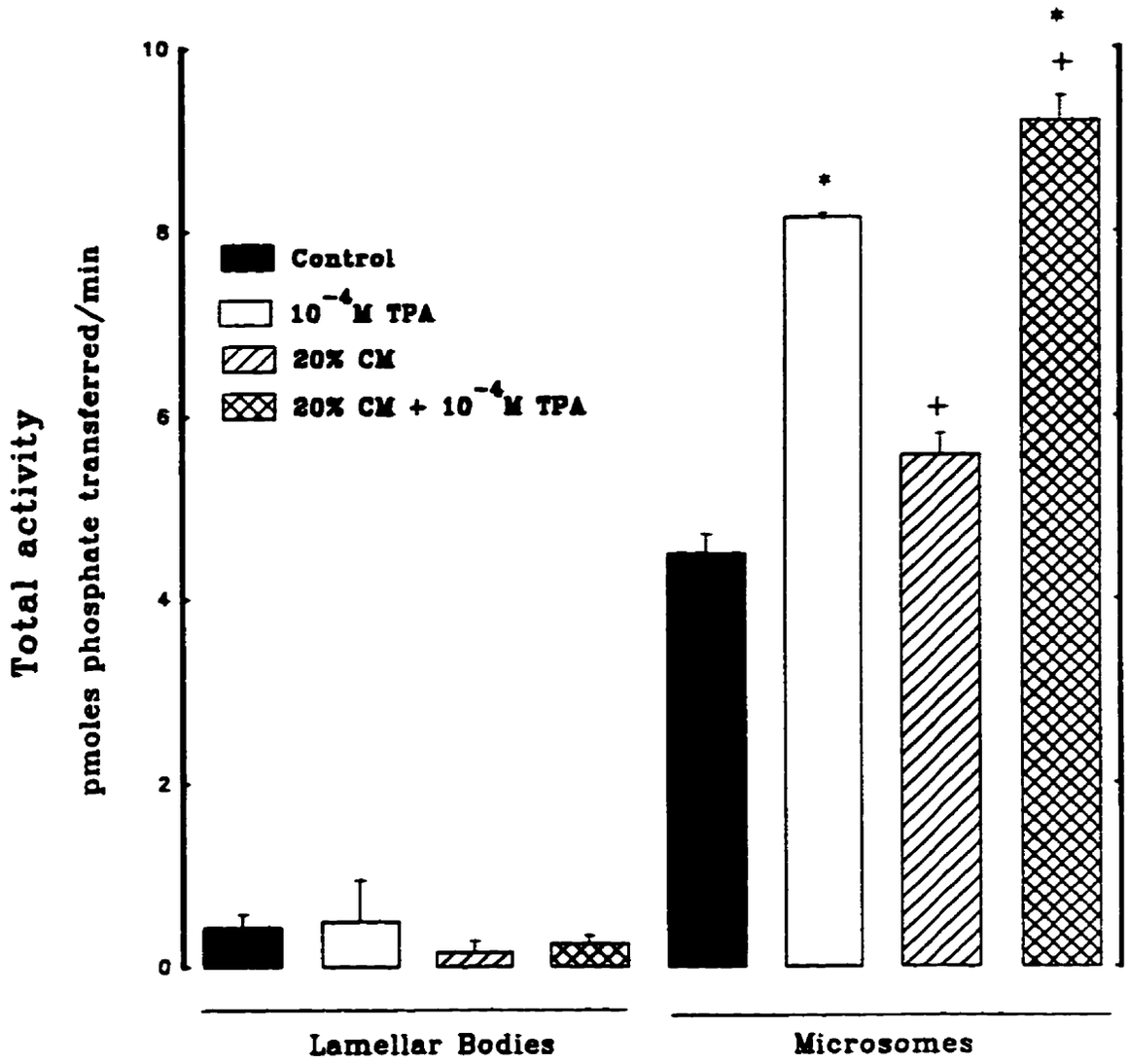


Figure 10: Effect of TPA on the Ca^{2+}-PS-dependent protein kinase C activity in the lamellar body fraction with incremental amounts of microsomal fraction. Cell samples were preincubated with either MEM or CM in MEM for 24 hours, gently rinsed and transferred to fresh medium with or without TPA (10μM) for 4 hours. Cells were subsequently removed, homogenized and lamellar bodies and microsomes isolated as previously described in Materials and Methods. Each point represents the mean ± standard error of the mean of at least three replicates. Regression lines were computer-fit.

Fig 10. Effect of 4βphorbol ester on Ca2+-PS-dependentprotein kinase C activity in lamellar bodies isolated from fetal rabbit Type II alveolar cells.

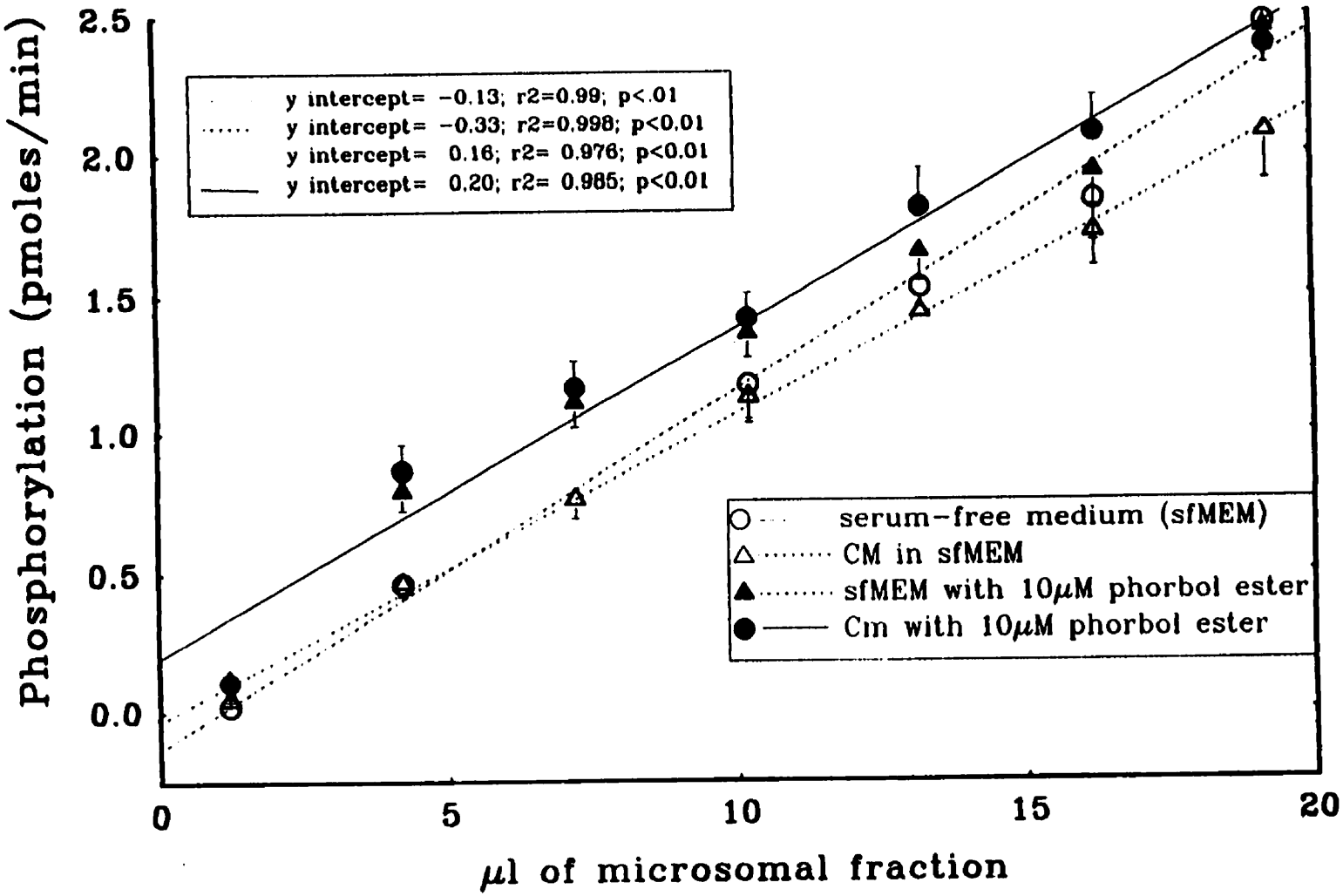


Figure 11: Distribution of [^{3}H]choline radioactivity in the subcellular compartments of fetal type II alveolar cells after simultaneous incubation with 10^{-4} TPA and the radioactive precursors inorganic [^{32}P] and [^{3}H]choline. The pattern of distribution was identical for both precursors and for the sake of simplicity only [^{3}H] is shown. results are expressed as a percentage of the total activity found in the homogenate and represent the mean ± standard deviation of a minimum of four determinations. The symbol * denotes a significant difference ($p<0.01$) between the experimental and control fractions. *Mito*^{1}, mitochondrial fraction isolated as the pellet from centrifugation at 10,000g; *LB's*, lamellar body fraction collected at the interface of a discontinuous sucrose gradient after centrifugation at 68,000g; *Mito*^{2}, a refined mitochondrial fraction pelleted beneath the sucrose interface of the preceding step; *Micro*, microsomal fraction, collected as the pellet of a 100,000g centrifugation step; *Cytosol*, the supernate of the preceding step; Membr, denotes the cumulative radioactivity in all membranous fractions. • indicates a significant difference ($p<0.05$) from the serum-free controls.

Fig 11. Distribution of radioactivity from [^{3}H]choline in fetal type II cell subcellular fractions after exposure to 10^{-4}M 4β phorbol ester

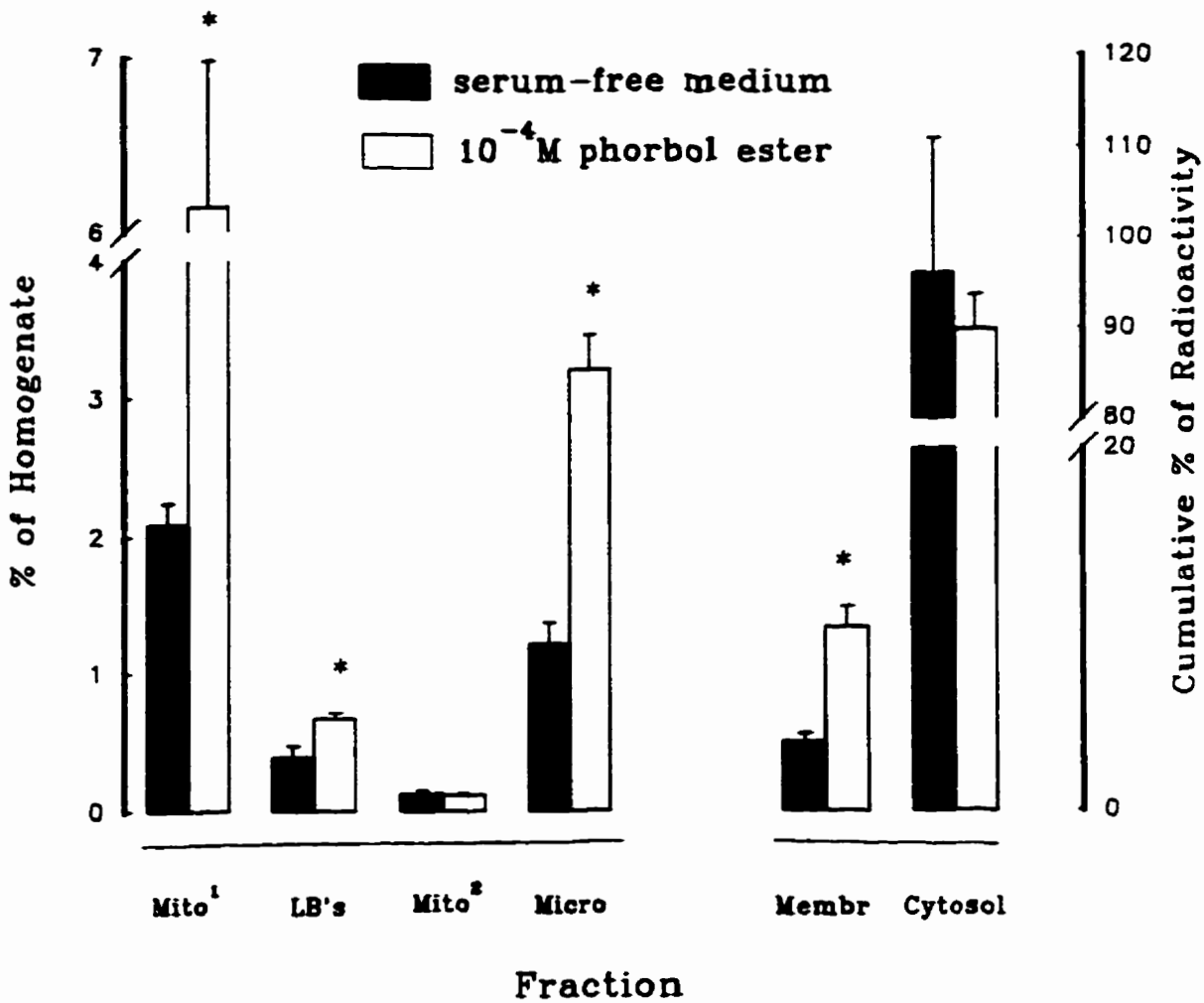


Figure 12: Distribution of [^{32}P] and [^{3}H]choline radioactivity in material released by isolated fetal type II alveolar cells. The cells were prelabelled for 24 hours with radioactive precursors, gently rinsed and incubated with or without 10^{-4} TPA in fresh media. After 4 hours the media was collected, mixed with adult rabbit lung homogenate and subjected to the subcellular fractionation procedure as described in the Materials and Methods. Results are expressed as a percentage of the total radioactivity recovered from all the fractions and represent the mean ± standard error of the mean of a minimum of four determinations. The designation of the fractions are identical to those in Figure 11. The * indicates a significant difference ($p<0.05$) from the serum-free controls.

Fig 12. Distribution of [^{32}P] and [^{3}H]choline in material released by isolated fetal rabbit type II alveolar cells.B

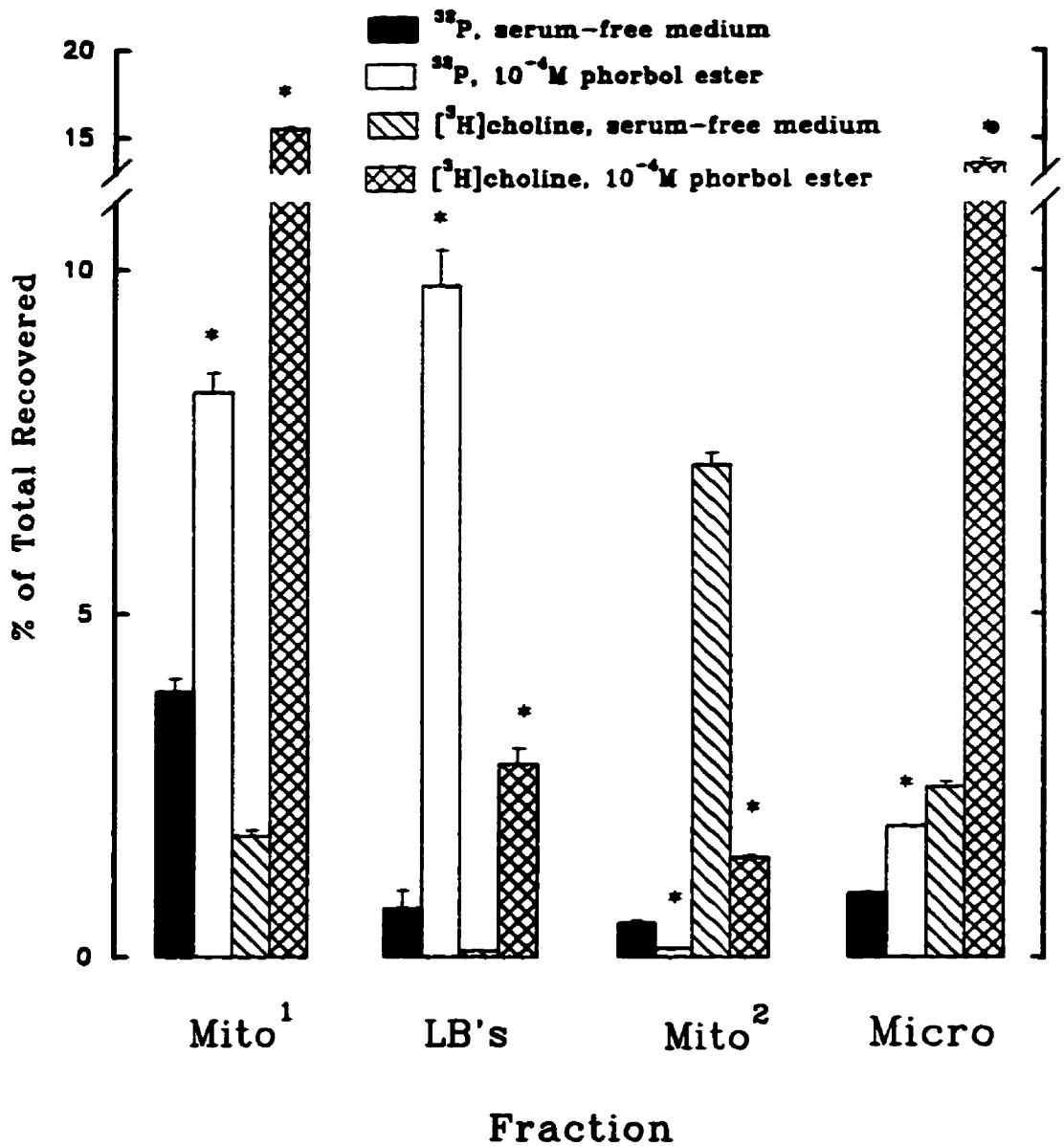
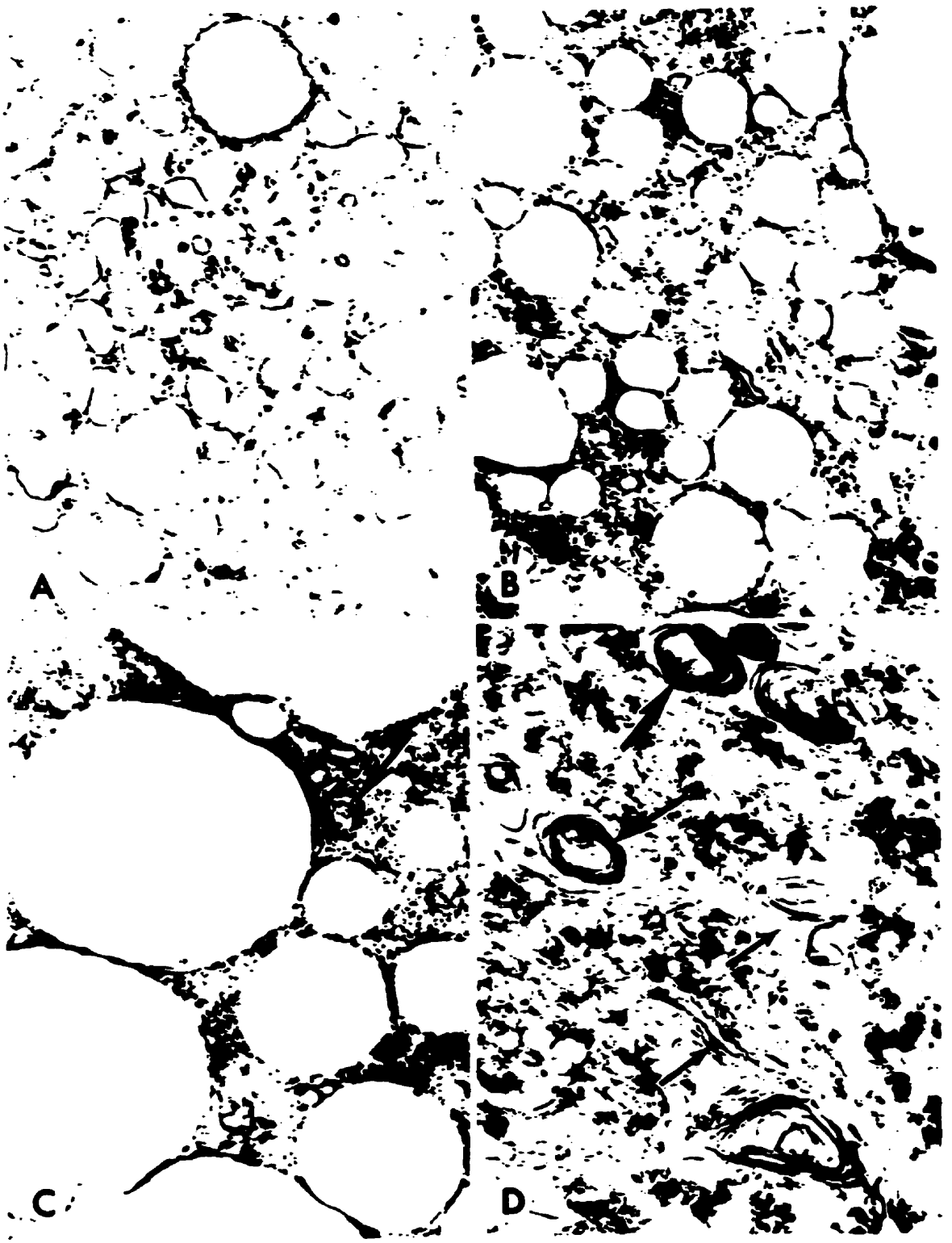


Figure 13: An electron micrograph of material collected from the culture medium of fetal type II cells exposed to 10^{-4} TPA. A (x11850). Relatively small vesicular membranous forms were observed. B (x11265). Large liposomal-like structures were also present. C (x17250). Large liposomal-like structures were bounded by membranes. Small lamellar structures were infrequently observed to co-precipitate with the large forms. D (x 17250). Lamellar structures that co-migrated with micellar liposomal structures with different morphologies. Some display a diffuse membranous nature (small arrows), in contrast to others that had their membranes condensed onto each other (large arrows).

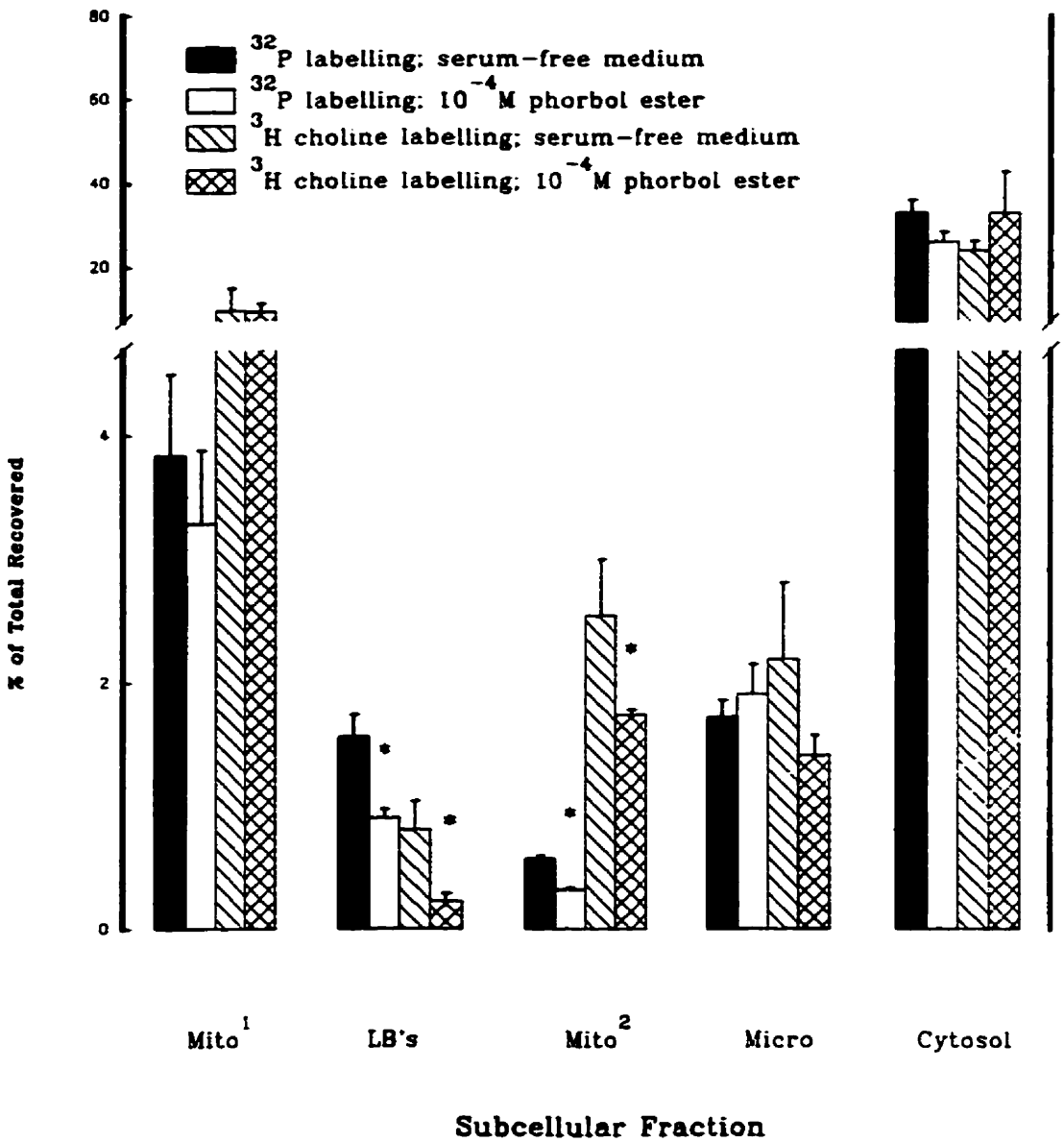

A
B
C
D

4.2d Remaining Radioactivity after TPA induced Release

The cells used in the above release study were washed, scraped, homogenised and fractionated using the same procedure. Radioactivity associated with the fractions was counted and is shown in Figure 14. The lamellar body and refined mitochondrial fractions contained significantly less radioactivity after TPA exposure than the identical fractions from unstimulated cells. Homogenate, cytosol, microsomal and crude mitochondrial fractions displayed no significant differences in radioactivity.

Figure 14: Subcellular distribution of [^{32}P] and [^{3}H]choline radioactivity remaining in the fractions following exposure to 10^{-4} TPA. After the cells were treated as in figure 7, they were scraped and subcellular fractions collected as described previously. Results are expressed as a percentage of the total radioactivity recovered from the cells and represent the mean ± standard error of the mean of a minimum of four determinations.

Fig 14. Subcellular distribution of radioactivity from [^{3}H]choline and [^{32}P] labelled fetal alveolar type II cells exposed to phorbol ester



DISCUSSION

Investigations of lung function have revealed the paramount importance of pulmonary surfactant in maintaining and stabilizing the lung during expansion and particularly at minimum lung volume. The significant role of pulmonary surfactant precipitated many studies which have provided insights into its composition and synthesis. However the mechanisms that regulate alveolar surfactant levels are yet to be elucidated. Alveolar levels of surfactant were originally thought to result from a receptor-coupled ligand-stimulated secretion mechanism. However recent evidence suggests that regulation of alveolar surfactant levels is far more complex (Rooney, 1985; Chander and Fisher, 1990; Bourbon, 1991; Mason, 1992). Feedback mechanisms have also been found to contribute to maintenance of alveolar levels. For example the presence of phospholipid and the surfactant protein SP-A in the alveolar space may inhibit secretion and/or *de novo* synthesis of phosphatidylcholine (Scott, 1992; Suwabe et al., 1992; Kresch et al., 1994).

The regulation of these processes controlling surfactant synthesis and secretion is therefore more complex. In fetal lung these processes are initiated in fetal type II cells during late gestation. The present study focuses on the regulation of surfactant secretory processes in the fetal lung.

Secretion of pulmonary surfactant by type II pneumocytes may be stimulated by a number of different agonists which function through different receptors (Rooney, 1985; Chander and Fisher, 1990; Wright and Dobbs, 1991).

Of these pathways for surfactant release, activation of protein kinase C (PKC) at some point after receptor activation produces release of surfactant-related material to a greater magnitude than by any other pathway (Mason, 1992). It has also been demonstrated that PKC activation in fetal type II cells induces release of surfactant-related phospholipids (Scott, 1994). These results suggest the involvement of PKC in surfactant secretion. They also suggest that PKC is activated in developing type II cells during the period of initiation of surfactant synthesis and secretion.

Previous studies have shown that activation of PKC is initiated by an increase in cytosolic calcium (Ca^{2+}), which appears to form a complex with enzyme. This binding increases the enzyme's affinity for diacylglycerol (DAG) and phosphatidylserine (PS), causing the enzyme to translocate to the membranous compartments of the cell resulting in formation of an activated enzyme complex consisting of PKC, Ca^{2+}, DAG and PS (Huang, 1989; Azzi et al., 1992; Hug and Sarre, 1993).

The assay used in this study is based on that developed by Hannun et al. (Hannun et al., 1985) and uses an environment of mixed micelles composed of octylphenoxypolyethoxyethanol (Triton X-100), PS, and tetradecanoylphorbol acetate (TPA). These micelles associate with the enzyme causing full activation and phosphorylation of a synthetic substrate. It should be noted that some amount of phosphorylation takes place in the absence of PS and Ca^{2+}. PKC activities reported in the present work represent the total phosphorylation minus

that observed without these co-factors and therefore represents Ca^{2+}-PS-dependent PKC activity only.

5.1a Phospholipid and Protein levels in the Developing Rabbit Lung

Table 2 indicates an increase in protein levels occurs in the developing rabbit lung on gestational day 24, apparently signifying a period of cell growth. This result corresponds with previous studies indicating that the appearance of major lung cell types, in particular type II alveolar cells does not occur until just after gestational day 24 in the rabbit (Kikkawa et al., 1968; Kikkawa et al., 1971). The increased phospholipid level which follows on day 27 is most likely the result of initiation of surfactant synthesis in the phenotypic type II cells. This is supported by the ratio of phospholipid to protein in the lamellar body subfraction. It is clear that this ratio doubles between gestational days 24 and 27 (Table 3), day 27 being the first day which secreted lamellar bodies are detectable within the presumptive alveoli (Faridy and Thliveris, 1987). Previous studies have shown increases in lavage phospholipid at this time (Rooney et al., 1976; Rooney and Gobran, 1977), particularly disaturated phosphatidylcholine (Oulton and Dolphin, 1988) which is considered to be a marker of surfactant.

Another increase in this ratio is observed between gestational day 30 and postnatal day 1, as gas exchange is initiated within the lung at birth. Figure 2 indicates that this ratio increased as the lung matured.

5.1b Phospholipid Composition in Lamellar Body Fraction

The lamellar body fraction is characterized by high levels of phosphatidylcholine and phosphatidylglycerol (Akino, 1992). In fractions isolated from whole lung of fetuses of 27 gestational days, and animals of postnatal day 1 and adults, levels of these two phospholipids are high (Table 4). Further evidence that this fraction represents intracellular-stored surfactant is provided by the high levels of disaturated phospholipid. Disaturated phospholipid accounted for greater than 48% of total phospholipids in all three age groups. This indicates that the fraction isolated as the lamellar bodies from neonatal rabbits contains surfactant-related material. Material isolated from the media of cultured cells of 24 gestational days fetal rabbits also displayed the typical vesicular appearance of lamellar bodies as depicted in the electron micrograghs (Fig. 8).

5.1c PKC Activity in Rabbit Lung

The addition of the PKC cofactors Ca^{2+} and PS significantly increased measurable phosphorylation in all fractions except the lamellar bodies. The lack of a significant effect in this fraction is likely not due to the inability of the added cofactors to activate the enzyme, but rather reflects the fact that the lamellar bodies have a high lipid content which may substitute for PS (Leach and Blumberg, 1989; Huang, 1989; Hug and Sarre, 1993), keeping the enzyme in an activated state. Unless specifically stated otherwise, future references to PKC activity refers to that which is Ca^{2+}-PS-dependent.

The time course of the enzyme activity shows that the enzyme phosphorylates substrate in a linear manner for the first 5 minutes of the assay. The length of the assay used in this study was therefore set at 3 minutes to ensure a linear response.

5.1d Subcellular Distribution of PKC Activity in Rabbit Lung

The majority of PKC activity in adult rabbit lung was present in the cytosolic fraction. Previous studies have similarly found up to 90% of PKC activity associated with the cytosolic fraction (Sano et al., 1985; Borner et al., 1992). PKC in this fraction is usually considered inactive as it requires a stimulus to translocate to cell membranes in order to be fully activated (Azzi et al., 1992; Hug and Sarre, 1993). Under the present assay conditions, full activation of cytosolic enzyme is achieved by supplying phorbol ester and phosphatidylserine. Consequently the activity recorded in this fraction represents a potential maximum which could act as a reserve, translocating to the membranes under appropriate stimulus conditions.

All particulate fractions assayed showed some enzyme activity. However the presence of PKC in these fractions does not necessarily indicate that the enzyme is in an active enzyme conformation *in vivo*. Studies have shown that dissociation of PKC from the membrane does not always follow removal of Ca^{2+} from the activated PKC complex (Wolf et al., 1985).

The highest specific activity among the particulate fractions was present in the lamellar bodies. The small difference between the activities measured with

and without co-factors required for activation suggests that PKC in the lamellar body fraction is already in an activated state. As noted above, this may be due to the high concentrations of phospholipid in this fraction. Although PS is generally regarded as one of the activating co-factors of PKC, studies have shown other phospholipids are capable of PKC activation (Hug and Sarre, 1993). Although protein in this fraction accounts for an extremely small portion of the total in adult lung, lamellar bodies contained approximately 4% of total PKC activity. The high levels of PKC activity relative to protein content support the hypothesis of a major role for PKC in the processes of the surfactant cycle.

5.1e PKC activity in fetal and neonate lung

5.1e.i Specific Activity

PKC specific activity at various time points in fetal, neonate and adult lung subcellular fractions displayed different patterns. The dramatic increase in specific activity observed in the homogenate between days 24 and 27 gestation corresponds to the period when type II cells first appear within the lung (Kikkawa et al., 1971). Additionally, DSPC content of lamellar bodies (Oulton and Dolphin, 1988) and phospholipid content in lung lavage increases on day 27 of gestation (Rooney et al., 1976; Rooney and Gobran, 1977). These changes signify lung maturation to a degree capable of supporting adequate gas-exchange for survival 24 hours later (Oulton and Dolphin, 1988).

The fraction isolated as the lamellar bodies also displayed a 3-fold increase in PKC activity during the period between day 24 and day 30 of

gestation before dropping dramatically on day 1 postnatal. This pattern suggests changes in PKC activity associated with initiation of surfactant synthesis.

The corresponding decrease observed in cytosolic specific activity over this period suggests PKC activation from the inactive pool during this critical period of development.

5.1e.ii Total Activity

Total activity in the homogenate and lamellar body fraction followed a similar pattern as specific activity. As noted above, this corresponds to the period during which the lung becomes functional. All other fractions displayed a biphasic response in which total activity decreased just prior to birth, and increased on postnatal day 1. A similar pattern has been observed for *in vivo* secretion rates of lamellar bodies by alveolar type II cells of rat lung (Faridy and Thliveris, 1987), as well as secretion of lavage lamellar body-like material into the fetal lung (Rooney and Gobran, 1977). Phospholipid content of the lamellar body fraction isolated from fetal rabbits also displays a similar biphasic pattern (Oulton et al., 1986; Scott et al. 1987).

Total activity was again largely concentrated in the cytosolic compartment. Since it is clear that PKC activation involves translocation of cytosolic enzyme (Nishizuka, 1988; Dekker and Parker, 1994), this compartment may possibly act as a reserve, translocating to the particulate fractions, including lamellar bodies, as required and according to appropriate stimulus.

5.1f Inhibition of PKC Activity

PKC inhibitors acridine orange, 9-aminoacridine and sangivamycin were used to characterize the activity in the lamellar body fraction. The microsomal fraction was chosen to contrast the effects of these compounds on PKC activity as it may provide a likely source of contamination during the lamellar body isolation procedure. PKC activity in the microsomal fraction was inhibited by all three compounds. Lamellar body activity was not affected by acridine orange or sangivamycin, and at low concentrations of 9-aminoacridine, PKC activity appeared to be increased.

The high concentration of phospholipid in the lamellar body fraction may play a role in reducing the effectiveness of the inhibitors. As previously noted, the lack of difference in enzyme activity with and without the added cofactors in the assay suggests PKC is already highly activated in lamellar body fraction. Acridine orange and 9-aminoacridine are competitive inhibitors with respect to lipid cofactors (Hannun and Bell, 1988). In addition acridine orange intercalates into lamellar body phospholipids (Fabisiak et al., 1987). Therefore the high lipid environment of this fraction may prevent the inhibitors from acting on the enzyme through surface dilution effects as they partition into the phospholipid micelles, thus decreasing their effective concentration in the lamellar body fraction (Hannun and Bell, 1988).

5.1g PKC Activity in Delipidated Fractions

As previously noted, enzyme assay of the particulate fractions may be subject to interference from the phospholipid component. This is particularly true for lamellar bodies where phospholipids account for greater than 90% of the weight. Delipidation by the method developed by Fiscus and Schneider (Fiscus and Schneider, 1966) removes virtually all of the phospholipid component (Chu and Rooney, 1985). Delipidation of the fractions decreased PKC activity in all fractions and could not be fully restored upon reconstitution of the fraction. However the lamellar body fraction retained a greater specific activity than any of the other intact fractions. This would suggest that PKC is present in the lamellar body fraction of day 27 gestation.

5.1h PKC Activity and Lamellar Bodies

The coupling of PKC activation to regulation of the surfactant synthesis and secretion leads to the question of whether PKC is associated with the lamellar body fraction. However, as observed in Figure 9, exposure of cells to conditioned media failed to produced a significant increase in Ca^{2+}-PS-dependent PKC activity in the lamellar body fraction. This result may be due in part to the interference of lipid component of the fraction. The microsomal Ca^{2+}-PS-dependent PKC activity however may serve to indicate that TPA produced an increased activity level of the enzyme in these cells. It was noted however that exposure of cells to fibroblast-conditioned medium (FCM) induced a significantly higher level of PKC activity, as reflected by the microsomal

response in Figure 9. Exposure of cells to FCM has previously been shown to accelerate the appearance of lamellar bodies in fetal type II cells (Scott, 1992; Scott and Das, 1993). Since enzyme activity was assayed under conditions of constant protein and cell numbers were monitored, a conclusion that differentiation of type II pneumocytes is accompanied by greater PKC activity is a logical progression.

To determine if any PKC activity could be ascribed to the lamellar body fraction, an addback assay was performed. The first step in this assay was determination of the amount of microsomal contamination that co-localized with lamellar body fraction. Previous studies have shown that the technique employed for lamellar body isolation results in 4.1% (or 1.025µl per 25µl of lamellar body fraction) (Oulton et. al., 1988) microsomal contamination based on the activity of the microsomal marker enzyme NADPH-cytochrome C reductase.

In cells exposed to serum-free medium or conditioned medium, the PKC activity was found to be due to contamination. However in cells exposed to TPA, a potent agonist of surfactant secretion, PKC activity was found to be present in this fraction. This suggests the association of PKC with the lamellar body fraction during the secretory response.

5.1i PKC and Synthesis of Surfactant-related Phospholipid

After concurrent incubation of cells with TPA and [^{3}H]-choline and [^{32}P]-inorganic phosphorus, cells were homogenised and fractions separated using

adult lung homogenate as a carrier. All particulate fractions except the mitochondrial fraction (Mito^2), which pelleted beneath the sucrose gradient, were found to contain significantly increased levels of the radiolabel. While the radiolabelled phosphorus may be incorporated into both protein and phospholipid, [^{3}H]-choline is probably associated entirely with the phospholipid fraction.

TPA-induced PKC activation had previously been shown to increase the rate of [^{3}H]-choline incorporation into DSPC by fetal alveolar type II cells (Scott, 1994). The material that co-migrated with the adult lamellar body fraction contained significantly greater amounts of both [^{3}H]-choline and [^{32}P]-inorganic phosphorus suggesting that PKC activation increases the amount of radiolabel incorporated into a fetal type II cell fraction having a similar density as adult lamellar bodies.

5.1j PKC and Release of Surfactant-related Phospholipid

Fetal type II cells, previously labelled by incubation with [^{3}H]-choline and [^{32}P]-inorganic phosphorus for 24 hours, were stimulated by exposure to TPA. The released material was isolated and subfractionated using adult lung homogenate as a carrier (see section 3.2j). Released material that co-migrated with the adult lamellar body fraction had significantly increased levels of both ^{3}H-choline and ^{32}P (Figure 12). Subfractionation of the cells remaining after TPA exposure suggested that the source of a significant quantity of the released material was the lamellar body compartment (Figure 14). A portion of the

released material was collected by centrifugation. Upon examination by electron microscopy, vesicles similar to those previously observed (Gross and Narine, 1989; Oulton et al., 1993; Scott et al., 1993) were detected. These structures suggest that fetal type II cells may be induced to release material similar to surfactant lamellar bodies by exposure to the PKC activator TPA.

PKC activation has previously been shown to increase secretion of DSPC in fetal type II cells (Scott, 1994).The nature of the material released however was not clear. Taken together, the results of the two preceding sections (5.1l and 5.1j) suggest that PKC activation is involved in both incorporation and release of material similar in structure and density to that of adult lamellar bodies. It is known that rabbit lung expresses several PKC isozymes (Wetsel et al., 1992). Although no direct conclusions may be reached concerning cell specific expression in the present study, it is reasonable to assume that the PKC enzyme family may have a multifunctional role in lung considering the large number of isoforms identified.

5.2 CONCLUSIONS

The dramatic increase in PKC activity observed in fetal alveolar type II cells during the latter stages of development suggests an important role for this enzyme in regulation of the surfactant cycle. The results obtained support the hypothesis that PKC activation is an important regulatory step in the secretion of surfactant-related material. This enzyme also appears to be involved in regulation of synthesis of surfactant-related material at least in fetal lung type II

alveolar cells. The high levels of PKC activity in lung, and particularly in lamellar bodies, suggests abundance of the enzyme in the lung tissue. Whole lung expresses a number of isozymes (Wetsel et al., 1992; Hug and Sarre, 1993), some of which are Ca^{2+}-PS-dependent while others are Ca^{2+}-independent. The presence of multiple isozymes of PKC in lung may partially explain the ability of PKC to regulate different functions within the lung. While the present studies utilised predominantly whole lung, the presence of both Ca^{2+}dependent and Ca^{2+}-independent activity suggest multiple enzyme functions. Furthermore these results, combined with the observations of radiolabel distributions in isolated fetal rabbit type II alveolar cells subcellular fractions also underscores the need for identification of the isozymes and clarification of their functions in developing and adult rabbit lung.

5.3 FUTURE DIRECTIONS

Recent studies indicate PKC to be a large family of isozymes. This family may be divided into two groups, Ca^{2+}-dependent PKC (cPKC) and Ca^{2+}-independent PKC (nPKC) with each group containing at least four isozymes (Azzi et al., 1992; Hug and Sarre, 1993) The cellular distribution of each isozyme differs both in terms of location and abundance. These differences appear to allow the enzyme to control separate processes within the same cell. Identification of the isozymes present in type II cells as well as their functions is possible with the use of antibodies. Subsequent studies will examine the

distribution, activity and functional control of PKC isozymes related to surfactant synthesis, secretion and reuptake in isolated fetal and adult type II cells.

REFERENCES

1. Adamson, I.Y.R., and D.H. Bowden. The type II cell as a progenitor of alveolar epithelial regeneration: a cytodynamic study in mice after exposure to oxygen. *Lab. Invest.* 30:35-42. 1974.
2. Aeberhard, E.E., C.T. Barret, S.A. Kaplan, and M.L. Scott. Stimulation of phosphatidylcholine synthesis by fatty acids in fetal rabbit type II pneumocytes. *Biochim. Biophys. Acta* 875:6-11. 1986.
3. Akino, T. Lipid components of the surfactant system. In *Pulmonary surfactant: from molecular biology to clinical practice*. Robertson, B., L.M.G. Van Golde, and Batenburg, Eds. pp 20-31. Amsterdam. Elsevier. 1992.
4. Avery, M.E., and J. Mead. Surface properties in relation to atelectasis and hyaline membrane disease. *Am. J. Dis. Child.* 97:517-523. 1959.
5. Azzi, A., D. Boscoboinik, and C. Hensey. The protein kinase C family. *Eur. J. Biochem.* 208:547-557. 1992.
6. Bachofen, H., P. Gehr, and E.R. Weibel. Alterations of mechanical properties and morphology in excised rabbit lungs rinsed with a detergent. *J. Appl. Physiol.* 1979.
7. Ballard, P.L., S. Hawgood, H. Liley, G. Wellenstein, L.W. Gonzales, B. Benson, B. Cordell and T. White. Regulation of pulmonary surfactant apoprotein SP 28-36 gene in fetal human lung. *Proc. Natl. Acad. Sci. USA* 83:9527-9531. 1986.
8. Bartlett, G.R. Phosphorus assay in column chromatography. *J. Biol. Chem.* 234:466-488. 1959.
9. Batenburg, J.J., W.J. Longmore, and L.M.G. Van Golde. The synthesis of phosphatidylcholine by adult rat lung alveolar type II epithelial cells in primary culture. *Biochim. Biophys. Acta* 529:160-170. 1978.
10. Batenburg, J.J., W.J. Longmore, W. Klazinga and L.M.G. van Golde. Lysolecithin acyltransferase and lysolecithin; lysolecithin acyltransferase in adult rat lung alveolar type II epithelial cells. *Biochim. Biophys. Acta* 573:136-144. 1979.
11. Batenburg, J.J. Biosynthesis of surfactant lipids. In *Pulmonary surfactant: from molecular biology to clinical practice*. Robertson. B., L.M.G. van Golde, and J.J. Batenburg, Eds. pp 255-281. Amsterdam. Elsevier. 1992.
12. Batenburg, J.J., and R.H. Elfring. Pre-translational regulation by glucocortcoid of fatty acid synthesis in type II cells from fetal rat lung. *FEBS-Lett.* 307:164-168. 1992.

13. Bazzi, M.D., and G.L. Nelsestuen. Differences in the effects of phorbol esters and diacylglycerol on protein kinase C. *Biochem.* 28:9317-9323. 1989.

14. Beer, D.G., M.S. Butler, G.R. Cunha, and A.M. Malkinson. Autoradiographic localization of specific 3H-dexamethasone binding in fetal lung. *Devel. Biol.* 105:351-364. 1984.

15. Bell, R.M. Protein kinase C activation by diacylglycerol second messengers. *Cell* 45:631-632. 1986.

16. Bent, A.E., J.H. Gray, E.R. Luther, M. Oulton, and L.J. Peddle. Fetal lung maturity: Relationship of gestational age and pregnancy complications to phosphatidylglycerol levels. *Am. J. Obstet. Gyne.* 142:664-669. 1982.

17. Berridge, M.J., and R.F. Irvine. Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature* 312:315-321. 1984.

18. Bligh, E.G., and W.J. Dyer. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911-917. 1959.

19. Boggaram, V., K. Qing, and C.R. Mendelson. the major apoprotein of rabbit pulmonary surfactant. Elucidation of primary sequence and cyclic AMP and developmental regulation. *J. Biol. Chem.* 263:2939-2947. 1988.

20. Boggaram, V., M.E. Smith, and C.R. Mendelson. Regulation of expression of the gene coding the major surfactant protein (SP-A) in human fetal lung in vitro. *J. Biol. Chem.* 264:11421-11427. 1989.

21. Borner, C., S.N. Guadagno, D. Fabbro, and I.B. Weinstein. Expression of four protein kinase C isoforms in rat fibroblasts. *J. Biol. Chem.* 267:12892-12899. 1992.

22. Bourbon, J.R. Nature, function and biosynthesis of surfactant lipids. In *Pulmonary surfactant: Biochemical, functional, regulatory, and clinical concepts.* Bourbon. J.R., Ed. pp 37-76. Boca Raton: CRC Press. 1991.

23. Bradford, M.M. A Rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 79:544-552. 1976.

24. Brown, L.A., and W.J. Longmore. Adrenergic and cholinergic regulation of lung surfactant secretion in the isolated perfused lung and in the alveolar type II cell in culture. *J. Biol. Chem.* 256:66-72. 1981.

25. Buckingham S. Studies on the identification of an antiatelectasis factor in normal sheep lung. *Am. J. Dis. Child.* 102:521-522. 1961.

26. Buckingham, S., and M.E. Avery. Time of appearance of lung surfactant in the foetal mouse. *Nature Lond.* 193:688:689. 1962.

27. Buckingham, S., H.O. Heinnemann, S.C. Sommers, and W.F. Macnary. Phospholipid synthesis in the large pulmonary alveolar cell: its relation to lung surfactants. *Am. J. Pathol.* 48:1027-1042. 1966.

28. Caniggia, I., I. Tseu, R.N.N. Han, B.T. Smith, A.K. Tanswell, and M. Post. Spatial and temporal differences in fibroblast behavior in fetal rat lung. *Am. J. Physiol.* 261:L424-L433. 1991.

29. Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847-7851. 1982.

30. Chander, A., W.D. Claypool, J.F. Strauss, A.B. Fisher. Uptake of liposomal phosphatidylcholine by granular pneumocytes in primary culture. *Am.J. Physiol.* 245:C397-C404. 1983.

31. Chander, A., and A.B. Fisher. Choline-phosphate cytidylyltransferase activity and phosphatidylcholine synthesis in rat granular pneumocytes are increased with exogenous fatty acids. *Biochim. Biophys. Acta* 958:343-351. 1988.

32. Chander, A., and A.B. Fisher. Regulation of lung surfactant secretion. *Am. J. Physiol.* 258:L241-L253. 1990.

33. Chevalier, G., and A.J. Collet. In vivo incorporation of 3H-choline, 3H-leucine and 3H-galactose in alveolar type II pneumocytes in relation to surfactant synthesis. A quantitative radiographic study in mouse by electron microscopy. *Anat. Rec.* 174:289-310. 1972.

34. Chu, A.J., and S.A. Rooney. Developmental differences in activation of cholinephosphate cytidylyltransferase by lipids in rabbit lung cytosol. *Biochim. Biophys. Acta* 835:132-140. 1985.

35. Clements, J.A., E.S. Brown, and P.R. Johnson. Pulmonary surface tension and the mucous lining of the lungs: some theoretical considerations. *J. Appl. Physiol.* 12:262-268. 1958.

36. Clements J.A., R.F. Hustead, R.P. Johnson, and I. Gribetz. Pulmonary surface tension and alveolar stability. *J. Appl. Physiol.* 16:444-450. 1961.

37. Clements, J.A. Functions of the alveolar lining. *Am. Rev. Respir. Dis.* 11:67-72. 1977.

38. Coalson, J.J., V.T. Winter, H.M. Martin, and R.J. King. Colloidal gold immunoultrastructural localization of rat surfactant. *Am. Rev. Respir. Dis.* 133:230-237. 1987.

39. Cockshutt, A.M., and F. Possmayer. Metabolism of surfactant lipids and proteins in the developing lung. In *Pulmonary surfactant: from molecular biology to clinical practice.* Robertson, B., L.M.G. Van Golde, and J.J. Batenburg, Eds. pp 339-378. Amsterdam. Elsevier. 1992.

40. Coonrod, J.D., R.L. Lester, and L.C. Hsu. Characterization of the extracellular bactericidal factors of rat alveolar lining material. *J. Clin. Invest.* 74:1269-1275. 1984.

41. Crapo, J.D., B.E. Barry, P. Gehr, M. Bachofen and E.R. Weibel. Cell number and cell characteristics of the normal lung. *Am. Rev. Respir. Dis.* 126:332-337. 1982.

42. Crecelius, C.A., and W.J. Longmore. Phosphatidic acid phosphatase activity in subcellular fractions derived from adult rat type II pneumocytes in primary culture. *Biochim. Biophys. Acta* 750:447:456. 1983.

43. Crecelius, C.A., and W.J. Longmore. Acyltransferase activities in adult rat type II pneumocyte derived subcellular fractions. *Biochim. Biophys. Acta* 795:238-246. 1984.

44. Dekker, L.V., and P.J. Parker. Protein kinase C- a question of specificity. *TIBS* 19:73-77. 1994.

45. Delahunty, T.J., and J.M. Johnston. The effect of colchicine and vinblastine on the release of pulmonary surface active material. *J. Lipid. Res.* 17:112-116. 1976.

46. Den Breejen, J.N., J.J. Batenburg, and L.M.G. van Golde. The species of acyl-CoA in subcellular fractions of type II pneumocytes isolated from adult rat lung and their incorporation into phosphatidic acid. *Biochim. Biophys. Acta* 1002:277-282. 1989.

47. Desai, R., T.D. Tetley, C.G. Curtis, G.M. Powell, and R.J. Richards. Studies on the fate of pulmonary surfactant in the lung. *Biochem. J.* 176:455-462. 1978.

48. Dobbs, L.G., and R.J. Mason. Stimulation of secretion of disaturated phosphatidylcholine from isolated alveolar type II cells by 12-O-tetradecanoyl-13-phorbol acetate. *Am. Rev. Respir. Dis.* 118:705-712. 1978.

49. Dobbs, L.G., and R.J. Mason. Pulmonary alveolar type II cells isolated from rats. Release of phosphatidylcholine in response to beta-adrenergic stimulation. *J. Clin. Invest.* 63:378-387. 1979.

50. Dobbs, L.G., J.R. Wright, S. Hawgood, R. Gonzalez, K. Venstrom, J. Nellenbogen. Pulmonary surfactant and its components inhibit secretion of phosphatidylcholine from cultured rat alveolar type II cells. *Proc. Natl. Acad. Sci. USA* 84:1010-1014. 1987.

51. Fabisiak, J.P., E.S. Vesell, and D.E. Rannel. Interactions of b-adrenergic antagonists with isolated rat alveolar type II pneumocytes. I Analysis, characterization and regulation of specific b -adrenergic receptors. *Pharmac. Exp. Ther.* 241:722-727. 1987.

52. Faridy E.E. and J.A. Thliveris. Rate of secretion of lung surfactant before and after birth. *Repir. Physiol.* 68:269-277. 1987.

53. Feldman D.A., C.R. Kovac, P.L. Dranginis and P.A. Weinhold. The role of phosphatidylglycerol in the activation of CTP:phosphocholine cytidylyltransferase from rat lung. *J. Biol. Chem.* 253:4980-4986. 1978.

54. Fiscus W.G. and W.C. Schneider. The role of phospholipids in stimulating phosphorylcholine cytidylyltransferase activity. *J. Biol. Chem.* 241:3324-3330. 1966.

55. Friedenauer, S., and H.H. Berlet. Sensitivity and variability of the Bradford protein assay in the presence of detergents. *Anal. Biochem.* 178:263-268. 1989.

56. George, T.P., S.C. Morash, H.W. Cook, D.M. Byers, F.B.S.C. Palmer, and M.W. Spence. Phosphatidylcholine biosynthesis in cultured glioma cells - evidence for channeling of intermediates. *Biochim. Biophys. Acta.* 1004:283-291. 1989.

57. Geiger, K., M.L. Gallagher, and J. Hedley-White. Cellular distribution and clearance of aerosolized dipalmitoyl lecithin. *J. Appl. Physiol.* 39:759-766. 1976.

58. Gilfillan, A.M., D.A. Smart, and S.A. Rooney. Phosphatidylglycerol stimulates cholinephosphate cytidylyltranferase activity and phosphocholine synthesis in type II pneumocytes. *Biochim. Biophys. Acta* 835:141-146. 1985.

59. Gilfillan, A.M., and S.A. Rooney. Functional evidence for adenosine A_2 receptor regulation of phosphatidylcholine in cultured type II pneumocytes. *J. Pharmacol. Exp. Ther.* 241:907-914. 1987.

60. Gilfillan, A.M., and S.A. Rooney. Functional evidence for involvement of P_2 purinoceptors in the ATP stimulation of phosphatidylcholine secretion in type II alveolar cells. *Biochim. Biophys. Acta* 959:31-37. 1988.

61. Goldenberg. V.E., S. Buckingham, and S.C. Sommers. Pilocarpine stimulation of granular pneumocyte secretion. *J. Histochem. Cytochem.* 16:102-109. 1969.

62. Griese, M., L.I. Gobran. and S.A. Rooney. ATP-stimulated inositol phospholipid metabolism and surfactant secretion in rat type II pneumocytes. *Am. J. Physiol.* 260:L586-L593. 1991.

63. Griese, M., L.I. Gobran, and S.A. Rooney. Signal-transduction mechanisms of ATP-stimulated phosphatidylcholine secretion in rat type II pneumocytes: interactions between ATP and the other surfactant secretagogues. *Biochim. Biophys. Acta* 1167:85-93. 1993.

64. Gross, N.J., and K.R. Narine. Surfactant subtypes in mice - characterization and quantitation. *J. Appl. Physiol.* 66:342-349. 1989.

65. Haagsman, H.P. and L.M.G. van Golde. Synthesis and assembly of lung surfactant. *Annu. Rev. Physiol.* 53:441-464. 1991.

66. Hallman, M., and L. Gluck. PG in lung surfactant. III Possible modifier of surfactant function. *J. Lipid Res.* 17:257-261. 1976.

67. Hallman, M., B.H. Feldman, E. Kirkpatrick, and L. Gluck. Absence of PG in respiratory distress syndrome in the newborn. Study of the minor surfactant phospholipids in newborns. *Pediatr. Res.* 11:714-728. 1977.

68. Hallman, M., B.L. Epstein, and L. Gluck. Analysis of labeling and clearance of lung surfactant phospholipids in rabbit. Evidence of a bidirectional flux between lamellar bodies and alveolar lavage. *J. Clin. Invest.* 68:742-751. 1981.

69. Hannun, Y.A., C.R. Loomis, and R.M. Bell. Activation of PKC by triton X-100 mixed micelles containing diacylglycerol and phosphatidylcholine. *J. Biol. Chem.* 260:10039-10043. 1985.

70. Hannun, Y.A., and R.M. Bell. Aminoacridines, potent inhibitors of protein kinase C. *J. Biol. Chem.* 263:5124-5131. 1988.

71. Hass, M.A., and W.J. Longmore. Regulation of lung surfactant cholesterol metabolism by serum lipoproteins. *Lipids* 15:401-406. 1980.

72. Hawco, M.W., P.J. Davis, and K.M.W. Keough. Lipid fluidity in lung surfactant: monolayers of saturated and unsaturated lecithins. *J. Appl. Physiol.* 51:509-521. 1981.

73. Hills, B.A. Monolayers. in The biology of surfactant. B.A. Hills. pp 145-183. Cambridge. Cambridge University Press. 1988.

74. Hitchcock, K.R. Hormones and the lung. I. Thyroid hormones and glucocorticoids in lung development. *Anat. Rec.* 194:15-40. 1979.

75. Huang, K.P. The mechanism of protein kinase C activation. *Trends in Neur. Sci.* 112:425-431. 1989.

76. Hug, H., and T.F. Sarre. Protein kinase C isoenzymes: divergence in signal transduction. *Biochem. J.* 291:329-343. 1993.

77. Hung, K-S. Innervation of rabbit fetal lungs. *Am. J. Anat.* 159:73-83. 1980.

78. Hunt, A.N., I.C.S. Normand, A.D. Postle. CTP: cholinephosphate cytidylyltransferase in human and rat lung - association in vitro with cytoskeletal actin. *Biochim. Biophys. Acta.* 1043:19-26. 1990.

79. Inoue, M., A. Kishimoto, Y.Takai, and Y. Nishizuka. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. II. Proenzyme and its activation by calcium-dependent protease from rat brain. *J. Biol. Chem.* 252:7610- 7616. 1977.

80. Jacobs, H., A. Jobe, M. Ikegami and D. Conaway. The significance of reutilization of surfactant phosphatidylcholine. *J. Biol. Chem.* 258:4156-4265. 1983.

81. Jacobs, H., M. Ikegami, A.H. Jobe, D.D. Berry, and S. Jones. Reutilization of surfactant phosphatidylcholine in adult rabbits. *Biochim. Biophys. Acta.* 837:77-84. 1985.

82. Kauffman, S.L., P.H. Burri, and E.R. Weibel. The postnatal growth of the rat lung. II. Autoradiography. *Anat. Rec.* 180:63-67. 1974.

83. Kikkawa, U., Y. Takai, Y. Tanaka, R. Miyake, and Y. Nishizuka. Protein kinase C as a possible receptor protein of tumor-promoting phorbol esters. *J. Biol. Chem.* 258:11442-11445. 1983.

84. Kikkawa, U., and Y. Nishizuka. The role of protein kinase C in transmembrane signalling. *Ann. Rev. Cell. Biol.* 2:149-178. 1986.

85. Kikkawa, Y., E.K. Motoyama, and L. Gluck. Study of lungs of fetal and newborn rabbits. *Am. J. Pathol.* 52:177-209. 1968.

86. Kikkawa, Y., M. Kaibara, E.K. Motoyama, M.M. Orzalesi, and C.D. Cook. Morphologic development of fetal rabbit lung and it's acceleration with cortisol. *Am. J. Path.* 64:423-442. 1971.

87. King, R.J., D.J. Klass, E.G. Gikas, and J.A. Clements. Isolation of apoproteins from canine surface active material. *Am. J. Physiol.* 224:788-795. 1973.

88. Kirazov, L.P., L.G. Venkov, and E.P. Kirazov. Comparison of the Lowry and the Bradford protein assays as applied for protein estimation of membrane fraction. *Anal. Biochem.* 208:44-48. 1993.

89. Klaus, M.H., J.A. Clements, and R.H. Havel. Composition of surface active material isolated from beef lung. *Proc. Natl. Acad. Sci. USA* 47:1858-1859. 1961.

90. Kraft, A.S., and W.B. Anderson. Phorbol esters increase the amount of Ca, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* 301:621-623. 1983.

91. Kresch, M.J., L.A. Cipriani, H. Lu, and C. Christian. Development al regulation of re-uptake of phosphatidylcholine by type II alveolar epithelium. *Biochim. Biophys. Acta* 1210:167-173. 1994.

92. Leach, K.L. and P.M. Blumberg. Tumour promoters, their receptors and their actions. In *Inositol lipids in cell signaling*. Michell, R.H., A.H. Drummond and C.P. Downes, Eds. pp 179-206. San Diego. Academic Press. 1989.

93. Liggins, G.C. Premature delivery of foetal lambs infused with glucocorticoids. *J. Endocrinol.* 45:515-523. 1969.

94. Liggins, G.C., J. Shellenberg, M. Manzai, J.A. Kitterman, and C.H. Lee. Synergism of cortisol and thyrotropin-releasing hormone on lung maturation in fetal sheep. *J. Appl. Physiol.* 65:1880-1884. 1988.

95. Macklin, C.C. The Pulmonary alveolar mucoid film & pneumocytes. *Lancet* 1:1099-1104. 1954.

96. Magoon, M.W., J.R. Wright, A. Baritussio, M.C. Williams, J. Goerke. Subfractionation of lung surfactant. Implications for metabolism and surface activity. *Biochim. Biophys. Acta* 750:18-31. 1983.

97. Marino, P.A., and S.A. Rooney. Surfactant secretion in newborn rabbit lung slice model. *Biochim. Biophys. Acta* 620:509-518. 1980.

98. Mason, R.J., J. Nellenbogen, J.A. Clements. Isolation of disaturated phosphatidylcholine with osmium tetraoxide. *J. Lipid Res.* 17:281-284. 1976.

99. Mason, R.J. Importance of the acyl dihydroxyacetone phosphate pathway in the synthesis of phosphatidylglycerol and phosphatidylcholine in alveolar type II cells. *J. Biol. Chem.* 253:3367-3370. 1978.

100. Mason, R.J., M.C. Williams, J.H. Widdicombe, M.J. Sanders, D.S. Misfeldt, and L.C. Berry. Transepithelial transport by pulmonary alveolar type II cells in primary culture. *Proc. Natl. Acad. Sci. U.S.A.* 79:6033. 1982.

101. Mason, R.J. Surfactant secretion. In *Pulmonary surfactant: from molecular biology to clinical practice.* Robertson, B., L.M.G. Van Golde, and J.J. Batenburg, Eds. pp 295-312. Amsterdam. Elsevier. 1992.

102. McMurray, W.C. Lecithin biosynthesis in liver mitochondrial fractions. *Biochem. Biophys. Res. Commun.* 58:467-474. 1974.

103. Mead J. Mechanical Properties of lungs. *Physiol. Rev.* 41:281-330. 1961.

104. Mendelson, C.R., and V. Boggaram. Hormonal control of the surfactant system in fetal lung. *Annu. Rev. Physiol.* 53:415-430. 1991.

105. Mescher, E.J., L.G. Dobbs, and R.J. Mason. Cholera toxin stimulates secretion of saturated phosphatidylcholine and increases cellular cyclic AMP in isolated rat alveolar type II cells. *Exp. Lung Res.* 5:173-182. 1983.

106. Nicholas, T.E., J.H.T. Power, and H.A. Barr. The pulmonary consequences of a deep breath. *Respir. Physiol.* 49:315-324. 1982.

107. Nishizuka, Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature* 334:661-665. 1988.

108. O'Neill, S., E. Lesperance, and D.J. Klass., Rat lung lavage surfactant enhances bacterial phagocytosis and intracellular killing by alveolar macrophages. *Am. Rev. Respir. Dis.* 130:1117-1122. 1984.

109. Oulton, M., M.Fraser, M. Dolphin, R. Yoon, and G. Faulkner. Quantification of surfactant pool sizes in rabbit lung during perinatal development. *J. Lipid Res.* 27:602-612. 1986.

110. Oulton, M., and M. Dolphin. Subcellular distribution of disaturated phosphatidylcholine in developing rabbit lung. *Lipids* 23:55-61. 1988.

111. Oulton, M., J.M. McDonald, D.T. Janigan, and G.T. Faulkner. Mouse alveolar surfactant characterizations of subtypes prepared by differential centrifugation. *Lipids* 28:715-720. 1993.

112. Oyarzun, M.J., and J.A. Clements. Control of lung surfactant by ventilation, adrenergic mediators, and prostaglandins in the rabbit. *Am. Rev. Respir. Dis.* 117:879-891. 1978.

113. Pattle, R.E. Properties, function and origin of the alveolar lining layer. *Nature Lond.* 175:1125-1126. 1955.

114. Pattle R.E., and L.C. Thomas. Lipoprotein composition of the film lining the lung. *Nature Lond.* 189:844, 1961.

115. Pian, M., L.G. Dobbs, and N. Duzgunes. Positive correlation between cytosolic free calcium and surfactant secretion in cultured rat alveolar cells. *Biochim. Biophys. Acta* 960:43-53. 1988.

116. Possmayer, F., F.G. Casola, F. Chen, P. MacDonald, M.A. Ormseth. T. Wong. P.G.R. Harding, and S. Takmakjian. Hormonal induction of pulmonary maturation in the rabbit fetus. Effects of maternal treatment with estradiol-17b on the endogenous levels of cholinephosphate, CDP-choline and phosphatidylcholine. *Biochim. Biophys. Acta* 664:10-21. 1981.

117. Post, M., J.J. Batenburg, E.A.J.M. Schuurmans, and L.M.G. van Golde. The rate limiting step in the biosynthesis of phosphatidylcholine by alveolar type II cells from adult rat lung. *Biochim. Biophys. Acta* 712:390-394. 1982.

118. Post, M., E.A.J.M. Schuurmans, J.J. Batenburg, and L.M.G. van Golde. Mechanisms involved in the synthesis of disaturated phosphatidylcholine by alveolar type II cells isolated from rat lung. *Biochim. Biophys. Acta* 750:68-77. 1983.

119. Post, M., and B.T. Smith. Effect of fibroblast-pneumocyte-factor on the synthesis of surfactant phospholipids in type II cells from fetal rat lung. *Biochim. Biophys. Acta* 793:297-299. 1984.

120. Post, M., J.S. Torday, and B.T. Smith. Alveolar type II cells isolated from fetal rat lung organotypic cultures synthesize and secrete surfactant-associated phospholipids and respond to fibroblast-pneumocyte factor. *Exp. Lung Res.* 7:53-65. 1984a.

121. Post, M., J.J. Batenburg, B.T. Smith, and L.M.G. van Golde. Pool sizes of precursors for phosphatidylcholine formation in adult rat lung type II cells. *Biochim. Biophys. Acta* 795:552-557. 1984b.

122. Post, M., J. Flores, and B.T. Smith. Inhibition of lung maturation by monoclonal antibodies against fibroblast-pneumocyte factor. *Nature* 308:284-286. 1984c.

123. Radford E.P. Method for estimating respiratory surface area of mammalian lungs from their physical properties. *Proc. Soc. Exp. Biol. Med.* 87:58-61. 1954.

124. Radford, E.P. Influence of physiochemical properties of the pulmonary surface on stability of alveolar spaces and on static hysteresis of lungs. *Proc. Int. Congr. Physiol. Sci.* 12th Leiden 1962. p. 275-280.

125. Radika, K. and F. Possmayer. Inhibition of foetal pulmonary choline-phosphate cytidylyltransferase under conditions favouring protein phosphorylation. *Biochem. J.* 232:833-840. 1985.

126. Rice, W.R., G.F. Ross, F.M. Singleton, S. Dingle, and J.A. Whitsett. Surfactant-associated protein inhibits phospholipid secretion from type II cells. *J. Appl. Physiol.* 63:692-698. 1987.

127. Rice, W.R., C.C. Dorn, and F.M. Singleton. P_2-purinoceptor regulation of surfactant phosphatidyl secretion. *Biochem. J.* 266:407-413. 1990.

128. Rooney, S.A., T.S. Wai-Lee, L. Gobran, and E.K. Motoyama. Phospholipid content, composition and biosynthesis during fetal lung development in the rabbit. *Biochim. Biophys. Acta* 431:447-458. 1976.

129. Rooney, S.A., and L.I. Gobran. Alveolar lavage and lavaged tissue phosphatidylcholine composition during fetal rabbit development. *Lipids* 12:1050-1054. 1977.

130. Rooney, S.A., I. Gross, and J.B. Warshaw. Thyrotropin releasing hormone stimulates surfactant secretion in the fetal rabbit. *Am. Rev. Respir. Dis.* 117:386-390. 1978.

131. Rooney, S.A., L.I. Gobran, P.A. Marino, W.M. Maniscalco, and I. Gross. Effects of betamethasone on phospholipid content, composition, and biosynthesis in the fetal rabbit lung. *Biochim. Biophys. Acta* 572:64-76. 1979.

132. Rooney, S.A. The surfactant system and lung phospholipid biochemistry. *Am. Rev. Respir. Dis.* 131:439-460. 1985.

133. Sano, K., D.R. Voelker, and R.J. Mason. Involvement of protein kinase C in pulmonary surfactant secretion from alveolar type II cells. *J. Biol. Chem.* 260:12725-12729. 1985.

134. Scott, J.E., F. Possmayer, and P.G.R. Harding. Alveolar pre-type II cells from fetal rabbit lung, isolation and characterization. *Biochem. Biophys. Acta* 753:195-204. 1983.

135. Scott, J.E., F. Possmayer, M.A. Quirie, A.K. Tanswell, and P.G.R. Harding. Alveolar pre-type II cells from the fetal rabbit lung: Characterization of the

production of disaturated phospholipid during phospholipid during cellular differentiation. *Biochem. Biophys. Acta* 879:292-300. 1986.

136. Scott, J.E., M. Oulton, M.R. Boylan, M. Dolphin, M.O. Fraser, and S. Temple. Profile of phospholipase A2 activity in subcellular fractions and lamellar bodies of developing neonatal and adult rabbit lung. Correlation with intracellular levels of disaturated phosphatidylcholine. *Biochem. Biophys. Acta* 921:75-81. 1987.

137. Scott, J.E. Phosphatidylcholine synthesis, secretion and reutilization during differentiation of the surfactant-producing type II alveolar cell from fetal rabbit lungs. *Exp. Lung Res.* 18:563-580. 1992.

138. Scott, J.E., S.-Y. Yang, E. Stanik, and J.E. Anderson. Influence of strain on [3H]thymidine incorporation, surfactant-related phospholipid synthesis, and cAMP levels in fetal type II alveolar cells. *Am. J. Respir. Cell Mol. Biol.* 8:258-265. 1993.

139. Scott, J.E. Influence of protein kinase C activation by 4-phobol ester or 1-oleoyl-2-acetylglycerol on disaturated phosphatidylcholine synthesis and secretion, and protein phosphorylation in differentiating rabbit type II alveolar cells. *Biochim. Biophys. Acta* 1221:297-306. 1994.

140. Scott. J.E., and R.M. Das. Perinatal lung development following maternal exposure to methylmercuric chloride. *Pediatr. Pulmon.* 17:11-21. 1994.

141. Sharma, A., L.W. Gonzales, and P.L. Ballard. Hormonal regulation of cholinephosphate cytidylyltransferase in human fetal lung. *Biochim. Biophys Acta* 1170:237-244. 1993.

142. Smith, B.T. Lung maturation in the fetal rat: acceleration by injection of fibroblast-pneumocyte factor. *Science* 204:1094-1095. 1979.

143. Smith, B.T. and K. Sabry. Glucocorticoid-thyroid synergism in lung maturation: a mechanism involving epithelial-mesenchymal interaction. *Proc. Natl. Acad. Sci. USA* 80:1951-1954. 1983.

144. Smith, D.M. and K.R. Hitchcock. Thyroid hormone binding to adult rat alveolar type II cells: an autoradiographic study. *Exp. Lung Res.* 5:141-153. 1983.

145. Snyder, J.M. and S.A. Magliato. An ultrastructural, morphometric analysis of rabbit fetal lung type II cell differentiation in vivo. *Anat. Rec.* 229:73-85. 1991.

146. Sorokin, S.P. the cells of the lung. in *Morphology of experimental carcinogenesis.* Hanna, M.G., and J.W. Deatherage, Eds. p 3-42. Netteshiem. Oak Ridge, Tenn. 1970.

147. Sueishi, K., K. Tanaka, and T. Oda. Immunostructural study of surfactant system. Distribution of specific proteins of surface active material in rabbit lung. *Lab. Invest.* 37:136-142. 1977.

148. Suwabe, A., R.J. Mason, D. Smith, J.A. Firestone, M.D. Browning, and D.R. Voelker. Pulmonary surfactant secretion is regulated by the physical state of extracellular phosphatidylcholine. *J. Biol. Chem.* 267:19884-19890. 1992.

149. Taeusch, H.W., M. Heitner and M.E. Avery. Accelerated lung maturation and increased survival in premature rabbits treated with hydrocortisone. *Am. Rev. Respir. Dis.* 105:971-973. 1972.

150. Takai, Y., A. Kishimoto, M. Inoue, and Y. Nishizuka. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. I. Purification and characterization of an active enzyme from bovine brain. J. Biol. Chem. 252:7603-7609. 1977.

151. Tio, D., A. Tayag, M. Rehn, and D. Warburton. Role of inositol trisphosphate isomer formation in type II pneumocyte signal transduction. *Am. J. Respir. Cell Mol. Biol.* 4:514-518. 1991.

152. Touchstone, J.C., J.C. Chen and K.M. Beaver. Improved separation of phospholipids in thin layer chromatography. *Lipids* 15:61-62. 1980.

153. Tsilibary, E.C., and M.C. Williams. Actin and secretion of surfactant. *J. Histochem. Cytochem.* 31:1298-1304. 1983.

154. Ueda, S., K. Kawamura, N. Ishii, S. Matsumoto, K. Hayashi, M. Okayasu, M. Saito, I. Sakurai. Ultrastructural studies on surface lining of the lungs: Part IV. Resected human lungs. *J. Jap. Med. Soc. Biol. Interface.* 16:34-60. 1985.

155. Vaskovsky, V.E., and E.Y. Kostetsky. Modified spray for the detection of phospholipids on thin layer chromatograms. J. Lipid Res. 9:396-399. 1968.

156. Von Neergaard, K. New interpretations of basic concepts of respiratory mechanics. Correlation of pulmonary recoil force with surface tension in the alveoli. *Z. Gesamte Exp. Med.* 66:373-394.1929.

157. Walters, D.V. The role of pulmonary surfactant in transepithelial movement of liquid. In *Pulmonary surfactant: from molecular biology to clinical practice*. B. Robertson, L.M.G. Van Golde. and J.J. Batenburg, Eds. pp 193-213. Amsterdam. Elsevier. 1992.

158. Warburton, D., L. Parton, S. Buckley, and L. Cosico. Verapamil: a novel probe of surfactant secretion from rat type II pneumocytes. *J. Appl. Physiol.* 66:1304-1308. 1989a.

159. Warburton, D., S. Buckley, and L. Cosico. P1 and P2 purinergic receptor signal transduction in rat type II pneumocytes. *J. Appl. Physiol.* 66:901-905. 1989b.

160. Weinhold, P.A., M.E. Rounsifer, S.E. Williams, P.G. Brubaker, and D.A. Feldman. CTP:phosphorylcholine cytidylyltransferase in rat lung. The effect

of free fatty acids on the translocation of activity between microsomes and cytosol. *J. Biol. Chem.* 259:10315-10321. 1984.

161. Wetsel, W.C., W.A. Khan, I. Merchenthaler, H. Rivera, A.E. Halpern, H.M. Phung, A. Negro-Vilar and Y.A. Hannun. Tissue and cellular distribution of the extended family of protein kinase C isozymes. *J. Cell Biol.* 117:121-133. 1992.

162. Whitsett, J.A., and T.E. Weaver. Structure, function and regulation of pulmonary surfactant proteins. In *Pulmonary surfactant: Biochemical, functional, regulatory, and clinical concepts.* Bourbon. J.R., Ed. pp 77-105. Boca Raton: CRC Press. 1991.

163. Widdicombe, J.G., and R.J. Pack. the clara cell. *Eur. J. Respir. Dis.* 63:202-220. 1982.

164. Williams, M.C. Conversion of lamellar body membranes into tubular myelin in alveoli of fetal rat lungs. *J. Cell Biol.* 72:260-277. 1977.

165. Wirtz, H.R., and L.G. Dobbs. Calcium mobilization and exocytosis after one mechanical stretch. *Science* 250:1266-1269. 1990.

166. Wolf, M., P. Cuatrecasas, and N. Sahyoun. Interaction of protein kinase C with membranes is regulated by Ca^{2+}, phorbol esters, and ATP. *J. Biol. Chem.* 260:15718-15722. 1985.

167. Woodcock-Mitchell, J., A. Burckchart, J.J. Mitchell, D.E. Rannels, J.E. Chin, and R.B. Low. Keratin species in type II pneumocytes in culture and during lung injury. *Am. Rev. Respir. Dis.* 134:566-571. 1986.

168. Wright, J.R., and J.A. Clements. Metabolism and turnover of lung surfactant. *Am. Rev. Respir. Dis.* 135:426-444. 1987.

169. Wright, J.R., and L.G. Dobbs. Regulation of pulmonary surfactant secretion and clearance. *Annu. Rev. Physiol.* 53:395-414. 1991.

170. Wu, B., Y. Kikkawa, M.M. Orzalesi, E.K. Motoyama, M. Kaibara, C.J. Zigas, and C.D. Cook. The effect of thyroxine on the maturation of fetal rabbit lungs. *Biol. Neonate* 22:161-166. 1973.

171. Xu, Z., D.A. Smart, and S.A. Rooney. glucocorticoid induction of fatty-acid synthase mediates the stimulatory effect of the hormone on choline-phosphate cytidylyltransferase activity in fetal rat lung. *Biochim. Biophys. Acta* 1044:70-76. 1990.

172. Young, S.L., S.A. Kremers, J.S. Apple, J.D. Crapo, and G.W. Brumley. Rat lung surfactant kinetics: biochemical and morphometric correlation. *J. Appl. Physiol.* 51:248-253. 1981.

173. Zimmermann L.J., W.S. Lee, B.T. Smith, and M. Post. Cyclic AMP-dependent protein kinase does not regulate CTP:phosphocholine cytidylyltransferase activity in maturing type II cells. *Biochim. Biophys. Acta* 1211:44-50. 1994.