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Tetrahydrocannabinol and Lung Surfactant Metabolism in Isolated Fetal Type II Alveolar Cells

BY

Tracy C. Cherlet

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

PC	Phosphatidylcholine
DSPC	Disaturated phosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
PI	Phosphatidylinositol
PG	Phosphatidylglycerol
CPCT	CTP:phosphocholine cytidylyltransferase
LMW	Low molecular weight (referring to CPCT)
HMW	High molecular weight (referring to CPCT)
PKC	Protein kinase C
THC	Δ^9-tetrahydrocannabinol
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
LDH	Lactate dehydrogenase
TPA	12-O-tetradecanoylphorbol-13-acetate
SEM	Standard error of the mean
PLC	Phospholipase C
MEM	Minimal essential medium
CDP-choline	Cytidine diphosphocholine
DAG	Diacylglycerol
PA	Phosphatidic acid
2-AG	2-arachidonylglycerol
Anandamide	N-arachidonylethanolamide
DHAP	Dihydroxyacetone phosphate
G-3-P	Glycerol-3-phosphate
1-AG-3-P	1-acylglycerol-3-phosphate
PLA_2	Phospholipase A_2
FPF	Fibroblast pneumocyte factor

ABSTRACT

Type II alveolar cells are the primary site of synthesis of disaturated phosphatidylcholine (DSPC), the major component of pulmonary surfactant. DSPC is thought to convey the surface tension lowering properties of surfactant in preventing alveolar collapse at end-expiration. Recent research indicates that lipid soluble agents may affect fetal lung development following maternal exposure. Since surfactant is primarily lipid in nature, potential exists that any one of these agents may alter surfactant metabolism. Δ^9-tetrahydrocannabinol (THC), a lipophilic cannabinoid found in marijuana, has been shown to affect many cellular processes including enzyme activities, alterations in the structure of cytoskeletal elements and macromolecular synthesis. Recently, a cannabinoid receptor has been identified in type II cells. The present study examines the effects of THC on surfactant phospholipid synthesis and secretion in isolated fetal type II cells. Freshly isolated and cultured fetal rabbit type II cells were incubated with THC and [^{3}H]choline over various times. Intracellular levels of radiolabeled DSPC were determined. [^{3}H]DSPC levels in both freshly isolated and cultured type II cells decreased upon cellular exposure to THC in a time and dose-dependent manner. Further studies examined the mechanism through which THC affects the synthesis of DSPC. Pulse-chase studies indicate that exposure to THC decreases intracellular phosphocholine levels but increases cytidine diphosphocholine (CDP-choline) and DSPC levels compared to control values. Evidence indicates that CTP:phosphocholine cytidylyltransferase (CPCT) is the rate-limiting step in

DSPC biosynthesis. Since CPCT catalyzes the formation of CDP-choline from phosphocholine, an increase in the activity of this enzyme would account for our observations. Results from enzyme assays suggest that CPCT activity increases by 30% in fetal lung cytosolic fractions upon exposure to THC. Thus, THC appears to increase surfactant phospholipid synthesis through activation of CPCT. Studies then focused on the effects of THC on release of surfactant DSPC from cultured and freshly isolated type II cells. Samples were pre-labeled with [^{3}H]choline, exposed to THC over various time periods and release of [^{3}H]DSPC determined. Results indicated that cellular exposure to THC increases surfactant secretion from cultured and freshly isolated fetal type II cells. These studies demonstrate that THC affects surfactant phospholipid metabolism, although further studies are required to elucidate the mechanism(s) through which this drug affects type II cell function.

INTRODUCTION

The Respiratory System - Overview

The respiratory system functions to filter inspired air and conduct it to the lungs for gas exchange. Once air has entered the respiratory system via the nose or mouth, it is channeled through the pharynx, trachea, bronchi and bronchioles to the alveoli. Alveoli are the terminal air spaces of the respiratory system and are the actual sites of gas exchange. At this level a thin membrane, the air-blood barrier, separates blood from air. This membrane consists of the attenuated alveolar epithelial cell, basement membrane and the vascular endothelial cell. In order for oxygen-carbon dioxide exchange to occur, gases must traverse this barrier.

The epithelium of the distal conducting airways and the alveoli of the lung consists of several cell types specialized for different functions such as gas exchange, lung expansion, pulmonary defense and clearance of particulate matter. Clara cells, macrophages, fibroblasts and alveolar type I and II cells are a few examples of some 40 different cell types identified within the lung. Of special interest to the present work are alveolar type II cells or simply, type II cells. Type II cells, located within the alveoli, are the primary sites of pulmonary surfactant synthesis. Surfactant, a phospholipid-rich material, functions to reduce surface tension at the air-liquid interface, thereby preventing alveolar collapse at end expiration. The following brief description outlines the general structure and function of cells present in the distal conducting airways and alveoli.

Subsequently, literature concerning pulmonary surfactant metabolism will be presented. Finally, background studies on marijuana in general and its relation to lung and lung surfactant will be reviewed to focus our studies on the potential affects of Δ^9-tetrahydrocannabinol (THC) on lung.

2.1 Structure of Distal Airways and Alveoli

2.1 A) Distal airways

2.1.a.i) Clara cells

Nonciliated Clara cells may be present within the distal conducting airways interspersed among ciliated cells. In humans, Clara cells may be recognized as low cuboidal cells with a round apical surface projection. These cells serve as progenitors of the epithelium of the small conducting airways, giving rise to other Clara cells or undergoing terminal differentiation into ciliated cells. Clara cells have also been implicated as primary sites of xenobiotic metabolism via a cytochrome pathway (Plopper, 1984). Studies by Ward et al. (1985) suggest also that these cells synthesize, store and secrete a group of apoproteins similar to those found in type II cells. Although the role of these proteins in fluid of the extracellular lining of bronchioles is unknown, evidence indicates that they may be involved in pulmonary surfactant biology (reviewed by Singh, 1997).

2.1.a.ii) Ciliated cells

Extending from the basement membrane to the epithelial surface of the conducting airways are ciliated columnar cells. These cells have a basally located nucleus and numerous cilia projecting from their apical surface into the airway lumen. Through a coordinated wave like motion, cilia propel mucus and its trapped particulate matter towards the pharynx. More recently, ciliated cells have been shown to release a carbohydrate-rich material that lines the apical surface of epithelial cells (Varsano, 1987).

2.1 B) Alveoli

Four major cell types are present at the alveolar level in the lung. Macrophages and fibroblasts embody only a small number of the total lung cell population. Alveolar type I and type II cells comprise 8% and 16%, respectively (Crapo, 1982).

2.1.b.i) Fibroblasts

The fibroblast is a spindle-shaped, elongated cell with an oval nucleus containing one or more nuclei. In the lung, fibroblasts are present within the interstitium of the alveoli and conducting passages. A primary role for interstitial fibroblasts is maintenance of the alveolar compartment through their anatomic location and by their production of collagens and other matrix compartments. Fibroblasts also play an important role during early lung development. The stimulation of fetal lung fibroblasts by glucocorticoids has been shown to induce

production of a low molecular weight polypeptide, fibroblast pneumocyte factor (FPF), that stimulates biochemical activity of type II cells (Smith, 1979). This protein acts on the type II cell, stimulating choline incorporation into disaturated phosphatidylcholine (DSPC), the major constituent of pulmonary surfactant (Post, 1986).

2.1.b.ii) Macrophages

Macrophages, found free in the alveolar space often in close apposition to the epithelium, are characterized by numerous lysosomes and filopodic processes. They are mobile cells, moving through the lung interstitium and along the alveolar epithelial surfaces. Alveolar macrophages have a wide range of functions but appear to play central roles in the pulmonary defense system as well as in maintenance of normal lung structure and surfactant processing (Wright, 1987; Crystal, 1991). Macrophages serve these important roles by phagocytizing particulate matter, killing inhaled microrganisms, functioning as an accessory cell in immune responses, recruiting and activating inflammatory cells, as well as by maintaining and repairing lung parenchyma (Hocking, 1979; Crystal, 1991).

2.1.b.iii) Alveolar Type I Cells

Alveolar type I cells, also known simply as type I cells, are extremely attenuated squamous cells that cover about 93% of the alveolar surface (Crapo, 1982). These cells are relatively simple, containing a small nucleus, a few small mitochondria, some cisternae of endoplasmic reticulum with ribosomes and a

Golgi apparatus. Type I cells are highly specialized to facilitate gas exchange. As the cells are extremely thin and adjacent type I cells are linked together by tight junctions, oxygen-carbon-dioxide exchange occurs freely across their membranes and cytoplasm. These cells are terminally differentiated cells. Their renewal following lung injury depends on the replication and differentiation of type II cells (Adamson, 1975).

2.1.b.iv) Alveolar Type II cells

Alveolar type II cells also line the walls of the alveoli. These cells are cuboidal in shape and are interspersed among type I cells. While there are greater numbers of type II than type I cells in the lung (16% versus 8% of total lung cell population), the former cells cover only about 7% of the alveolar air surface (Crapo, 1982). Adjacent type I and II cells are connected by tight junctions which serve to prevent transepithelial leakage of molecules larger than 1000kDa (Mason, 1991). Morphologically, these cells are characterized by the presence of apical microvilli and parallel membrane lamellae stacked upon one another. These lamellae, or lamellar bodies, are rich in phospholipids characteristic of those found in surfactant isolated from the alveolar space. Type II cells proliferate to repair injured alveolar epithelium and subsequently differentiate into either new type II cells or type I cells (Evans, 1975). Most importantly however, the type II cell has been recognized as the source of pulmonary surfactant. Surfactant is a phospholipid-rich material that is responsible for the reduction of surface tension in the alveolus (King, 1984). Thus, it allows for adequate lung expansion,

prevents pulmonary collapse and facilitates gas exchange required to maintain blood oxygen levels.

2.2 Development and Maturation of the Lungs

Development of human lungs begins near the end of the fourth week after fertilization. Maturation of the lungs may be divided into three periods, covering the fifth week to birth. The pseudoglandular period ranges from 5 to 17 weeks and is characterized by lungs that contain all major pulmonary elements (i.e. primitive trachea and bronchial tree), excluding those involved in gas exchange. The canalicular period, which occurs 16 to 25 weeks postconception, is characterized by the appearance of terminal conducting units including respiratory bronchioles and alveolar ducts. Generally, infants born prematurely during this stage of lung development will not survive. Subsequently, the saccular period (26-40 weeks) ensues with the appearance of terminal sacs and capillary networks in sufficient numbers to support respiration and gas exchange. During this period, the air-blood barrier begins to thin and type II cells are identifiable. However, substantial amounts of pulmonary surfactant are not detectable until 30-32 weeks gestation (Moore, 1998). In the neonatal period, the lungs undergo extensive remodeling and development. It has been estimated that approximately 95% of alveoli develop postnatally (Moore, 1998).

The importance of fully developed lungs is clearly demonstrated in fetuses born prematurely at 24 to 26 weeks postconception. The lungs of infants born at

this time are usually incapable of providing adequate gas exchange due to a limited alveolar surface area as well as underdevelopment of vascularity in the alveolar walls (Moore, 1998). These characteristics, coupled with low surfactant levels leads to the inability to expand the lungs thereby precipitating neonatal respiratory distress syndrome (NRDS). NRDS, characterized by increased lung liquid content, decreased lung compliance and eventual alveolar collapse, is one of the most common complications of prematurely born infants. Some 30,000 infants succumb to this disease each year in the USA (Endocrine, 1996), making it one of the leading causes of neonatal morbidity and mortality.

2.3 Surfactant

Type II cells are the primary sites of surfactant production. Surfactant is composed mostly of lipid (90%), but also contains proteins (8%) and carbohydrates (2%) (King, 1984). Of the lipid portion, over 85% is phospholipid and of this, 70-85% is phosphatidylcholine (PC). Over 60% of the PC consists of DSPC which is predominantly dipalmitoylphosphatidylcholine (DPPC) (King, 1984). DPPC, the single most abundant phospholipid, is largely responsible for the surface-active properties of lung surfactant (Clements, 1977).

Over the years, there has been an increasing interest in the surfactant proteins. Presently, four surfactant-associated proteins have been identified: two hydrophilic proteins (SP-A and SP-D) and two hydrophobic proteins (SP-B and SP-C). SP-A, the most abundant surfactant protein, has been implicated as having

a regulatory role in surfactant metabolism, being involved in pulmonary defense and being important in surfactant function (McCormack, 1998). SP-B and SP-C are necessary for formation of tubular myelin (see below) and promote the rapid adsorption of lipids to the air-water interface in the alveolus. A detailed description of surfactant protein structure and function may be found in a review by Hawgood and Poulain (1995).

2.3 A) Surfactant function

Several functions have been associated with pulmonary surfactant, including roles in pulmonary defense and transepithelial translocation. Surfactant facilitates migration of alveolar macrophages to inflamed tissues via chemotactic factors (Hoffmann, 1987) and may enhance the killing of bacteria by macrophages (LaForce, 1973). If surfactant is not present to increase surface pressure within the lung, fluid accumulates in the interstitial tissue and leads to pulmonary oedema, as seen in neonatal respiratory distress syndrome (nRDS) (Walters, 1992).

The function most frequently associated with surfactant and indeed its primary function, is its ability to reduce surface tension in alveoli, thereby preventing alveolar collapse at end expiration. As previously indicated, DPPC appears largely responsible for this function. DPPC is amphipathic in nature in that it contains hydrophobic as well as hydrophilic regions. Thus, when exposed to the air-liquid interface in the alveolus, DPPC molecules align with their polar head groups interacting with the aqueous hypophase lining the alveolus while the

nonpolar fatty acid groups extend into the alveolus. As alveolar size decreases during expiration, DPPC molecules effectively lower surface tension by compressing and excluding water from between the polar head groups. Films of DPPC can be compressed to surface tensions as low as $1 mN \cdot m^{-1}$ and show remarkable stability (Goerke, 1989) compared to other phospholipids. It is this stability at low surface tensions (high transmural pressure) that is important in preventing pulmonary collapse at end expiration.

2.3 B) Synthesis

Surfactant is synthesized in the endoplasmic reticulum of type II cells, stored as lamellar bodies and subsequently released into the alveolar lumen in response to a variety of stimuli. Although PC and its derivatives constitute the major part of surfactant, other phospholipids such as phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) are also components (Akino, 1992). The following outline will deal only with a review of the general plan of synthesis of surfactant-related phospholipids, predominantly DSPC (refer to figure 1).

The parent molecule involved in the synthesis of all glycerophospholipids is phosphatidic acid (PA). The major proportion of PA originates from the process of glycolysis. In this well characterized cycle, glucose is phosphorylated, isomerized and finally cleaved to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP). Through the action of triose phosphate isomerase, glyceraldehyde 3-phosphate may be transformed into DHAP.

Acylation and reduction of DHAP produces 1-acylglycerol-3-phosphate (1-AG-3-P) which subsequently undergoes a secondary acylation by 1-AG-3-P acyltransferase to produce PA. In type II cells, it is estimated that approximately 60% or more of the PA is produced by this process (Mason, 1978). However, DHAP may also be reduced to glycerol-3-phosphate (G-3-P) by the action of G-3-P dehydrogenase. Through an acylation process, G-3-P acyltransferase may convert G-3-P to 1-AG-3-P, in turn contributing to the PA pool. An additional source of G-3-P is derived by the phosphorylation of glycerol by glycerol kinase. Although adult type II cells contain glycerol kinase (Fisher, 1982), glycerol plays only a minor role in the production of PC due to low circulating concentrations of glycerol in blood and low glycerol uptake by the type II cells (Rhoades, 1974). However, glycerol may be an important substrate for G-3-P during the neonatal period (Mims, 1971) as blood concentrations of glycerol increase directly after birth (Persson, 1966).

The formation of PA represents a divergent point for synthesis of PC and the acidic phospholipids (see figure 1). The synthesis of PC requires the formation of diacylglycerol (DAG), whereas the synthesis of PG, PI and PS proceed through the production of cytidine diphosphodiacylglycerol (CDP-DAG) by the enzymatic action of phosphatidate cytidylyltransferase. CDP-DAG reacts with the hydroxyl group of a polar alcohol. If the alcohol is serine or inositol, then PS or PI is produced, respectively. PS may be decarboxylated to PE, which in turn can be methylated to PC. PG is produced from CDP-DAG by the action of glycerophosphate phosphatidyltransferase and phosphatidylglycerophosphatase. It

is worth noting that unlike other tissues, lung contains relatively high levels of PG and this is reflected in the high surfactant PG levels. PG levels act as markers of lung maturity as PG synthesis increases just prior to birth (Casola, 1980; Hallman 1980; Oulton, 1986).

The production of PC within the cell requires hydrolysis of PA to DAG by phosphatidate phosphatase. DAG and cytidine diphosphocholine (CDP-choline) are enzymatically converted by cholinephosphotransferase to PC. CDP-choline is produced by the phosphorylation of choline to phosphocholine, which subsequently reacts with CTP to produce CDP-choline. Phosphocholine is converted to CDP-choline by CTP:phosphocholine cytidylyltransferase (CPCT) (E.C. 2.7.7.15) which is thought to be the rate-limiting step in PC biosynthesis (Tokmakjian, 1981; Post, 1984; Zimmerman, 1993). The PC formed is generally of two forms based on the configuration of fatty acids esterified to the glycerol backbone. Initial evidence supported the view that DAG configured in a disaturated state could not be used as substrate by cholinephosphotransferase (Possmayer, 1977), suggesting that virtually all the DSPC of surfactant arose from 1-saturated-2-unsaturated phosphatidylcholine (Smith, 1978). A deacylation-reacylation process replaced the unsaturated fatty acid with palmitoyl (Post, 1983). However, several groups have now shown that under certain conditions cholinephosphotransferase may in fact utilize dipalmitoylglycerol to produce DSPC directly (Miller, 1981; Van Heusden, 1981; Ide, 1982). Therefore, DSPC may be synthesized directly from DAG configured in a disaturated state by

cholinephosphotransferase or it may be indirectly produced by deacylation and reacylation of PC.

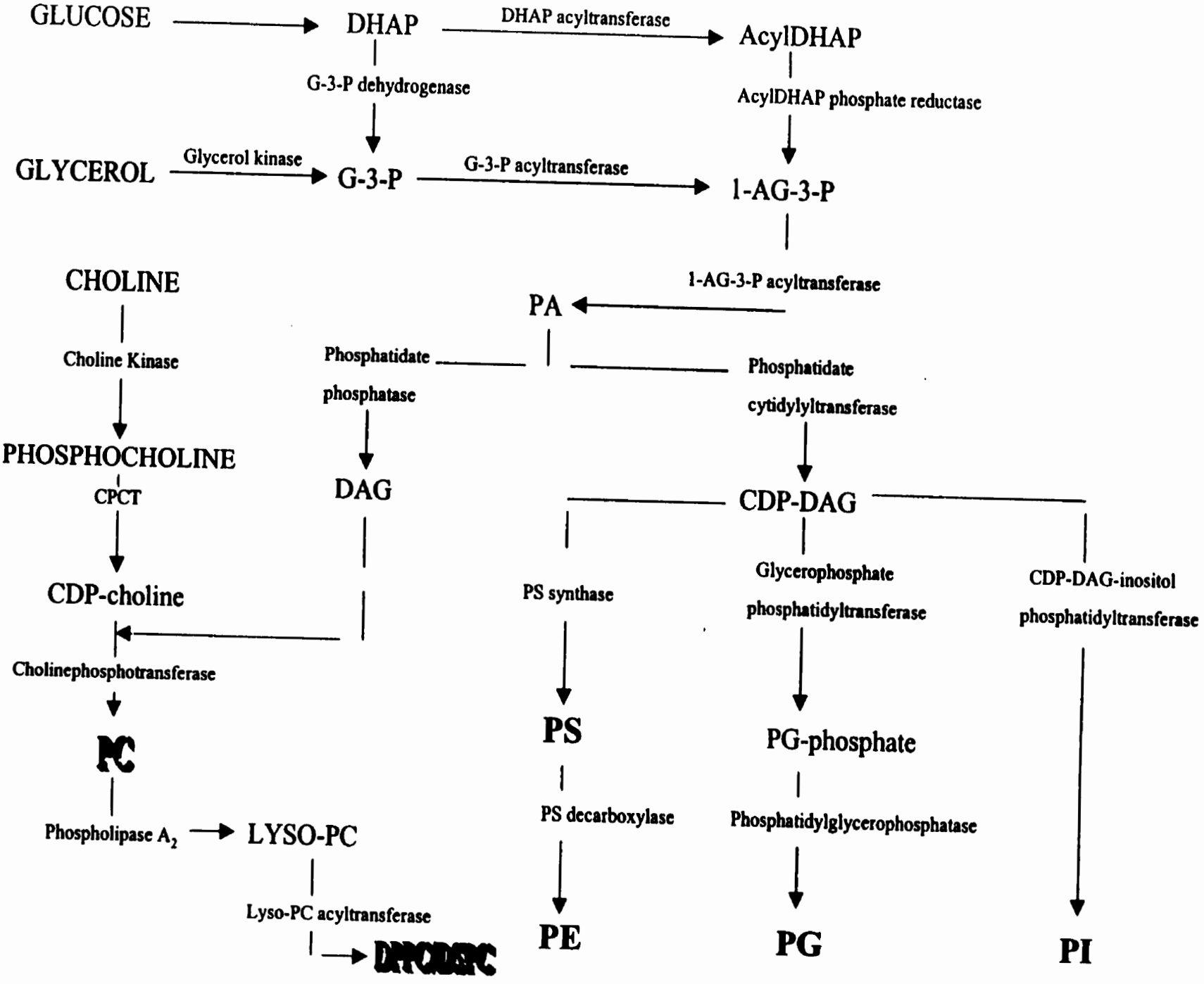
The mechanism(s) that govern the onset of surfactant and DSPC synthesis have been examined in detail. Major work has focused on the biochemical changes that occur in lung tissue during development, particularly during the initiation of surfactant synthesis. Glucocorticoids (Rooney, 1979; Smith, 1980), thyroid hormones (Liggins, 1988; Devaskar, 1996) and androgens (Smith, 1984) appear to influence fetal lung maturation, although the cellular mechanism(s) through which these hormones act is still not entirely clear. Glucocorticoids in particular do not appear to stimulate directly surfactant synthesis but rather act through epithelial-interstitial cell interactions. Fetal glucocorticoids stimulate fetal lung fibroblasts to synthesize FPF (Floros, 1985). When released by the fibroblast, FPF stimulates surfactant phospholipid synthesis in adjacent type II cells by activating the rate-limiting enzyme in surfactant phospholipid synthesis, CPCT (Post, 1986; MacDonald, 1995). For a more comprehensive review of the hormonal control of lung maturation, see papers of Smith (1984) and Mendelson (1991).

2.3 C) CTP:phosphocholine cytidylyltransferase (E.C. 2.7.7.15)

In all mammalian species studied, evidence indicates that production of surfactant phospholipids increases towards the end of gestation, reaching peak levels just prior to birth (Zimmerman, 1993). In the *de novo* synthesis of PC,

FIGURE 1: Biosynthesis of the surfactant-related phospholipids PC, DPPC, PS, PE, PG and PI in lung alveolar type II cells (modified from Samuels, 1997). 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
PA	Phosphatidic acid
CDP-DAG	Cytidine 5'-diphosphate diacylglycerol
DAG	Diacylglycerol
CPCT	CTP:phosphocholine cytidylyltransferase
CDP-choline	Cytidine 5'-diphosphate choline
PG-phosphate	Phosphatidylglycerol-phosphate
PC	Phosphatidylcholine
Lyso-PC	Lysophosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
PS	Phosphatidylserine
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol


GLUCOSE
DHAP
DHAP acyltransferase
AcylDHAP
G-3-P dehydrogenase
AcylDHAP phosphate reductase
GLYCEROL
Glycerol kinase
G-3-P
G-3-P acyltransferase
1-AG-3-P
CHOLINE
1-AG-3-P acyltransferase
PA
Choline Kinase
Phosphatidate phosphatase
Phosphatidate cytidylyltransferase
PHOSPHOCHOLINE
CPCT
DAG
CDP-DAG
CDP-choline
PS synthase
Glycerophosphate phosphatidyltransferase
CDP-DAG-inositol phosphatidyltransferase
Cholinephosphotransferase
PS
PG-phosphate
PC
PS decarboxylase
Phosphatidylglycerophosphatase
Phospholipase A2
LYSO-PC
Lyso-PC acyltransferase
PE
PG
PI

dramatic reductions in the level of phosphocholine have been observed between 25 and 27 days gestation in the rabbit (term gestation is 31 days), while CDP-choline levels exhibited only a moderate decrease (Tokmakjian, 1981). Additionally, it has been demonstrated by Tokmakjian et al. (1981) that the pulmonary level of PC almost doubles between 27 and 30 days gestation. Thus, as gestation progresses, large decreases in phosphocholine levels occur, CDP-choline levels decrease slightly and PC synthesis increases dramatically. Taken together, these alterations in pool sizes suggest that CPCT regulated conversion of phosphocholine to CDP-choline shifts in the direction of CDP-choline formation during pulmonary development. Further studies of isolated fetal type II cells indicate an increase in CPCT activity during late gestation (Zimmerman, 1993) coinciding with the increase in PC synthesis.

There are several ways in which CPCT activity may be modulated. In isolated type II cells, CPCT activity has been found associated with both cytosolic and microsomal fractions (Stern, 1976), although the majority of activity has been localized to the cytosol. Within the cytosol, CPCT exists as both an inactive low molecular weight form (LMW) and as an active high molecular weight form (HMW). The LMW is found predominately in the cytosol of fetal lung, whereas the HMW is found in adult lung cytosols. The HMW form of CPCT is also found associated with cellular membranes in both fetal and adult lung tissues. Activation of CPCT correlates with conversion from the LMW to the HMW form of the enzyme. Thus, the HMW form associated with cellular membranes represents stimulated CPCT while the LMW form associated with cellular

cytosols represents an inactive enzyme pool. Recently, Zimmerman et al. (1993) observed a decrease in cytosolic CPCT total activity with a concurrent increase in microsomal activity in developing fetal rat type II cells. These results suggest that activation of CPCT correlates not only with conversion from the LMW to the HMW form of the enzyme, but also with its translocation from cytosol to membranes. By utilizing cytosolic fractions obtained from isolated fetal type II cells, it has been demonstrated that CPCT may be maximally stimulated by anionic phospholipids, including PG, PI and PS (Stern, 1976; Zimmerman, 1995) as well as by oleic acid (Mallampalli, 1993; Zimmerman, 1995) and DAG (Utal, 1991). PA and PE stimulate CPCT to a small degree, while PC inhibits CPCT activity (Zimmerman, 1995). The inhibition of CPCT activity by PC is likely due to a negative feedback loop, in which the presence of the enzymes' end product inhibits further PC production. Considerable evidence suggests that CPCT activity is also regulated by phosphorylation-dephosphorylation (Hatch, 1992; Wang, 1993). The inactive form of the enzyme is highly phosphorylated, whereas the active form is dephosphorylated. The kinases involved in the phosphorylation of CPCT are still under great debate. Evidence indicates however, that cAMP-dependent kinases (Zimmerman, 1994; Wieprecht, 1996) and protein kinase C (Utal, 1991; Wieprecht, 1996) are not responsible for phosphorylating this enzyme *in vivo*. Recently, Wieprecht et al. (1996) identified phosphorylation sites of rat liver CPCT and some of these sites appear within consensus sequences for proline-directed protein kinases. Taken together, it appears that CPCT activity is regulated by specific activators and inhibitors, conversion between the LMW and

the HMW forms of the enzyme, translocation between cytosol and membranes as well as by phosphorylation and dephosphorylation .

The mechanism(s) by which CPCT activity increases in late gestation has long been an area of great debate. Hogan et al. (1994) demonstrated that CPCT mRNA levels increase three-fold between day 18 and day 21 of gestation in fetal rat lung. This increase in mRNA was associated with an increase in CPCT protein levels and enzymatic activity in microsomal fractions (Zimmerman, 1993; Hogan, 1994). Recently, it has been suggested that the increase in CPCT activity and mRNA levels is in part due to an increase in CPCT mRNA stability (Hogan, 1996), rather than an increase in transcription rate. Zimmerman et al. (1995) proposed that the increase in CPCT activity late in gestation was due to an increased capacity of cytosolic lipids to activate CPCT with advancing gestation. It is probable that the elevated CPCT activity in developing lungs may be attributed to increased mRNA levels as well as to increased activation of CPCT by cytosolic lipids.

2.3 D) Secretion

Surfactant is synthesized in the endoplasmic reticulum of type II cells, stored as lamellar bodies and secreted into the alveolar space. During breathing, continual turnover of surfactant in alveolar fluid occurs and therefore a continual rate of secretion is required. Wright et al. (1987) have estimated that *in vivo*, 10-15% of the stored intracellular surfactant pool is secreted per hour. Surfactant secretion, or the intracellular movement of lamellar bodies to the cell surface and

their subsequent exocytosis, is a difficult process to study, as the entire cycle appears to be tightly regulated (Scott, 1992; Kresch 1994). Studies have utilized several models to attempt to identify the mechanism(s) involved in secretion including organ cultures, perfused lung slices as well as adult and fetal lung cell culture. From these studies, it has become clear that secretion may be affected by periods of hyperventilation (Oyarzun, 1978), microtubule disruption by C_2 toxin and cytochalasins (Tsilibary, 1983; Rose, 1999), increases in cytosolic calcium (Dobbs, 1986), activation of protein kinase C (Scott 1994; Samuels, 1995) as well as by adrenergic (Mettler, 1981) and purinergic (Chander, 1995) receptor activation.

Large inflations and hyperventilation of intact or isolated lungs can increase the amount of surfactant in the alveolar space (Oyarzun, 1978). Although the mechanism through which this occurs has not been completely elucidated, two possible routes have been proposed. By applying hydrostatic pressure beneath membranes of isolated type II cells to induce stretch, Wirtz et al. (1990) observed transient increases in cytosolic calcium followed by stimulation of surfactant release. Using cultured fetal rabbit type II cells, Scott et al. (1993) observed increases in cellular cAMP levels when fetal cells were subjected to cyclic stretching similar to those which occur during episodes of fetal breathing movements. Therefore, ventilation induced increase in surfactant release is accompanied by increased cAMP and/or calcium levels.

Alterations in the cytoskeleton also play a role in secretion of surfactant. The intracellular movement of lamellar bodies to the cell surface for exocytosis is

thought to be mediated by alterations in microfilaments. Tsilibary et al. (1983) observed actin filaments in close proximity with lamellar bodies in perfused lung slices. Upon stimulation of secretion, there was a large decrease in the number of lamellar bodies per cell with a corresponding increase in the number of actin filaments. The loss of lamellar bodies could then be inhibited by disrupting actin filaments with cytochalasin D treatment. In contrast, a study by Rice et al. (1984) demonstrated a stimulatory effect of cytochalasins on release of [^{3}H]PC in isolated type II cells in culture. Recently Rose and colleagues (1999) observed a two to three-fold increase in surfactant secretion by freshly isolated type II cells following inhibition of actin polymerization. Although the discrepancies between these findings may be largely due to changes in cytoskeletal architecture in cultured type II cells (Mason, 1978) or to differences between microfilament inhibitors, a clear explanation has not yet been put forward.

β-adrenergic agonists have been shown to increase surfactant secretion in isolated type II cells (Mettler, 1981; Rice, 1985). The β-adrenergic agonist terbutaline, induces a release of [^{3}H]PC by isolated rat type II cells with concurrent increases in cytosolic cAMP and cAMP-dependent protein kinases (Rice, 1985). Cholera toxin, a substance which bypasses the receptor and directly activates adenylate cyclase, also stimulates release of surfactant-related material. This effect is accompanied by increased cytoplasmic cAMP activity (Mescher, 1983). Presumably, as is the case in other systems, the β-adrenergic receptor is coupled to adenylate cyclase through G proteins. Activation of adenylate cyclase

increases cAMP levels leading to phosphorylation of actin by cAMP-dependent protein kinases (figure 2). Phosphorylation of actin and/or other proteins leads to PC secretion, although few studies have focused on the distal steps in the secretory pathway.

Activation of protein kinase C (PKC) also leads to surfactant secretion by type II cells. TPA, or 12-O-tetradecanoylphorbol-13-acetate, is a well known activator of PKC (Linke, 1997) and has been shown to produce a dose-dependent stimulation of surfactant secretion (Scott, 1994). The specificity of phorbol esters for PKC in type II cells has been demonstrated by the ability of PKC inhibitors to down-regulate phorbol ester-mediated secretion. Major regulatory routes of surfactant secretion exists through activation of a Ca^{2+}-PS-dependent PKC (Scott, 1994; Samuels, 1995), although a PS-independent form of the enzyme may also play a role (Samuels, 1999). Once activated, PKC probably phosphorylates specific proteins that ultimately activate contractile proteins that move lamellar bodies to the apical surface for exocytosis (figure 2).

The calcium ionophores A23187 and ionomycin have also been shown to stimulate surfactant release *in vitro*, suggesting a role for Ca^{2+} in secretion in type II cells (figure 2) (Dobbs, 1986; Sano 1987). Release of surfactant from isolated type II cells is accompanied by calcium efflux (Dobbs, 1986; Warbourton, 1989). The stimulatory affect of calcium ionophores appears to be independent of extracellular calcium concentrations, as depletion of calcium from medium does not inhibit this effect (Dobbs, 1986). As calcium ionophores are known to release calcium from intracellular stores, it is thought that calcium is released into the

cytoplasm and subsequently secreted from the cell in conjunction with surfactant. By exposing fetal rat type II cells to verapamil, a calcium channel blocking agent, Warburton et al. (1989) observed a multiphasic concentration effect on surfactant secretion, with a stimulatory effect at low concentrations. Release of surfactant from verapamil-treated cells was accompanied by an increase in calcium efflux while intracellular calcium levels decreased. Taken together, these results suggest that intracellular calcium concentrations help to regulate the release of surfactant by type II cells.

Adenosine trisphosphate (ATP) is one of the most effective surfactant secretagogues, increasing PC secretion as much as 5-6 fold in isolated alveolar type II cells (Chander, 1995). The stimulatory effect of ATP on secretion appears to be mediated by both a $P2Y_2$ purinergic receptor coupled to phospholipase C (PLC) (Rice, 1990a) as well as by an A_{2b} receptor coupled to adenylate cyclase (Gobran, 1997) (figure 2). A_{2b} is a subtype of P_1 purinergic receptors that are stimulated by adenosine, analogues of adenosine and ATP in fetal type II cells (Gobran, 1997) to activate adenylate cyclase. Activation of adenylate cyclase results in cAMP formation and stimulation of cAMP-dependent protein kinases that phosphorylate specific proteins involved in surfactant secretion. Acting via $P2Y_2$ receptors, ATP has been shown to activate phospholipase C (PLC), leading to the formation of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Rice, 1990a; Griese, 1991). DAG activates PKC (Chander, 1995) and it in turn phosphorylates proteins. IP_3 mobilizes intracellular calcium, thereby activating calcium-dependent protein kinases.

However, blocking calcium mobilization from intracellular stores (Rice, 1990a; Rice, 1990b) does not change ATP-induced secretion, suggesting that surfactant release is not dependent on calcium release. UTP, another $P2Y_2$ receptor agonist, also stimulates release of surfactant by isolated type II cells, although UTP does not increase cAMP formation (Gobran, 1994; Gobran, 1997). Like ATP, receptor stimulation by UTP generates the second messengers IP_3 and DAG.

There is evidence that developmental changes occur in the response of type II cells to surfactant secretagogues. Recently, Gobran et al. (1997) have suggested that the $P2Y_2$ receptor-coupled ATP signaling pathway is not functional until the 4^{th} postnatal day. Later studies indicate that the response of newborn isolated type II cells is not due to insufficient expression of the $P2Y_2$ receptor genes, but rather diminished expression of specific PKC isozymes (Gobran, 1998). However, the physiological importance of this developmental delay in the response of type II cells to surfactant secretagogues is still unknown.

Release of surfactant by type II cells in response to many different stimuli linked to a variety of second messenger systems may be inhibited by components of pulmonary surfactant (Dobbs, 1987; Scott, 1992). This inhibitory effect provides a negative feedback loop required for the regulation of pulmonary surfactant secretion. It has been observed by Dobbs et al. (1987) that surfactant lipids inhibit stimulated secretion by as much as 40%. Later studies by Scott et al. (1992) indicate that both DPPC and PC reduce the release of PC from isolated fetal rabbit type II cells, although the former reduces release to a greater degree. SP-A inhibits secretion in a dose-dependent manner (Dobbs, 1987; Rice 1987)

and this inhibition appears to occur via a SP-A receptor located on alveolar type II cells (Strayer, 1996). Although the detailed mechanisms by which lipids and SP-A inhibit surfactant secretion is unknown, it appears that downstream exocytotic events are affected.

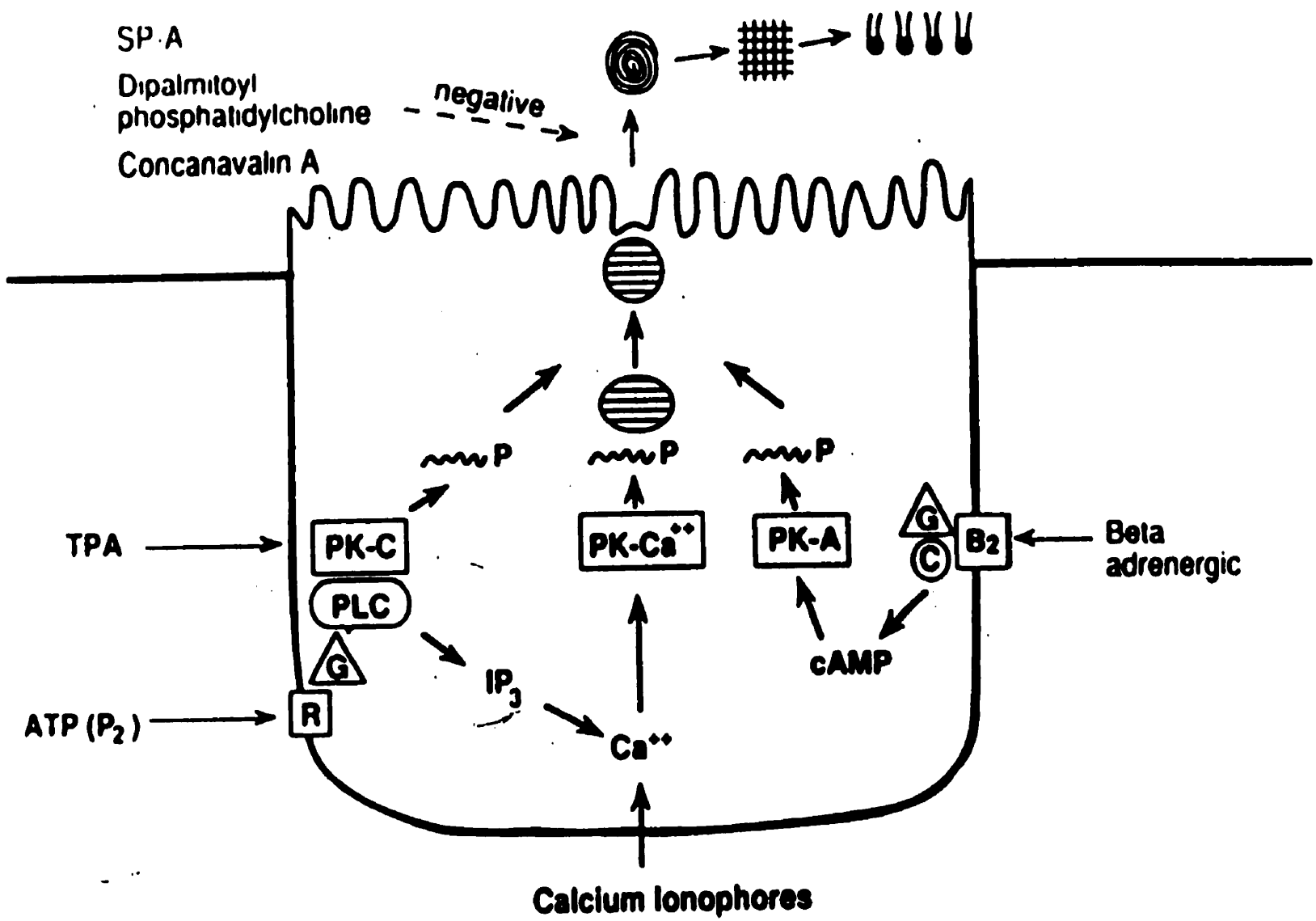
Once surfactant has been secreted from the alveolar cell, it undergoes a transformation into a three-dimensional, highly ordered lattice structure known as tubular myelin. Tubular myelin is believed to be the major precursor of pulmonary surfactant as it contains a high percentage of phospholipids. The events responsible for this transformation *in vivo* are not understood, but *in vitro* the formation of tubular myelin has been shown to require calcium, phospholipids and surfactant proteins SP-A and SP-B (Suzuki, 1989).

2.3 E) Clearance of surfactant from the alveolar space

The ongoing secretion of surfactant must be balanced by an equally active clearance pathway to prevent excessive accumulation of surfactant in the alveolar lumen. To date, three routes of surfactant clearance have been identified. A small amount of surfactant may be removed from the whole lung via the airways and vascular circulation. Components of surfactant may also be degraded by alveolar macrophages or type II cells. The latter reincorporate selected components into newly synthesized material (Chander, 1987; Wright, 1991).

FIGURE 2: Secretion of surfactant from type II cells (Mason, 1998). This figure depicts four groups of agonists that result in release of surfactant from the type II cell. Interaction between second messengers is more complex than depicted and the distal events involved in secretion are still unknown. The abbreviations used in the figure are as follows:

TPA	12-O-tetradecanoyl phorbol acetate
ATP	Adenosine trisphosphate
PK-C	Protein kinase C
PLC	Phospholipase C
G	G-protein
R	Cell membrane receptor
IP_3	Inositol trisphosphate
Ca^{++}	Calcium ions
PK-Ca^{++}	Calcium dependent protein kinase
P	Phosphorylation
B_2	β-adrenergic receptor
C	Adenylate cyclase
cAMP	Cyclic adenosine monophosphate
PK-A	Protein kinase A
SP-A	Surfactant protein A


SP-A
Dipalmitoyl phosphatidylcholine
Concanavalin A
negative
P
P
P
TPA
PK-C
PLC
G
R
ATP (P_2)
IP_3
Ca^{++}
PK-Ca^{++}
PK-A
cAMP
G
C
B_2
Beta adrenergic
Calcium Ionophores

However, the major route of surfactant clearance is through internalization of surfactant by type II cells. By exposing isolated type II cells to radiolabeled PC, Chander et al. (1983) observed a time, temperature and concentration dependent uptake of [^{3}H]PC. As exogenous lipids are internalized, they are incorporated into lamellar bodies and eventually re-secreted. This uptake process in isolated type II cells is stimulated by SP-A, SP-B, SP-C and PG (Suzuki, 1989).

From previous studies, it is clear that the equilibrium between the synthesis, secretion and reutilization of surfactant is under strict control of a complex system of regulatory mechanisms allowing constant surfactant pool sizes within alveoli. Without tight regulation and feedback mechanisms between the various pathways in surfactant metabolism, insufficient or excessive levels of surfactant may arise leading to pulmonary complications.

2.4 MARIJUANA: *History of use as a drug, effects and toxicity; a review of current literature.*

Marijuana comprises the leaves, stems and flower clusters of the hemp plant *Cannabis sativa*. The plant contains 60 unique compounds known as cannabinoids, the most abundant and psychoactive being Δ^9-tetrahydrocannabinol (THC). For many centuries, marijuana has been used both as a recreational and medicinal drug. Recent reports have identified traces of marijuana in the tissues of an ancient Egyptian mummy dated back to 950 B.C. in a pattern that is

characteristic of drug inhalation (Nerlich, 1995). Today, considerable controversy exists regarding the possible role of marijuana as a therapeutic agent. Marijuana has been used to alleviate the nausea associated with chemotherapy, reduce migraine pain, control seizures in epileptic patients, decrease intraocular pressure in glaucoma and stimulate appetites in AIDS patients (Gurley, 1998; Voth, 1997). However, marijuana smoking exposes the user to 50% higher levels of the procarcinogen benz-α-pyrene than tobacco smoking (Hoffman, 1975). Smoking marijuana also results in a 5-fold increase in blood carboxyhemoglobin levels as compared to tobacco smoking and exposes the lung to 3 times the amount of tar (Wu, 1988). Consequently, the modern development of cannabinoids as therapeutic agents is restricted by their abuse potential, negative side effects and by difficulties in separating psychotropic from possible therapeutic effects.

Today, marijuana is one of the most commonly used illicit drugs in society and evidence indicates that its frequency of use is increasing (Hutchings, 1991). Studies of pregnant women indicate that at least 10 per cent report some marijuana use throughout their pregnancy (Fried, 1980). In blood plasma, THC has a half-life of 30 hours when administered intravenously and 23 hours after oral administration (Wall, 1981). The cannabinoids from marijuana, including THC, tend to be stored in fat and may be detected in body tissues for up to 30 days following a single exposure (Jones, 1980). Recently, such findings have renewed concerns over the developmental effects of marijuana use on the outcome

of pregnancy, as considerable prolonged fetal exposure may occur as a result of the large maternal tissue pool of the drug in regular marijuana users.

THC must pass through the placental membrane before entering the fetal circulation. THC crosses the placental barrier freely by passive diffusion. Thus, one would expect fetal and maternal drug levels to reach equilibrium. Recent evidence indicates, however, that lower levels of marijuana are present in fetal blood as compared to maternal levels (Szeto, 1993). This observation may be attributed to the extensive binding of THC to maternal plasma proteins. As a large portion of the drug is bound to maternal proteins, only small amounts may be available to diffuse across the placenta. In sheep, THC has been found to transfer slowly from maternal to fetal circulation, a finding that also has been attributed to THCs' binding to maternal plasma proteins (Abrams, 1985). However, fetal drug levels depend not only on the rate of placental transfer, but also on the rate of drug elimination from the mother. As placental transfer rate of THC is slow relative to the rate at which it is metabolized by the mother, drug concentrations may not reach high levels in the fetus. Nevertheless, potential for prolonged low level exposure exists.

Maternal marijuana use has been linked to several developmental effects on the growing embryo. Some evidence suggests marijuana impairs fetal growth and increases the frequency of preterm delivery (Hatch, 1986; Zuckerman, 1989). THC has also been linked to fetal resorption, growth retardation, neurobehavioural abnormalities in the neonate and increased fetal and embryonic mortality (Harclerode, 1980). Smoking marijuana may affect the fetus indirectly.

Fetal oxygenation may be restricted by elevated carbon monoxide levels in maternal blood. Marijuana also decreases the respiratory rate (Clapp, 1986) while increasing heart rate and blood pressure (Wu, 1988) of the mother thus, impairing pulmonary gas exchange and reducing placental blood flow to the fetus.

2.4 A) Cellular affects of cannabinoids

There has been a long-standing interest in determining the cellular mechanisms through which cannabinoids exert their effects. Since they are very hydrophobic, cannabinoids including THC have been considered substances that specifically alter membrane properties thereby affecting membrane-associated proteins. However, with the recent identification of two cannabinoid receptors, many cannabinoid effects are now believed to be mediated by specific receptor interactions.

2.4.a.i) Specific lipid and protein interactions

As cannabinoids and THC are very hydrophobic, they have a much higher affinity for biomembranes than for aqueous solutions. Using nuclear magnetic resonance techniques, it has been shown that THC assumes a characteristic orientation in DPPC bilayers, thereby inducing membrane perturbations (Makriyannis, 1989). These cannabinoid : lipid interactions may not only have affects on the biophysical properties of lipid bilayers, but also may induce changes in the function of surrounding membrane proteins. THC has been shown to affect the functioning of membrane-associated proteins that are involved in

signal transduction processes, such as adenylate cyclase (Hillard, 1986; Hillard, 1990). Hillard et al. (1990) observed an increase in adenylate cyclase activity in cardiac cell membranes upon exposure to THC concentrations of 1-3μM. As THC does not directly affect adenylate cyclase activity (Hillard, 1986; Hillard 1990), the increase in enzyme activity is thought to be secondary to changes in the physical properties of the membrane phospholipid bilayer by THC. Membrane lipid perturbations caused by THC changes membrane protein activities that are linked to adenylate cyclase (Hillard, 1990). Howlett and Fleming (1984) demonstrated that at low THC concentrations (<1μM), adenylate cyclase activity is inhibited in neuroblastoma cell membranes while at high concentrations THC stimulates activity. In these studies, the inhibition of adenylate cyclase by low drug concentrations represents a receptor-mediated event whereas stimulation at higher concentrations is a result of membrane perturbations.

THC may also directly affect cellular activities by its lipophilic nature. Hillard et al. (1994) demonstrated that THC increases activity of PKC isolated from rat forebrain cytosol in a dose-dependent manner. Stimulation of phospholipase A_2 (PLA_2) upon exposure to THC in a cell-free system (Evans, 1987) is thought to be the result of THC interacting with hydrophobic regulation sites on PLA_2. Although cannabinoids and THC have also been shown to increase unesterified arachidonic acids levels in cerebral cortex slices (Reichman, 1988) and to disrupt microtubules and microfilaments in nerve cells (Tahir, 1992), the mechanism through which they act remains elusive.

2.4.a.ii) Cannabinoid receptors

Although many effects may be explained by cannabinoid lipid solubility, some now appear to be mediated through specific receptors. Matsuda et al. (1990) first characterized and cloned a cannabinoid receptor in rat cerebral cortex. This receptor, later referred to as CB_1, contains seven transmembrane segments, several glycosylation sites and highly conserved residue sequences among the G-protein class of receptors. CB_1 is largely localized in the brain specifically in the hippocampus, associational cortical regions, cerebellum and basal ganglia. This distribution pattern correlates well with the known effects of cannabinoids on memory, perception and control of movement. However, CB_1 has also been detected in rat testis (Gerard, 1991) and in freshly isolated lung type II cells (Rice, 1997). An isoform of CB_1, CB_{1A}, has recently been identified as the spliced form of the human and rat CB_1 gene transcript (Shire, 1995). However, CB_{1A} appears to be a relatively minor component of the total central cannabinoid receptors.

Discovery of the CB_1 receptor prompted a search for an endogenous cannabinoid receptor agonist. Two such endogenous ligands, N-arachidonyl-ethanolamide (anandamide) and 2-arachidonylglycerol (2-AG) have recently been isolated and characterized from whole porcine brain (Devane, 1992) and canine gut (Mechoulam, 1995), respectively. Anandamide appears to be largely localized in the brain and liver, although trace amounts may be detected in spleen and pancreas (Willoughby, 1997; Watanabe, 1998). Recently, high levels of 2-AG have been identified in rat brain while substantial levels have been detected in liver, spleen, kidney and lung (Kondo, 1998).

A second receptor subtype, CB_2, has also been identified and localized in tissue structures of the immune system, such as those of the spleen (Munro, 1993) and Peyers' patches of the gut. At the cellular level, CB_2 receptors have been found on B-cells, monocytes and T-cells (Galiegue, 1995). Although CB_2 shares only a low overall homology with CB_1 (44%) (Munro, 1993), both receptors are members of the G-protein linked super-family of receptors. In comparison to other G-protein coupled receptors however, the pharmacology of the cannabinoid receptors are very poorly understood. Both CB_1 and CB_2 receptors have been shown to inhibit adenylate cyclase via a pertussis toxin-sensitive G protein (Condie, 1996), although CB_1 receptors may stimulate this enzyme under certain conditions (Rhee, 1998). CB_1 but not CB_2 receptors have been shown to inhibit calcium entry through voltage-dependent calcium channels (Caulfield, 1992; Mackie, 1992) and stimulate nitric oxide release in the central nervous system of leeches (Stefano, 1997). In immune cells, CB_2 has been linked to decreased levels of interleukin 2 (Condie, 1996).

Cannabinoid receptors have also been identified throughout development, suggesting that they may play a role in normal fetal development. During the embryonic development of the rat, Buckley et al. (1998) have identified the expression of CB_1 receptor mRNA as early as gestational day 11 in the brain of the embryo. At embryonic day 15, CB_1 receptor expression appears in a small number of airway passages within the lung. By day 17, however, this expression becomes more widespread throughout the bronchi, reaching peak values at day 21. These observations led Buckley and colleagues to conclude that CB_1

receptors may be involved in preparing the lungs for birth. The presence of these receptors within fetal tissues encouraged researchers to examine their role in fetal development. In recent studies involving rats, anandamide and 2-AG were shown to retard development of embryos into blastocysts (Paria, 1995; Paria, 1998). The adverse affects of the endogenous cannabinoids may be reversed *in vitro* by CB_1 antagonists, suggesting a role for this receptor in embryonic development. In the same studies, the CB_1 receptor agonist, CP-55,940 was shown to prevent implantation of blastocysts into the uterine wall; this effect was reversed by coadministration of CP-55,940 with SR141716A, a receptor antagonist.

OBJECTIVES

While marijuana is known to impair fetal growth and increase the frequency of preterm delivery, little is known about its affects on the developing respiratory system. The respiratory system is critical in allowing the neonate to meet the challenge of a new environment, as the transition from a liquid-filled fetal lung to an air-breathing postnatal lung depends on proper and adequate lung development. Fetal drug exposure may alter normal lung development, thereby posing serious problems to the newborn. Research has suggested that fetal lung development is particularly sensitive to detrimental effects of various agents ranging from endogenous steroids to environmental pollutants. In many cases, these agents are lipid soluble and some of their affects may be due to their solubility. Given the increasing frequency of marijuana use in conjunction with the recent identification of cannabinoid receptors within the lung, potential exists that lipophilic cannabinoids may alter surfactant metabolism and ultimately function. The present work focuses on the effects of THC on fetal and adult lung cell function, with particular emphasis on the surfactant system. Using *in vitro* models based on isolated surfactant-producing adult and fetal lung type II cells, the objectives of this work are:

(I) To determine if THC (the principle active ingredient of marijuana) affects the synthesis and secretion of surfactant by isolated adult and fetal type II cells.

(II) To determine the cellular mechanism(s) through which THC affects the intracellular and extracellular processing of surfactant in isolated adult and fetal rabbit type II cells.

MATERIALS AND METHODS

4.1 MATERIALS

Time pregnant New Zealand White Rabbit does were obtained from St. Andrews Rabbitry, St. Andrews, Manitoba, Canada. Cell culture materials (solutions, flasks, and serum) were obtained through Gibco (Burlington, ON, Canada). Thin layer chromatography plates were obtained from Fischer Scientific Canada (Edmonton, AB, Canada). Biochemicals and lactate dehydrogenase kits were from Sigma Chemical Company (St. Louis, MO, USA). Radioactive materials ([^{3}H]choline chloride, specific activity 75 Ci/mmole and [^{14}C]phosphorylcholine, specific activity 50mCi/mmole) were obtained through New England Nuclear (Boston, MA, USA). Tetrahydrocannabinol and cannabinol were kindly supplied by the Bureau of Drug Surveillance, Ottawa, Canada. Centrifugation steps were carried out using the Joaun CR3000, International Equipment Company (IEC) centrifuge IEC B-20A and the Beckman L8-M ultracentrifuge. The IEC and Beckman centrifuges required rotors A-237 and 80T1, respectively.

4.2 METHODS

4.2 A) Isolation of rabbit pre-type II cells

Fetal pre-type II cells were isolated as described previously by Scott et al. (1983), with some modifications. Pregnant New Zealand White rabbits (time of mating designated as day 0) were killed on the 24th gestational day with 2ml of

Euthanyl (240mg/ml sodium pentobarbital) injected into the lateral ear vein. Fetuses were removed by hysterotomy, decapitated, and placed in ice-cold, sterile Hanks Balanced Salt Solution (HBSS) (pH 7.1). Using sterile techniques, fetal lungs were removed from the thorax using scissors and forceps. Extraneous tissue was discarded. To break up tissue aggregates, lungs were chopped using a Sorval Tissue Chopper (Sorval Instruments, Newton, CT). Tissue was trypsinized for 35 minutes at 37°C in a trypsinization flask with 250ml of trypsin/EDTA (0.05%/0.02%). Approximately 50ml of minimal essential medium (MEM) containing 10% stripped newborn calf serum (sNCS) (v/v) was added to the cell suspension to stop protease action. The suspension was filtered through three layers of 150um Nitex gauze and centrifuged for 10min at 250xg and surpernate discarded. The pellet was resuspended gently in 50ml of MEM containing 10% sNCS (v/v) using a 10ml pipet being careful not to damage the cells. Cell suspension was plated into five 75-cm^2 incubation flasks and incubated for 1 hour in an atmosphere of 5% CO_2 at 37°C to allow fibroblasts to adhere. Medium containing unattached cells was collected and cell number determined with a Coulter cell counter. Cells were replated in 25cm^2 culture flasks at a density of $1x10^5$ cells/flask. Medium was changed after 24 hours and every 48 hours thereafter. All cells were used within 5-6 days of isolation.

4.2 B) Synthesis and release of surfactant by isolated fetal rabbit pre-type II cells

DSPC is a phospholipid unique to pulmonary surfactant. Therefore, it is considered a marker of surfactant synthesis and secretion. To determine if THC affects synthesis of DSPC in cultured fetal type II cells, isolated rabbit pre-type II cells were grown to confluence over a period of 6 days. Stock THC was prepared by dissolving 25mg of agent in 6.45mls of 100% ethanol and diluted to concentrations of 10^{-7}-10^{-4}M in MEM with 10% sNCS. Cell cultures were exposed to 1uCi/ml [^{3}H]choline and each concentration of THC for 24 hours. Prior to extraction for DSPC analysis (see below), cells were removed by trypsinization and medium was collected. Samples were frozen separately at –85°C until further use.

4.2 C) Isolation of DSPC

A total phospholipid extract was obtained by the protocol of Bligh and Dyer (1959). One volume of sample fraction was mixed with six volumes of chloroform:methanol (1:2) and vortexed. Two volumes of chloroform plus 2.6 volumes of 1% potassium chloride were added to separate organic and aqueous phases. The top aqueous fraction was removed with a Pasteur pipet and discarded. The bottom organic fraction was dried under air and resuspended in 200ul of chloroform:methanol (20:1). This fraction was used as the source of PC and DSPC.

The method of Mason et al. (1976) was used to isolate DSPC from total lipid extract. To obtain both PC and DSPC levels, samples were resuspended in

200ul of choloroform:methanol (20:1), divided in half and dried under air. DSPC was isolated from the organic lipid extract by reaction with 0.5ml of carbon tetrachloride containing 3.5mg OsO_4 for 15 minutes at room temperature. OsO_4 is a powerful oxidant that reacts with double bonds to form complexes (Mason, 1976) and since DSPC has only saturated fatty acids, OsO_4 does not alter its structure. Both DSPC and PC samples are treated identically from this point. After air drying, samples were once again resuspended in 200ul of chloroform:methanol (20:1). Ten percent of each sample was plated onto single-channeled thin layer chromatography plates (Whatman, LK5D, Fischer Scientific, Edmonton, AB). A PC standard was prepared using 30mg of PC in 3mls of chloroform:methanol (20:1) and plated onto every fifth channel. Plates were run in the solvent system described by Skipski and Barclay (1969) (chloroform:methanol:water; 75:25:4) until the solvent front reached the top of the plate. Plates were dried and standards visualized with iodine vapour. Spots were scrapped into scintillation vials and 5ml of Ready Protein scintillation cocktail (Beckman Instruments, Palo Alto, CA) and 200ul water were added. Samples were counted for [^{3}H] activity using a LS 5801 scintillation counter and H# for quench compensation (Beckman Instruments, Palo Alto, CA).

4.2 D) Pulse-chase experiment

In order to determine the effect of THC on the incorporation of [^{3}H]choline into intermediates in the *de novo* pathway for DSPC synthesis, pulse-chase experiments were performed. Isolated fetal rabbit type II cells were grown

to confluence over a period of five days. Cultures were pre-labeled with 8uCi of [^{3}H]choline in 4ml of MEM with 10% sNCS (v/v) for a period of 1 hour. Cultures were washed twice with 2ml of HBSS and incubated with 10^{-4} M THC in MEM over various time periods. Control samples were incubated in MEM. Media were decanted and cultures frozen at -80°C. Samples were resuspended in 2ml of HBSS. DSPC and PC were isolated following total phospholipid extraction as described above.

Choline, phosphocholine and CDP-choline were isolated as described by Tokmakjian et al. (1981). Aqueous fractions of the chloroform:methanol extractions were dried under air and resuspended in 200ul of ethanol:water (1:1). Choline, phosphocholine and CDP-choline standards were prepared by dissolving 1mg of each in 1ml of ethanol:water (1:1). Standards were plated onto single-channeled thin layer chromatography plates (Whatman, LK5D, Fischer Scientific, Edmonton, AB) along with a fixed percentage of each sample. Plates were run in a solvent system of 50ml ethanol, 50ml 0.87% NaCl, and 5ml NH_4OH until the solvent front reached the top of the plate. Plates were allowed to dry overnight at 80°C. Standards were visualized with iodine vapour and scrapped into scintillation vials. To each sample, 200ul water and 5ml of Ready Protein scintillation cocktail (Beckman Instruments, Palo Alto, CA) were added. Samples were counted for [^{3}H] activity using a LS 5801 scintillation counter and H# for quench compensation (Beckman Instruments, Palo Alto, CA).

4.2 E) Determination of cellular THC toxicity

Release of lactate dehydrogenase (LDH) is an important indicator of cell damage prior to death (Scott, 1994). Medium was collected from control and THC treated cell cultures from 9 and 48 hour incubations. LDH activity was measured using a kit from Sigma Chemical Company (St. Louis, MO). With this kit, LDH activity is detected when reagent absorbance increases at 340nm due to the reduction of nicotinamide adenine dinucleotide when LDH converts lactate to pyruvate.

4.2 F) Cytosolic and microsomal fractionation of rabbit lung

New Zealand White rabbits of known gestational time were sacrificied by injection of 3ml of euthanyl into the lateral ear vein. Fetuses were rapidly removed by hysterotomy, decapitated, and placed into cold sterile HBSS. Fetal lungs were removed and extraneous tissue dissected free. Adult lungs were removed from the chest cavity and placed into cold sterile HBSS.

Adult and fetal lungs were homogenized in 0.26M sucrose containing 1mM phenylmethylsulfonylfluoride (PMSF), a protease inhibitor, and centrifuged at 2200xg for 10 minutes to precipitate nuclei. Supernate was centrifuged at 10,000xg for 15 minutes to collect mitochondria. This supernate was centrifuged at 100,000xg for 60 minutes. The pellet was resuspended in 0.26M sucrose as the microsomal fraction and supernate was the cytosolic fraction. Protein was quantified as described below and adjusted to a final concentration of 5mg/ml.

4.2 G) Protein quantification

Protein assays were performed using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Mississauga, ON) which is based on the method of Bradford (Bradford, 1976). Sample protein binds to Commassie blue dye producing a color change whose intensity may be compared spectrophotometrically (595nm) to a known concentration of a bovine serum albumin standard.

4.2 H) Enzyme assays

CTP:phosphocholine cytidylyltransferase (CPCT) has been identified as the rate-limiting step in the *de novo* synthesis of phosphatidylcholine. Consequently, agents which alter PC synthesis may affect CPCT activity. CPCT was assayed using the method of Chan et al (1983) based on the conversion of [^{14}C]cholinephosphate to [^{14}C]CDP-choline. 100-150ug of protein was incubated with $Mg(Ac)_2$ (100mM), CTP (50mM), Tris-HCl (1M, pH 6.5), and phosphocholine (3mM, 2 Ci/mole with 0.1mM EGTA). Since PG has been shown to be an activator of CPCT (Zimmerman, 1995), 10^{-4}M THC was added to the reaction mixture alone or in combination with 100ug of PG. Each replicate was made up to a final volume of 100ul with ddH_2O. Reaction was initiated by addition of enzyme. Incubation periods of 10, 20, 30, 45, 60 and 90 minutes were used and samples were incubated at 37°C in a shaking water bath. The reaction was terminated by placing the samples in boiling water for 5 minutes.

Samples were centrifuged at 12,800xg for 5 minutes and 25ul was plated onto dry thin layer chromatography plates (Whatman, LK5D, Fischer Scientific,

Edmonton, AB) along with 30ul of a 5mg/ml CDP-choline standard. Plates were allowed to dry and subsequently developed in a solvent system of ethanol:water:ammonia (100:50:2) until the solvent front reached the top of plate. After drying, plates were exposed to iodine vapour. Spots representing authentic CDP-choline were scrapped into scintillation vials and 200ul of water and 5ml of Ready Protein Scintillation cocktail were added. Radioactivity was determined on a scintillation counter (model LS 5800, Beckman). Quench was determined using H# (Beckman Instant, Palo Alto, Ca.).

4.2 I) Synthesis of surfactant by freshly isolated type II cells

In order to determine if THC affects the synthesis of surfactant-related material, fetal rabbit pre-type II cells were isolated as described above. As Rice et al. (1997) were unable to detect cannabinoid receptors in cultured alveolar type II cells, experiments were performed on freshly isolated cells. Samples consisting of $2.0x10^5$ cells were incubated in MEM with 10% sNCS (v/v) in an atmosphere of 5% CO_2 at 37°C with 3uCi/ml of [^{3}H]choline and 10^{-7} to 10^{-4}M THC for various time periods. Cells were collected by centrifugation at 300xg for 10 minutes and supernate discarded. DSPC was isolated from total phospholipids as described above.

4.2 J) Secretion of surfactant DSPC from isolated fetal type II cells

To determine if THC induces release of DSPC from fetal type II cells, isolated rabbit pre-type II cells were pre-labeled with [^{3}H]-choline for 24 hours. Pre-labeling medium was removed and cells collected by trypsinization (10ml of trypsin/EDTA) and centrifugation (250xg for 10 minutes). Cell pellet was resuspended in a known volume of MEM containing 10% sNCS (v/v) and cell number determined with a Coulter cell counter. Samples consisting of $2x10^5$ cells were incubated for 3 hours with THC (10^{-7}M - 10^{-4}M) or TPA (10^{-5}M) as a positive control at 5% CO_2 and 37°C. After centrifugation at 300xg for 10 minutes, media were collected and DSPC isolated from total phospholipid extract as described below.

4.2 K) Release of surfactant DSPC from freshly isolated fetal type II cells

Experiments to determine effects of THC on secretion of surfactant-related material by freshly isolated type II cells were next employed. Fetal pre-type II cells were isolated from 24 gestational day rabbit as previously described. Isolated cells were immediately assayed. Cell suspensions were pre-labeled with 3uCi/ml [^{3}H]choline for 24 hours at 37°C and 5% CO_2 with gentle stirring. Samples consisting of $2x10^5$ cells were exposed to 10^{-5}M TPA (positive control) and 10^{-4}M THC over 1, 3 and 5 hours in an atmosphere of 37°C and 5% CO_2. Samples were centrifuged at 1400rpm to pellet cellular debris. Medium was removed and 1ml of chloroform/methanol (1/2) was added. DSPC was isolated from medium as previously described.

4.2 L) Statistical analysis

Where appropriate, statistical analysis was made by either post hoc application of Duncans' New Multiple Range Test (Ott, 1977) or through analysis of co-variance. A significant difference was determined to be $p<0.05$.

RESULTS

To examine the effects of THC on the incorporation of [^{3}H]choline into DSPC by cultured fetal rabbit type II alveolar cells, cultures were grown to confluence over 6 days. Cells were exposed to [^{3}H]choline (1uCi/ml or 2uCi/ml to final concentrations of 12.5uCi/mole or 25uCi/mole) in the presence of 10^{-7}-10^{-4}M THC over 24 hours. Radiolabeled DSPC in cellular and medium components was determined. THC (10^{-5}-10^{-4}M) significantly ($p<0.05$) reduced [^{3}H]choline incorporation into cellular DSPC (figure 3), suggesting the synthesis of this phospholipid is reduced upon exposure to THC. In contrast, radiolabel associated with DSPC released into the medium displayed a significant increase ($p<0.05$) in samples exposed to 10^{-4}M THC as compared to the corresponding control value.

Since THC appeared to inhibit the synthesis of DSPC in isolated type II cells, we examined the effects of THC on the accumulation of radiolabel in intermediates of the *de novo* synthesis pathway of DSPC. Results are shown in figures 4-8. Radioactivity associated with choline in isolated fetal rabbit type II cells following exposure to 10^{-4}M THC is shown in figure 4. Radioactive choline levels were significantly higher ($p<0.05$) in cultures exposed to THC over 30 minutes, 1 and 3 hours compared to the corresponding control values not exposed to THC. However, significantly lower ($p<0.05$) levels of [^{3}H]choline were observed in cell cultures exposed to THC after 3 hours compared to corresponding control values. The accumulation of [^{3}H]choline in phosphocholine in isolated fetal rabbit type II cells is shown in figure 5. 10^{-4}M THC induced a significant

decrease ($p<0.05$) in levels of phosphocholine present in cultured fetal rabbit type II cells at 30 minutes, 20 and 48 hours. In cell cultures exposed to THC, radioactivity in CDP-choline was significantly greater ($p<0.05$) at all time points as compared to control cultures (figure 6). The accumulation of [^{3}H]choline in PC in cultured fetal type II rabbit cells is shown in figure 7. There was a significant increase ($p<0.05$) in radioactivity associated with PC isolated from cells exposed to THC for 30 minutes, 1, 9 and 20 hours compared to untreated cells. Similarly, THC induced a significant increase ($p<0.05$) in radiolabel in DSPC after exposure to the cells for 3, 9 or 20 hours compared to the control values (figure 8).

Release of cellular lactate dehydrogenase (LDH) is generally considered to be an indicator of cellular damage in cultured cells (Scott, 1994). To determine whether 10^{-4}M THC induced release of LDH, medium was collected from cultures exposed to THC for 9 or 48 hours and LDH activity was determined. No significant changes in LDH activity occurred among control values over 48 hours (figure 9). A significant decrease ($p<0.05$) in activity was observed in medium collected from cell cultures exposed to THC for 9 hours compared to the corresponding control value (figure 9). No significant changes between experimental and control cultures was observed after 48 hours exposure to THC.

FIGURE 3: Accumulation of radiolabeled DSPC in cultured fetal rabbit type II cells and release of [^{3}H]DSPC by these cells following exposure to 10^{-7}-10^{-4}M THC over 24 hours. Cultured fetal rabbit type II cells were grown to confluence over a period of 6 days. Cells were exposed to 1uCi/ml [^{3}H]choline and 10^{-7}-10^{-4}M THC for a period of 24 hours. Medium was removed from cultures and saved. Cells were removed by trypsinization. DSPC was isolated from both fractions as described in Materials and Methods. Results are expressed as the radioactivity per flask (mean ± SEM) for a minimum of five replicates. * indicates significant differences ($p<0.05$) from the corresponding control value as determined by Duncans' New Multiple Range Test.

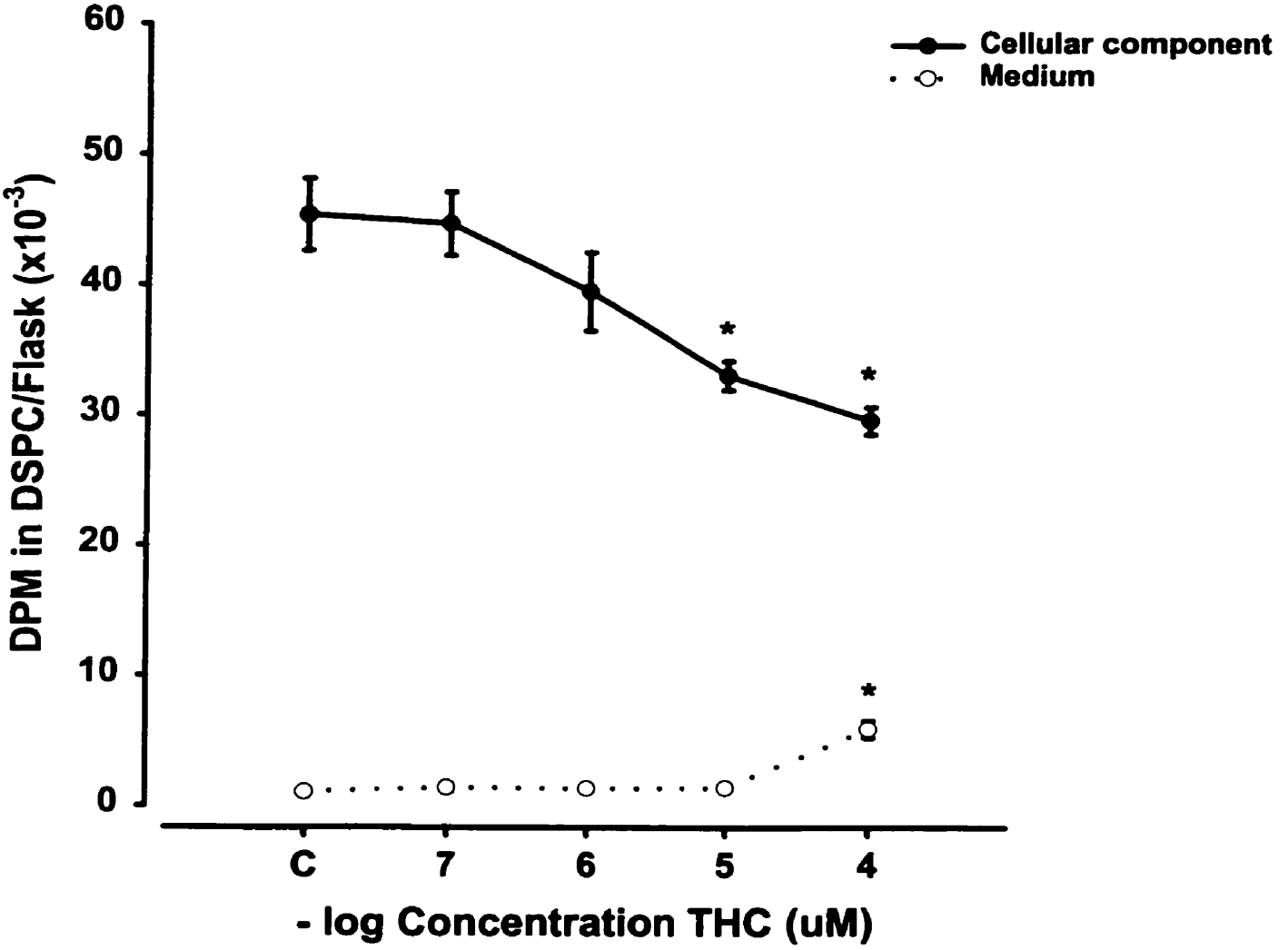

60
50
40
30
20
10
0
DPM in DSPC/Flask (x10-3)
Cellular component
Medium
*
*
*
C
7
6
5
4
- log Concentration THC (uM)

FIGURE 4: Radioactivity associated with choline in isolated fetal rabbit type II cells following exposure to 10^{-4}M THC for 30 minutes, 1, 3, 9, 20 and 48 hours. Cell cultures were grown to confluence over a period of 5 days. Cells were pre-labeled with [^{3}H]choline (2uCi/ml) for 1 hour. Samples were exposed to 10^{-4}M THC in MEM and uptake of radiolabeled choline by the cells determined. Control samples were not exposed to THC. Results are expressed as the radioactivity per flask (mean ± SEM) for a minimum of five replicates. * indicates significant differences ($p<0.05$) from the corresponding control values from cell cultures not exposed to THC determined by Duncans' New Multiple Range Test.

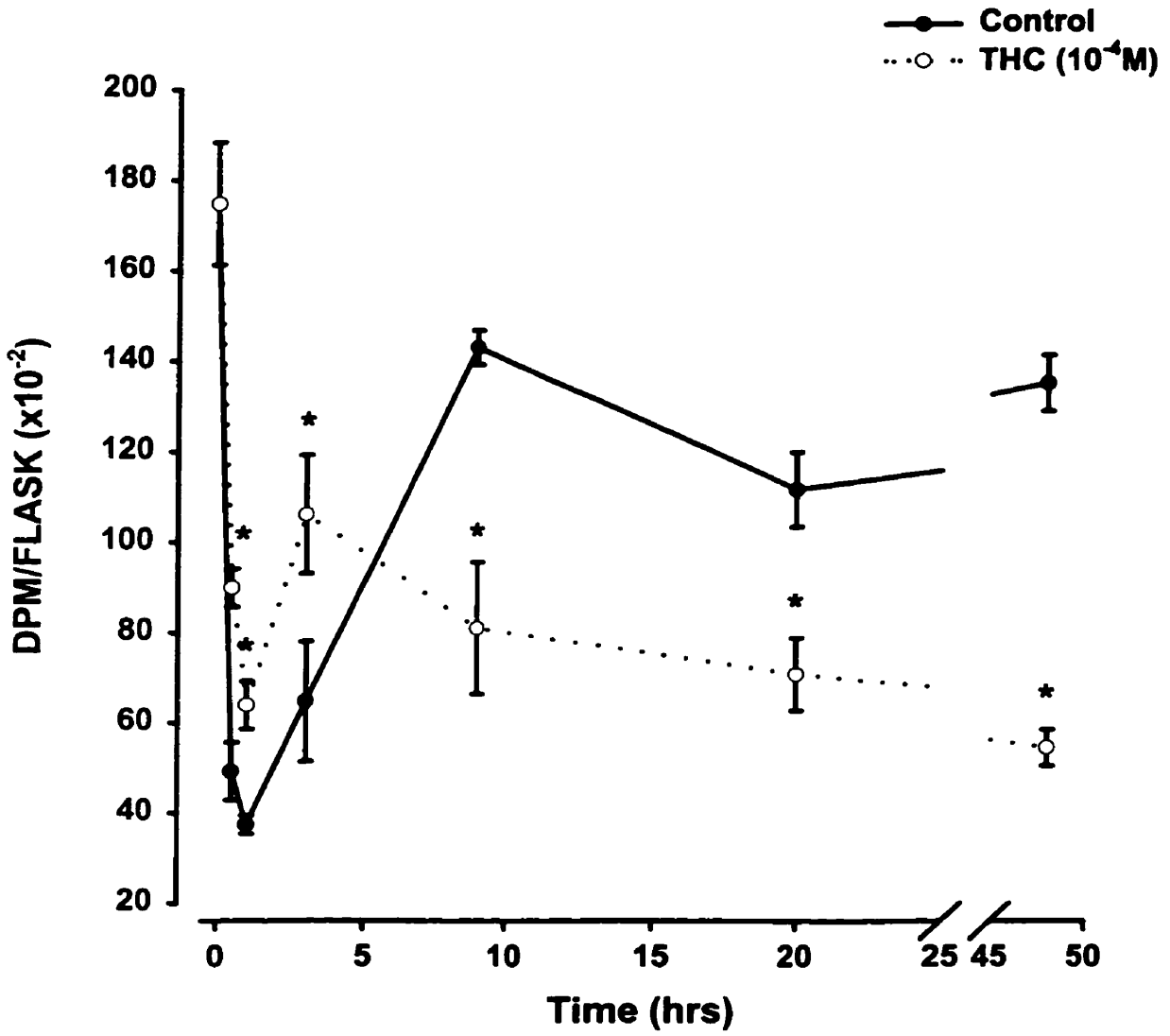

Control
THC (10^{-4}M)
DPM/FLASK ($x10^{-2}$)
200
180
160
140
120
100
80
60
40
20
0
5
10
15
20
25
45
50
Time (hrs)

FIGURE 5: Accumulation of [^{3}H]choline in phosphocholine in isolated fetal rabbit type II cells following exposure to 10^{-4}M THC for 30 minutes, 1, 3, 9, 20 and 48 hours. Cultured fetal rabbit type II cells were pre-labeled with [^{3}H]choline for 1 hour. Samples were exposed to 10^{-4}M THC in MEM and intracellular [^{3}H]phosphocholine isolated. Results are expressed as the radioactivity per flask (mean ± SEM) for a minimum of five replicates. * indicates significant differences ($p<0.05$) from the corresponding control value obtained for cells not exposed to THC as determined by Duncans' New Multiple Range Test.

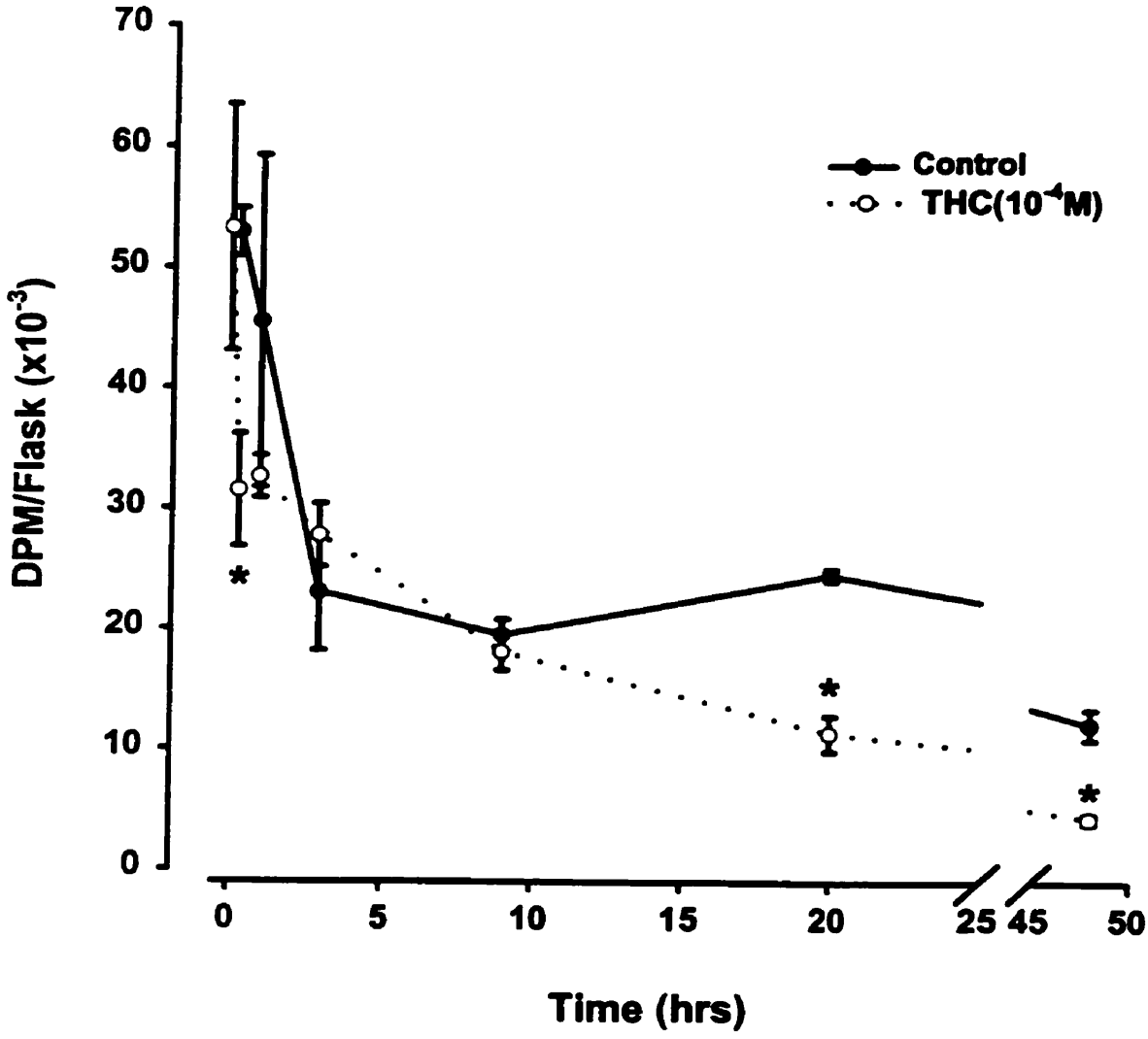

70
60
50
40
30
20
10
0
DPM/Flask (x10^{-3})
Control
THC(10^{-4}M)
*
*
*
0
5
10
15
20
25
45
50
Time (hrs)

FIGURE 6: Accumulation of [^{3}H]choline in CDP-choline in isolated fetal rabbit type II cells following exposure to 10^{-4}M THC for 30 minutes, 1, 3, 9, 20 and 48 hours. Cultures were pre-labeled with [^{3}H]choline for 1 hour. Cells were exposed to 10^{-4}M THC in MEM and radiolabeled CDP-choline isolated. Results are expressed as the radioactivity per flask (mean ± SEM) for a minimum of five replicates. * indicates significant differences ($p<0.05$) from the corresponding control values from cells not exposed to THC as determined by Duncans' New Multiple Range test.

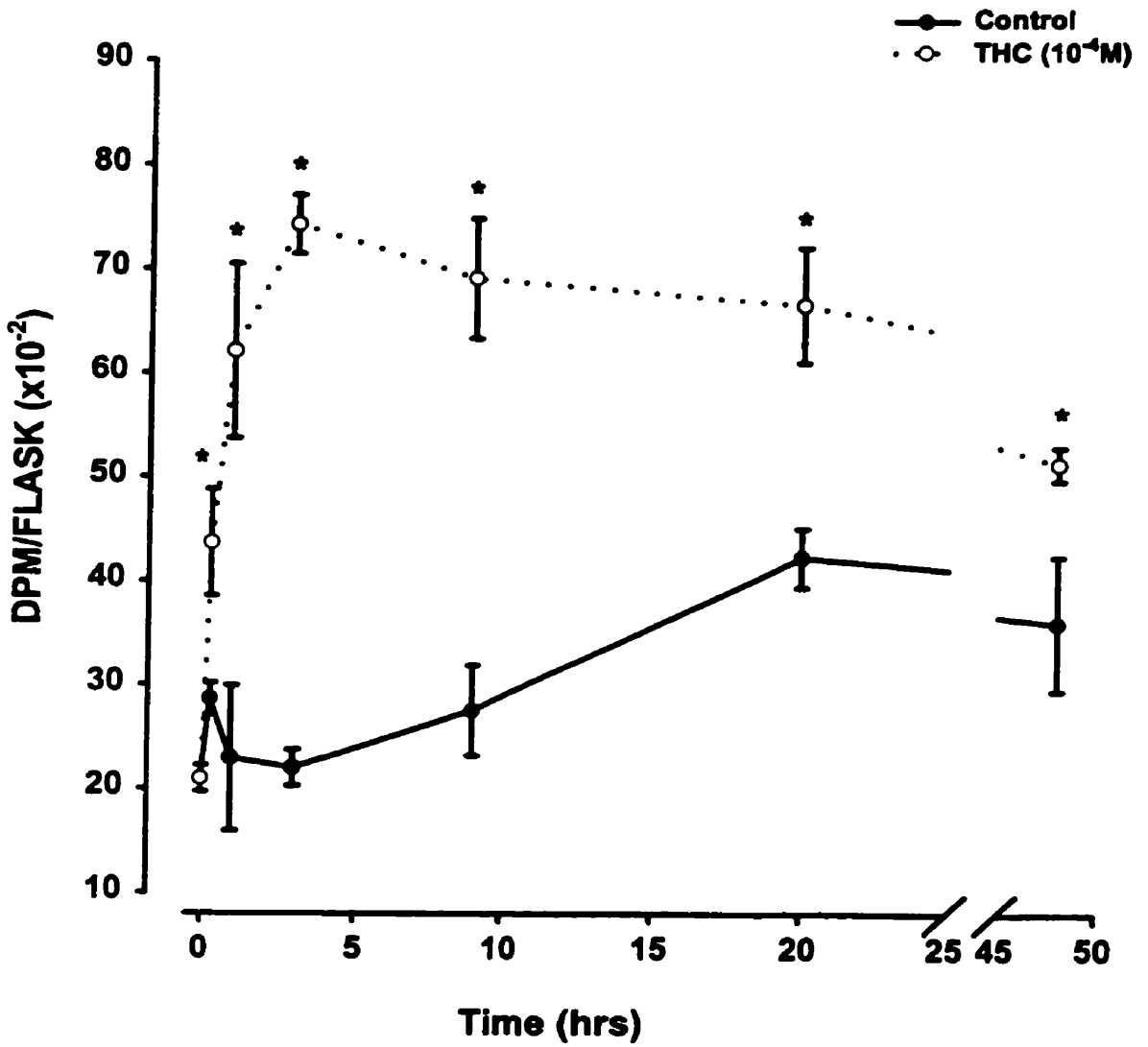

Control
THC (10^{-4}M)
DPM/FLASK ($x10^{-2}$)
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
45
50
Time (hrs)

FIGURE 7: Accumulation of [^{3}H]choline in PC in isolated fetal rabbit type II cells following exposure to 10^{-4}M THC for 30 minutes, 1, 3, 9, 20 and 48 hours. Cultures were pre-labeled with [^{3}H]choline for 1 hour. Cells were exposed to 10^{-4}M THC in MEM and radiolabeled PC isolated. Results are expressed as the radioactivity per flask (mean ± SEM) for a minimum of five replicates. * indicates significant differences ($p<0.05$) from the corresponding control values from cells not exposed to THC as determined by Duncans' New Multiple Range test.

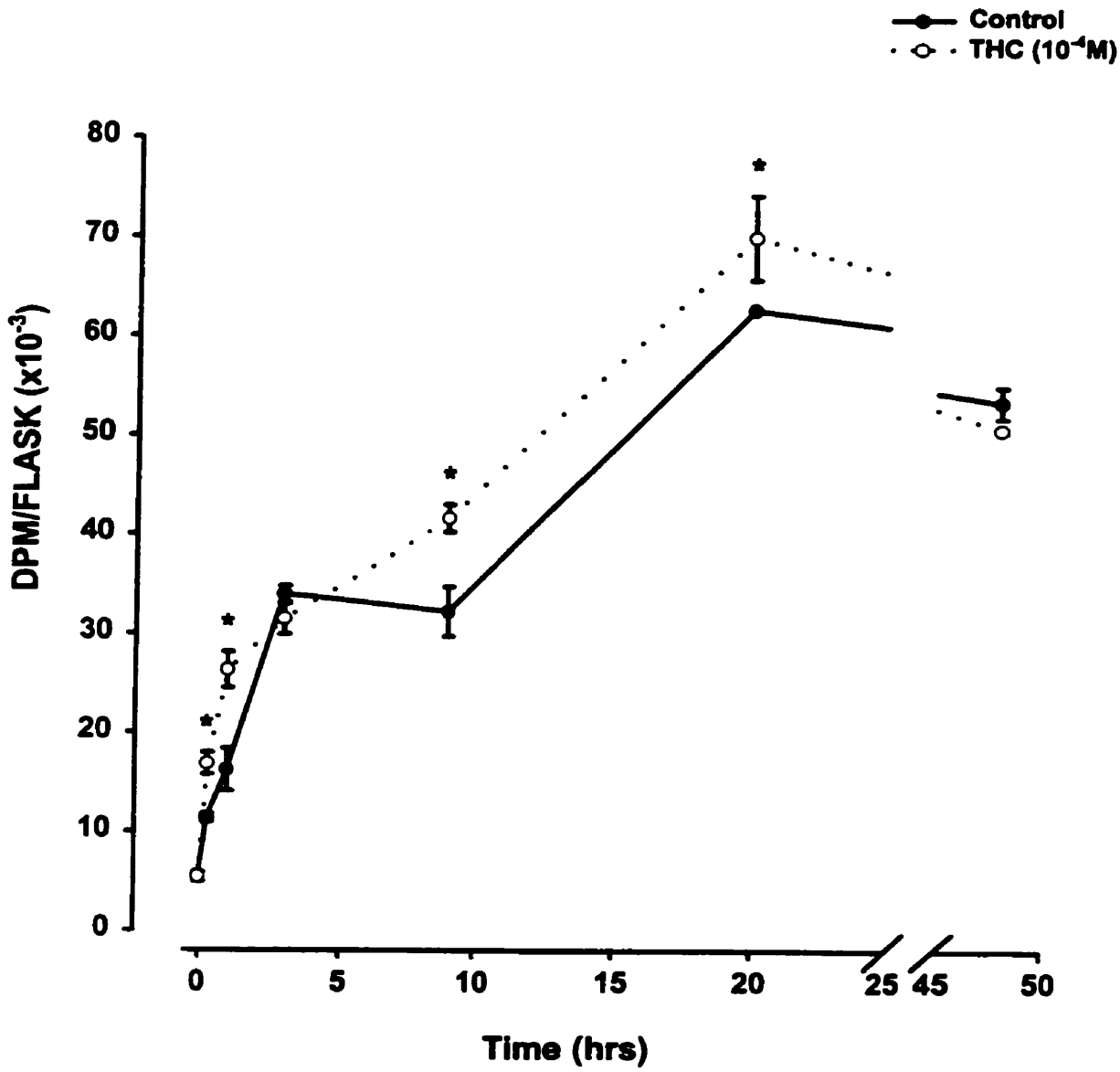

Control
THC (10^{-4}M)
DPM/FLASK ($x10^{-3}$)
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
45
50
Time (hrs)

FIGURE 8: Accumulation of [^{3}H]choline in DSPC in isolated fetal rabbit type II cells following exposure to 10^{-4}M THC for 30 minutes, 1, 3, 9, 20 and 48 hours. Cultures were pre-labeled with [^{3}H]choline for 1 hour. Cells were exposed to 10^{-4}M THC in MEM and radiolabeled DSPC isolated. Results are expressed as the radioactivity per flask (mean ± SEM) for a minimum of five replicates. * indicates significant differences ($p<0.05$) from the corresponding control values from cells not exposed to THC as determined by Duncans' New Multiple Range test.

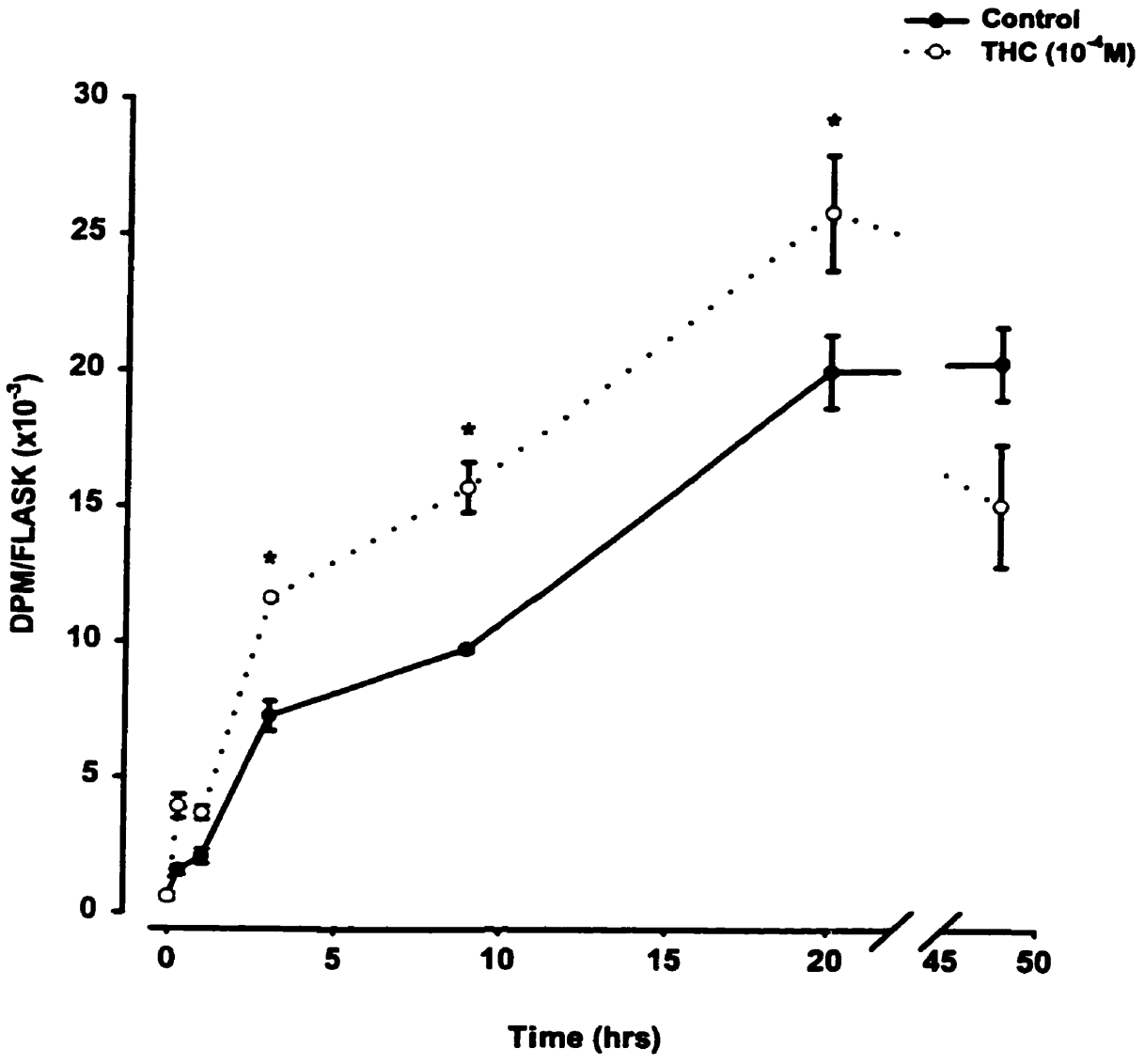
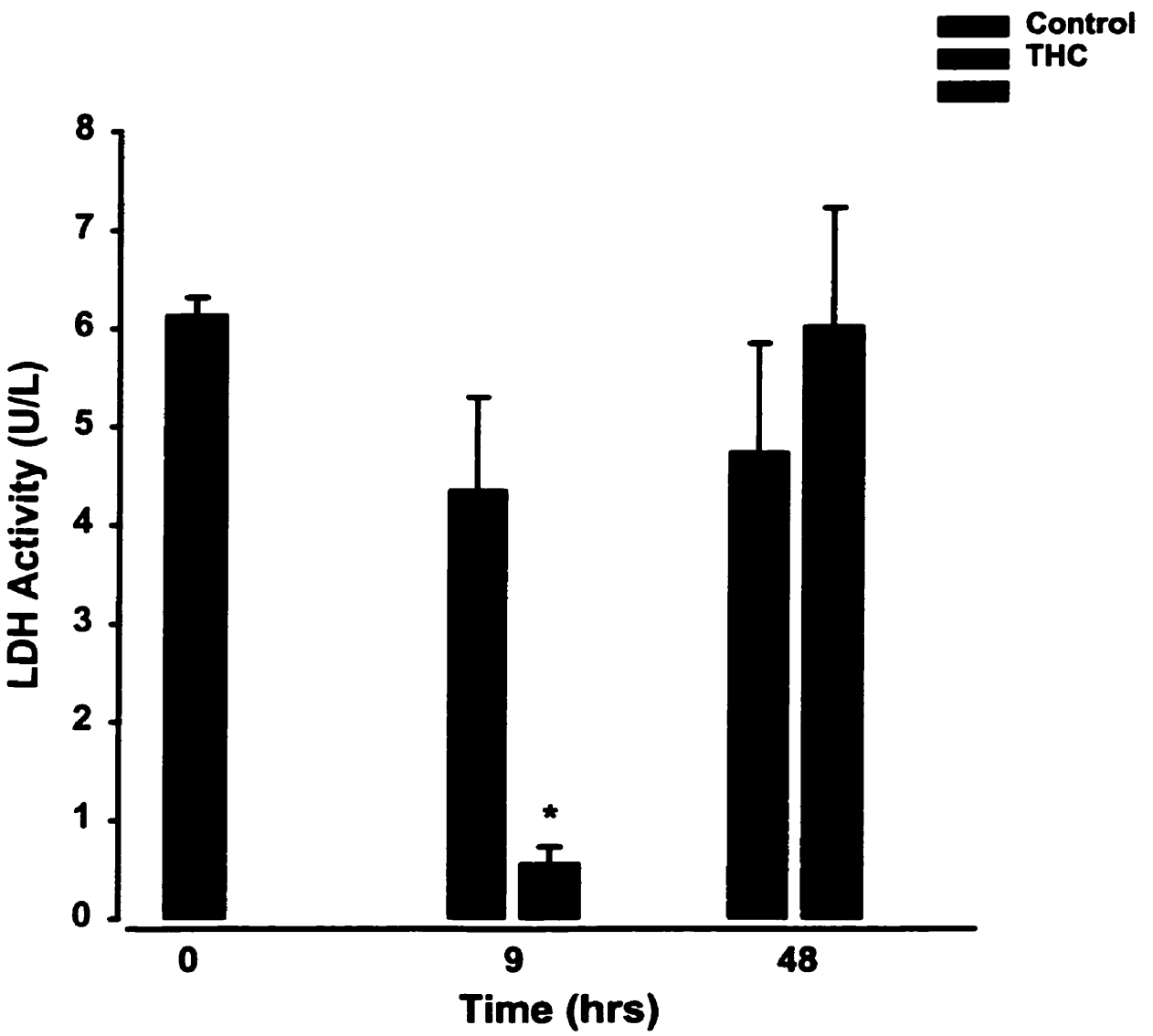

Control
THC (10^{-4}M)
DPM/FLASK ($x10^{-3}$)
30
25
20
15
10
5
0
0
5
10
15
20
45
50
Time (hrs)

FIGURE 9: Lactate dehydrogenase activity (U/L) in culture medium collected from control cell cultures or cultures exposed to 10^{-4}M THC over a period of 48 hours. Cell cultures were grown to confluence over 5 days. Cells were exposed to 10^{-4}M THC in MEM over 48 hours. Medium was collected from 0, 9 and 48 hour cell cultures. Lactate dehydrogenase activity was determined as described in Materials and Methods. Results are expressed as the mean ± SEM for a minimum of five replicates. * indicates significant differences ($p<0.05$) compared to the corresponding control value.


Control
THC
LDH Activity (U/L)
8
7
6
5
4
3
2
1
0
*
0
9
48
Time (hrs)

From our pulse-chase experiments, it is evident that while radiolabeled phosphocholine levels decrease radiolabeled CDP-choline, PC and DSPC levels increase upon cellular exposure to 10^{-4}M THC. Thus, an examination of the affects of THC on CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme in DSPC synthesis, was pursued. CPCT catalyzes the conversion of phosphocholine to CDP-choline in the presence of cytidine trisphosphate. If THC increases the activity of CPCT, the changes we observed in the levels of phosphocholine, CDP-choline, PC and DSPC may be accounted for. To determine if THC affects CPCT activity in adult lung, cytosolic fractions were incubated with 10^{-4}M THC, 100ug PG (a known activator of the enzyme) or a combination of PG and 10^{-4}M THC for 90 minutes. Conversion of radiolabeled phosphocholine into CDP-choline was measured. CPCT activity was constant over this time period (figure 10). No differences were detected between control and PG or THC exposed samples (figure 10).

It has previously been shown in fetal lung that the majority of CPCT activity is located within the cytosol of type II cells, although some activity is also found associated with membranes (Stern, 1976). To determine if THC affects enzyme activity within cytosolic fractions prepared from 24th gestational day fetal lung, samples were assayed in the presence or absence of THC over 90 minutes. Analysis of covariance revealed a significant increase (slope: $F=5.777(1,36)$; y intercept: $F=21.605(1,36)$) ($p<0.05$) in the rate of [^{14}C]CDP-choline formation in the presence of THC in fetal lung. Consistent with our previous findings, no

significant changes in CPCT activity were detected in cytosolic fractions of adult rabbit lung (slope: F=0.062(1,36); y intercept: F=15.56(1,36)) (figure 11). CPCT specific activity in cytosolic and membrane fractions from fetal rabbit lungs exposed to PG, 10^{-4}M THC or PG and 10^{-4}M THC is shown in figure 12. In these experiments, addition of PG to the enzyme assay mixture served as a positive control, as fetal CPCT activity has been shown to undergo activation with addition of this lipid (Zimmerman, 1995). As expected, PG produced a 25% increase in CPCT specific activity in cytosol. A similar increase was observed in samples exposed to PG in combination with 10^{-4}M THC, while THC alone stimulated a 45% increase in CPCT specific activity. Interestingly, CPCT specific activity associated with microsomal fractions was 25% greater ($p<0.05$) than that of cytosolic fractions. No significant changes in enzyme activity were observed in microsomal fractions exposed to PG, THC or a combination of the two.

Total CPCT activity in cytosolic and membrane fractions of fetal rabbit lungs in the presence of PG, 10^{-4}M THC or PG and 10^{-4}M THC is shown in figure 13. CPCT total activity significantly increased ($p<0.05$) in all cytosolic fractions incubated with PG, THC and PG with THC compared to the corresponding control values. No significant changes in CPCT total activity were detected in fetal microsomal fractions. Fetal cytosolic control fractions had approximately three times the amount of CPCT total activity compared to the corresponding untreated microsomal fractions.

FIGURE 10: Rate of [^{14}C]CDP-choline formation in whole adult lung cytosolic fractions. Adult lung was homogenized in 0.26M sucrose and centrifuged. Supernate from the 100,000xg centrifugation was taken as the cytosolic fraction (see Methods). CPCT activity was determined at 0, 10, 20, 30, 45, 60 and 90 minutes in the presence of 3mM [^{14}C]phosphocholine. Samples contained PG (100ug), 10^{-4}M THC or a combination of PG and THC. Results are representative of four independent experiments and are expressed as the rate of CDP-choline formation per mg of cytosolic protein (mean ± SEM) for a minimum of three replicates.

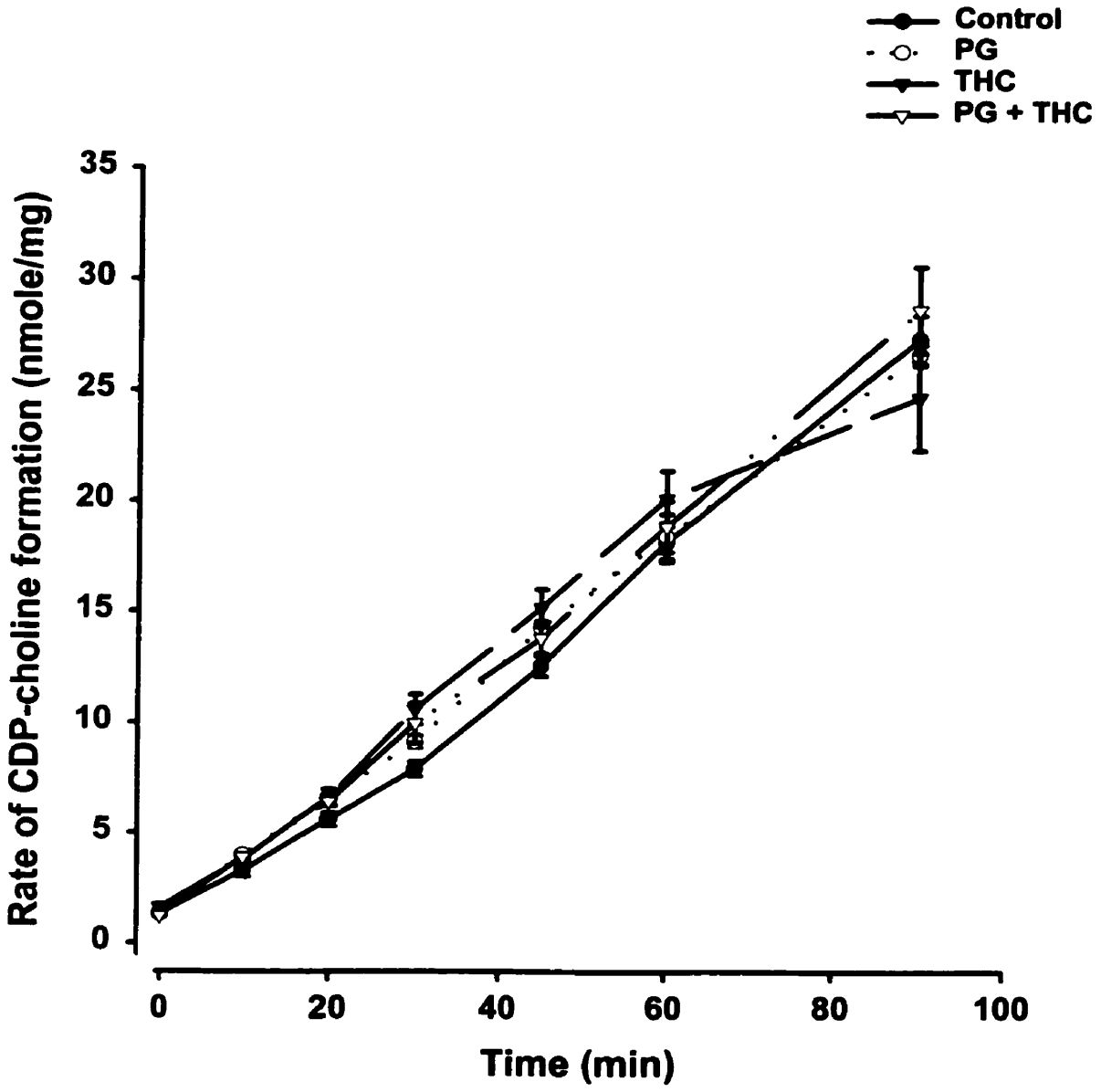

Control
PG
THC
PG + THC
Rate of CDP-choline formation (nmole/mg)
35
30
25
20
15
10
5
0
0
20
40
60
80
100
Time (min)

FIGURE 11: Rate of [^{14}C]CDP-choline formation in fetal and adult lung rabbit cytosols. Whole fetal (24 day of gestation) and adult rabbit lungs were collected, homogenized in 0.26M sucrose and centrifuged. Supernate from the 100,000xg centrifugation was used as the cytosolic fraction (see Methods). CPCT activity was determined at 0, 10, 20, 30, 45, 60 and 90 minutes in the presence or absence of 10^{-4}M THC. Results are expressed as the nmoles of CDP-choline formed per mg of cytosolic protein. Analysis of co-variance was used to identify differences in activity.

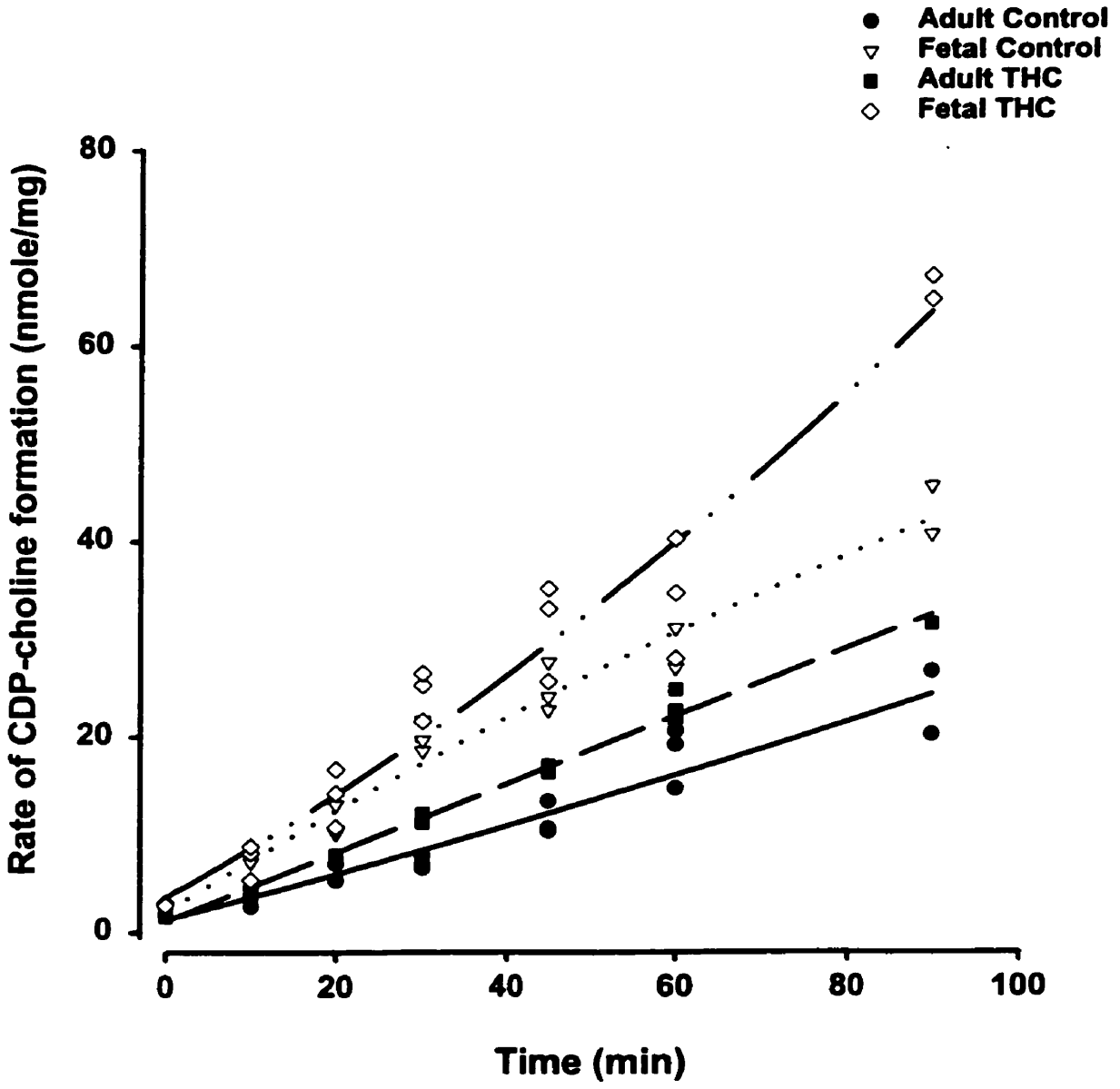

Adult Control
Fetal Control
Adult THC
Fetal THC
Rate of CDP-choline formation (nmole/mg)
80
60
40
20
0
0
20
40
60
80
100
Time (min)

FIGURE 12: Specific activity of CPCT in cytosolic and membranous fractions obtained from fetal rabbit lungs of 24 gestational days in the presence of PG (100ug), 10^{-4}M THC or a combination of the two. Fetal rabbit lungs were collected, homogenized and centrifuged. Supernate and pellet from the 100,000xg centrifugation were used as cytosolic and membranous fractions, respectively. Results are expressed as the rate of CDP-choline formed per mg of protein per minute (mean ± SEM) for a minimum of three replicates. * indicates significant differences ($p<0.05$) from the corresponding control values as determined by Duncans' New Multiple Range Test.

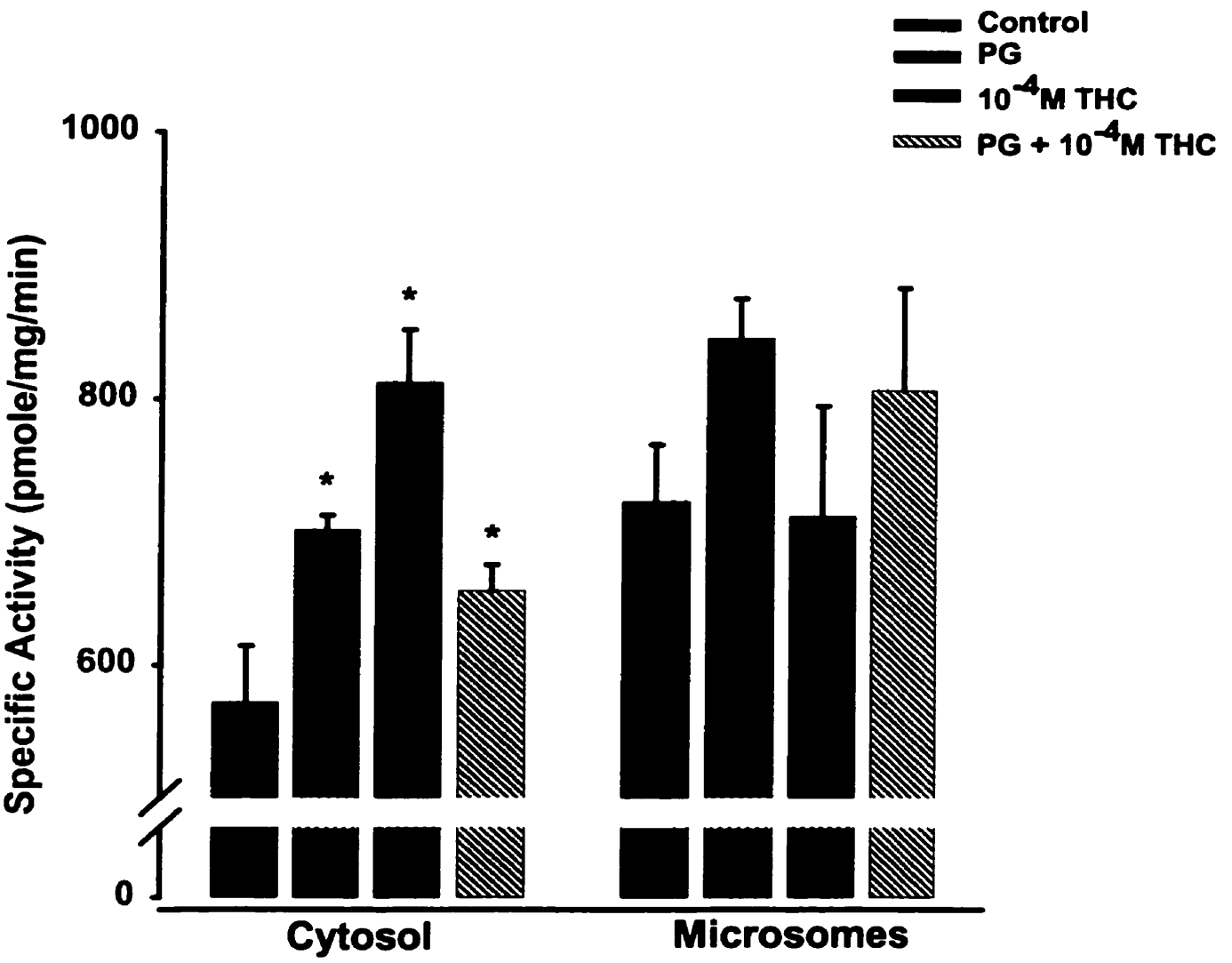
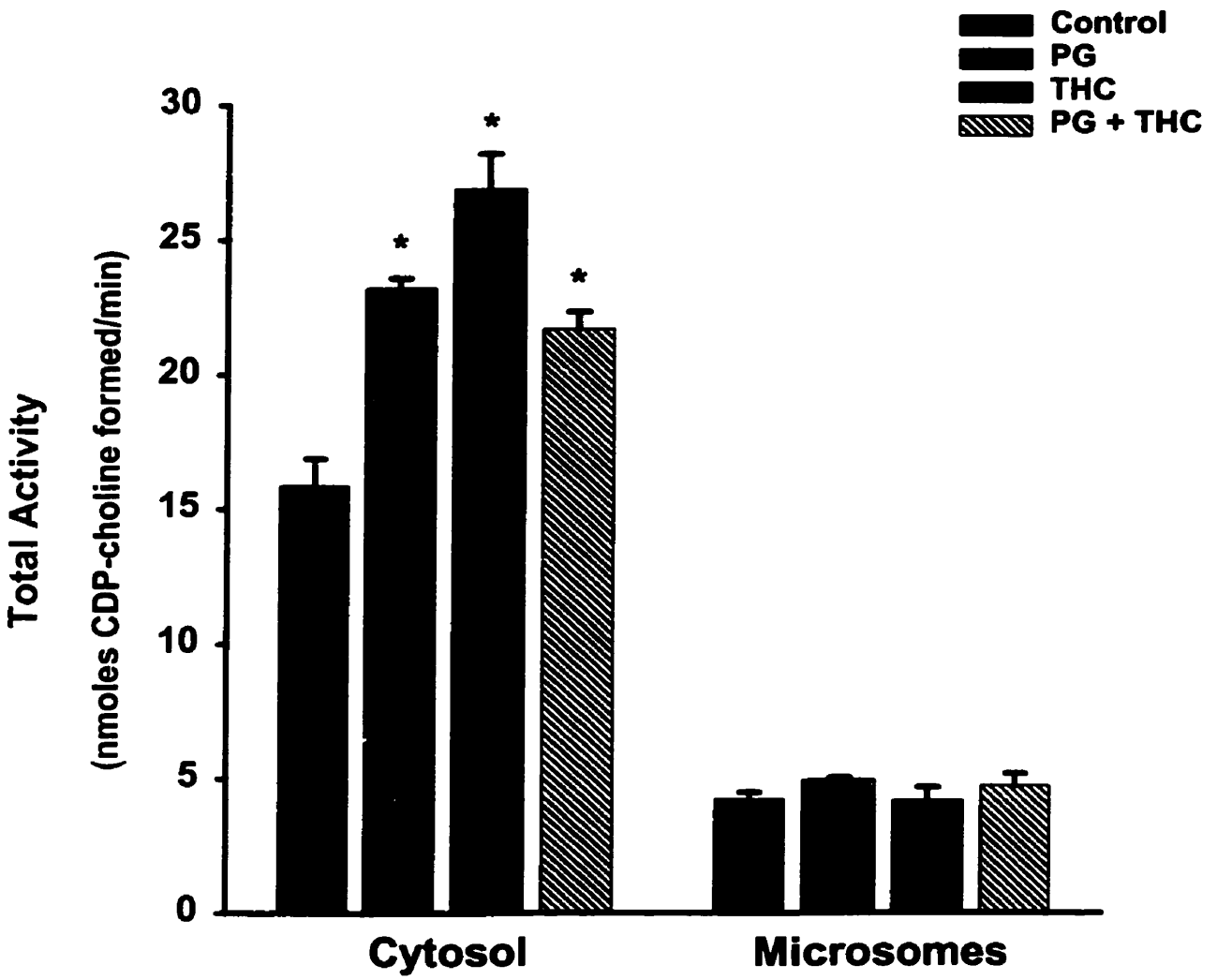

Control
PG
10-4M THC
PG + 10-4M THC
1000
800
600
0
Specific Activity (pmole/mg/min)
*
*
*
Cytosol
Microsomes

FIGURE 13: Total CPCT activity in cytosolic and membrane fractions from fetal rabbit lungs in the presence of PG (100ug), 10^{-4}M THC or a combination of PG and THC. Results are expressed as the rate of CDP-choline formed per minute (mean ± SEM) for a minimum of three replicates. * indicates significant differences ($p<0.05$) determined by Duncans' New Multiple Range Test.


Control
PG
THC
PG + THC
Total Activity
(nmoles CDP-choline formed/min)
30
25
20
15
10
5
0
*
*
*
Cytosol
Microsomes

During the course of these studies, Rice et al. (1997) demonstrated that CB_1 mRNA was present in freshly isolated fetal and adult type II cells but was lost in culture. While THC may also have a non-receptor based mechanism of action, we thought it was necessary in light of the new findings to examine THC effects in freshly isolated cells. Fetal rabbit pre-type II cells were isolated from gestational day 24 rabbits and used immediately. Cells were exposed to 10^{-7}-10^{-4}M THC in the presence of [^{3}H]choline over various time periods. Figure 14 demonstrates the intracellular accumulation of [^{3}H]DSPC. When exposed to THC over 1 and 3 hours, no significant changes were observed in intracellular [^{3}H]DSPC. However, exposure for 8 or 18 hours resulted in a significant decrease ($p<0.05$) in the accumulation of radiolabeled DSPC at 10^{-6}-10^{-4}M THC concentrations. These results contrast with our observations from cultured pulse-labeled type II cells, in which significant increases in cellular [^{3}H]DSPC was detected.

To study the effects of THC on release of surfactant-related DSPC from isolated fetal rabbit type II cells, cell cultures were grown to confluence over a period of 7 days. Cultures were pre-labeled with [^{3}H]choline for 24 hours, incubated with TPA (a known stimulator of DSPC release in type II cells) or THC and the medium collected. Figure 15 shows the radioactivity in DSPC released by these cells following exposure to THC (10^{-7}-10^{-4}M) or TPA (10^{-5}M) over a period of 3 hours. TPA induced a significant increase ($p<0.05$) of almost 30% in [^{3}H]DSPC release by fetal type II cells compared to the corresponding control

values. Exposure to THC (10^{-6} and 10^{-4}M) also induced a significant ($p<0.05$) release of [^{3}H]DSPC by these cells.

In order to determine whether the effects of THC on release of surfactant-related material by fetal type II cells involves cannabinoid receptors, a similar experiment to that above was performed on freshly isolated type II cells. Freshly isolated rabbit type II cells were pre-labeled with [^{3}H]choline for 24 hours. Samples were exposed to 10^{-5}M TPA or 10^{-4}M THC over 1, 3 or 5 hours. Radiolabel associated with DSPC in the culture medium was determined. TPA induced a significant release ($p<0.05$) of [^{3}H]DSPC over 3 and 5 hours, while THC stimulated release ($p<0.05$) of this material from type II cells over 1, 3 and 5 hours (figure 16).

FIGURE 14: Accumulation of radiolabeled DSPC in freshly isolated fetal rabbit type II cells following exposure to 10^{-7}-10^{-4}M THC over 1, 3, 8 and 18 hours. Fetal rabbit pre-type II cells were isolated as described in Materials and Methods. Cells were incubated immediately with radioactive precursor plus varying THC concentrations over 1, 3, 8 and 18 hours. Samples were centrifuged, medium removed and DSPC isolated from the cell pellet. Results are expressed as the radioactivity per replicate (mean ± SEM) for a minimum of four replicates. * indicates significant differences ($p<0.05$) as compared to the corresponding control values from samples not exposed to THC as determined by Duncans' New Multiple Range Test.

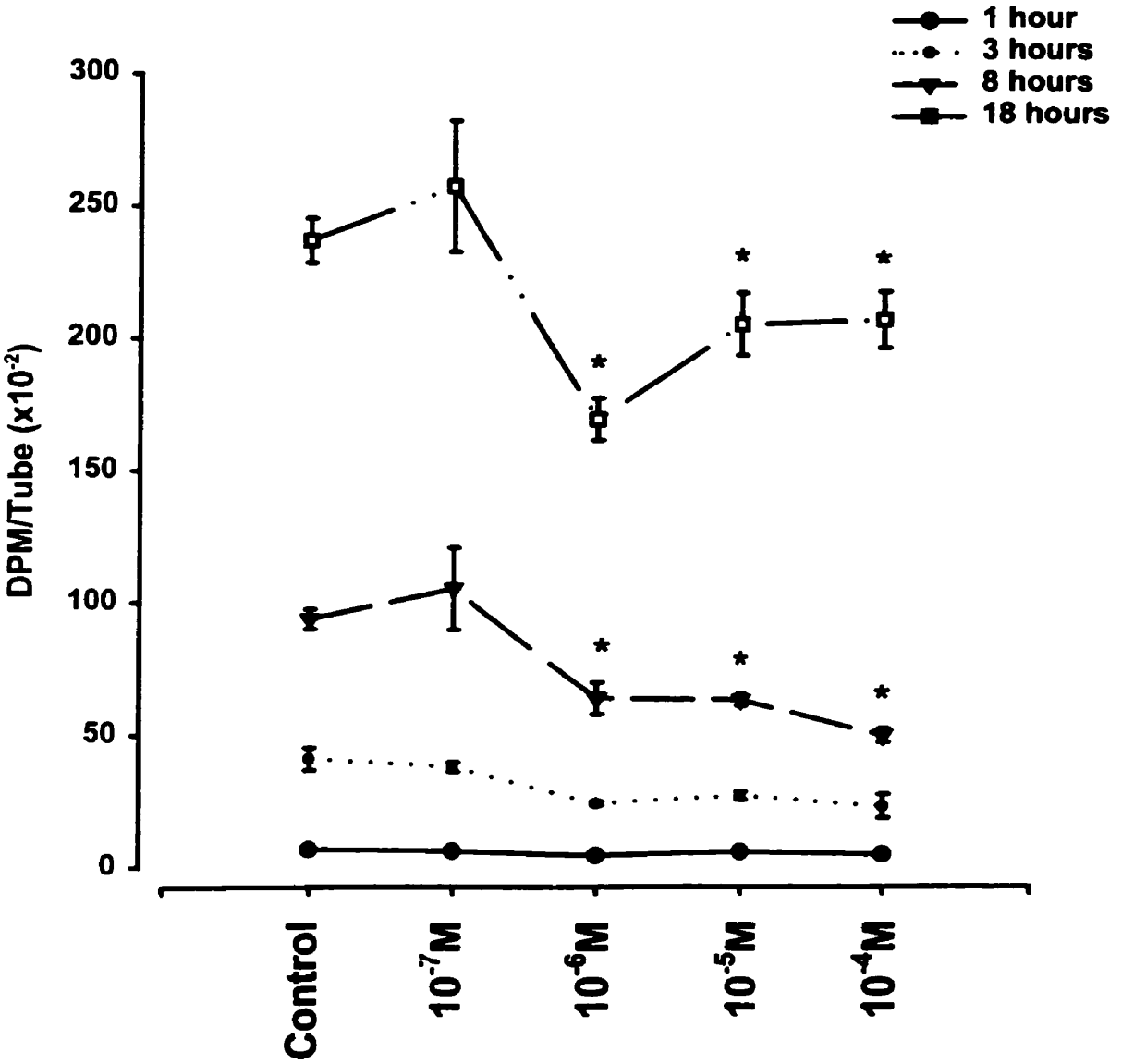

1 hour
3 hours
8 hours
18 hours
DPM/Tube (x10-2)
300
250
200
150
100
50
0
Control
10-7M
10-6M
10-5M
10-4M
*

FIGURE 15: Release of radiolabeled DSPC by cultured fetal rabbit type II cells following exposure to 10^{-5}M TPA or 10^{-7}-10^{-4}M THC over 3 hours. Fetal rabbit type II cells were grown to confluence over a period of 7 days. Cells were pre-labeled with 1uCi/ml [^{3}H]choline in MEM, exposed to TPA or THC and medium collected. DSPC was isolated. Results are representative of two independent experiments and are expressed as the radioactivity per replicate (mean ± SEM) for a minimum of four replicates. * indicates significant differences ($p<0.05$) from the corresponding control values determined by Duncans' New Multiple Range test.

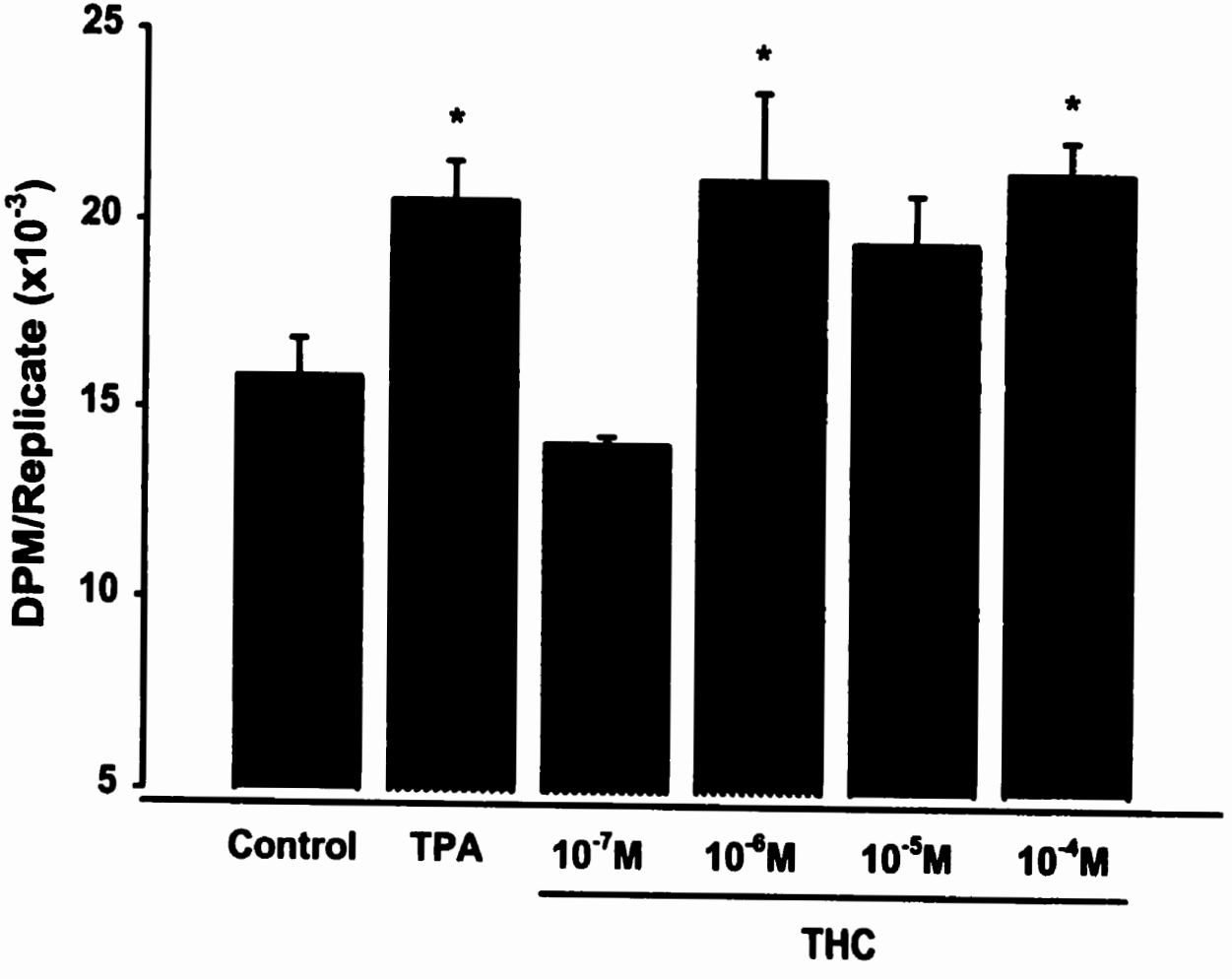
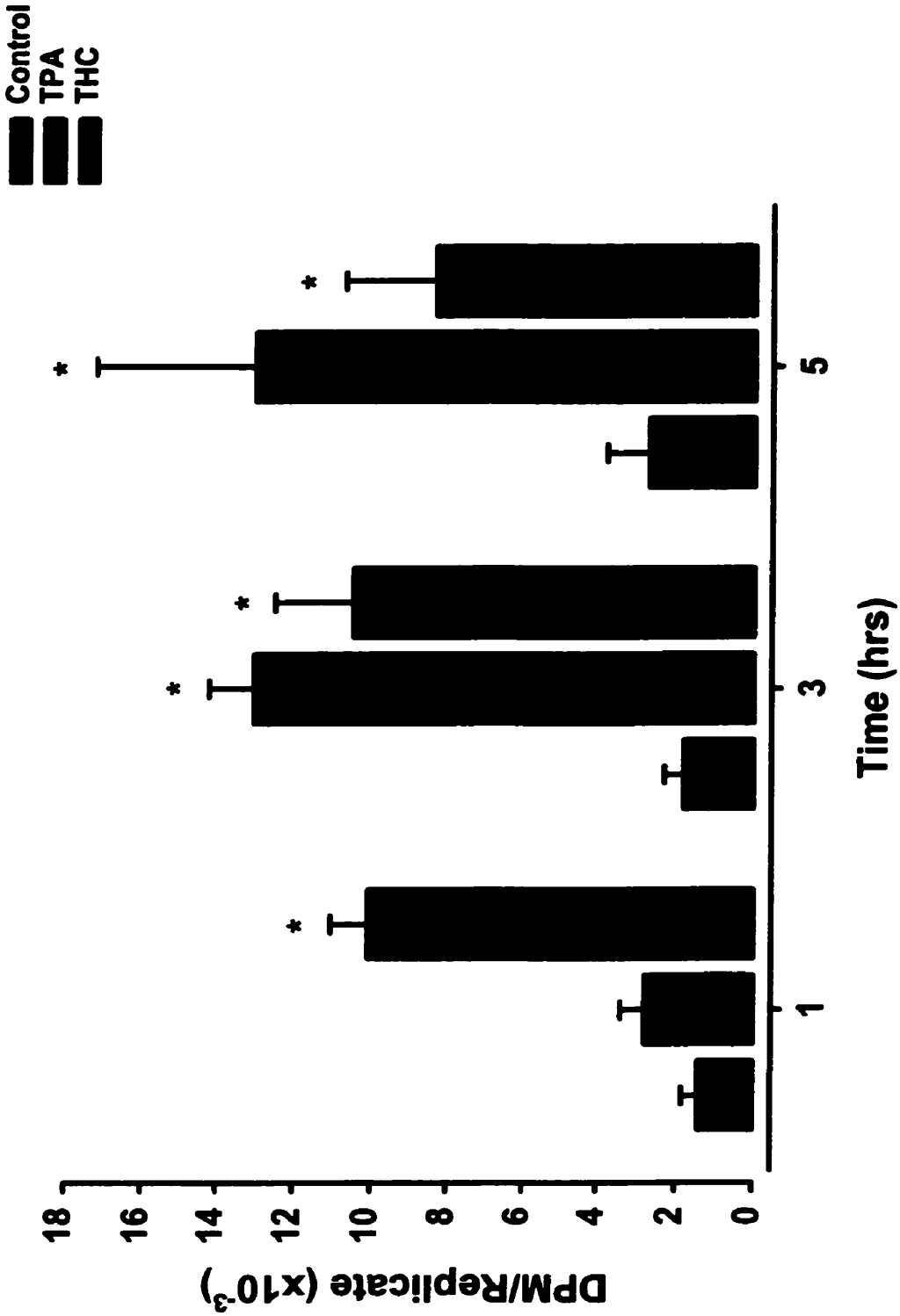

25
20
15
10
5
DPM/Replicate (x10-3)
*
*
*
Control
TPA
10-7M
10-6M
10-5M
10-4M
THC

FIGURE 16: Release of radiolabeled DSPC from freshly isolated fetal rabbit type II cells following exposure to 10^{-5}M TPA or 10^{-4}M THC for 1, 3 and 5 hours. Type II cells were isolated from fetal lungs as described in Materials and Methods. Cells were pre-labeled for 24 hours with 3uCi/ml [^{3}H]choline. Samples consisting of $2x10^5$ cells were incubated with TPA or THC over various times. Samples were centrifuged at 1400rpm and medium removed. DSPC was isolated from medium as described in Materials and Methods. Results are expressed as the radioactivity per replicate (mean ± SEM) for a minimum of four replicates. * indicates significant differences ($p<0.05$) from the corresponding control values determined by Duncans' New Multiple Range Test.


Control
TPA
THC
DPM/Replicate (x10-3)
0
2
4
6
8
10
12
14
16
18
1
3
5
Time (hrs)
*
*
*
*
*

DISCUSSION

Marijuana is one of the most abused illicit drugs in society (Hutchings, 1991). Smoked marijuana is known to be associated with impairment of attention, short-term memory loss as well as impairment of cognitive and psychomotor performance. However, little research has focused on potential risks of marijuana use on the respiratory system. Studies have suggested that regular marijuana smoking induces alveolar cell hyperplasia, bronchiolitis and fibrosis of distal respiratory airways (Fligiel, 1991). Marijuana smoke, like tobacco, has been shown to increase the risk of cancer in the upper respiratory airways (Nahas, 1992). Recently, Sherman et al. (1995) have suggested that exposure to marijuana is associated with DNA damage in alveolar macrophages. Effects of marijuana use on adult and fetal type II cell function, in particular the production and secretion of surfactant, have not been addressed. Of particular concern to investigators today are short-term and long-term effects of marijuana use. In the human population, direct or indirect effects of marijuana use alone are often difficult to determine since users are frequently multiple drug-dependent. This dependence often involves drugs such as cocaine, heroine or alcohol, all of which have well documented effects on certain organs or cell functions (Knight, 1994).

Surfactant, a phospholipid-rich material synthesized by type II cells, functions to stabilize the lungs at low surface tension. Surfactant phospholipid synthesis by human fetal lung begins during the 30th week of gestation and increases throughout gestation (Possmayer, 1985). Premature infants born with

insufficient surfactant levels potentially develop respiratory distress, which is characterized by increased lung liquid content, decreased lung compliance and eventual alveolar collapse. Normal development of the lung involves a complex series of multiple steps involving mesenchymal-epithelial interactions, hormone production and enzyme activation. Any drug or compound that affects any one of these steps may alter normal fetal lung development and thereby jeopardize the readiness of the lung to meet the challenge of breathing in an air environment. Marijuana smoke inhalation results in rapid distribution of its most psychoactive agent, Δ^9-tetrahydrocannabinol (THC), throughout the body. THC is highly lipophilic and in the pregnant woman can cross the placenta readily (Szeto, 1993). Consequently, THC can enter the fetal bloodstream thereby reaching the developing fetal lung. Furthermore, THC has a biological half-life of 8 days in fat (Nahas, 1992) and it takes 30 days to completely eliminate a single dose. With such a large maternal pool of THC in conjunction with a slow elimination rate, potential exists for prolonged fetal exposure in regular marijuana users. With the recent identification of CB_1 receptor mRNA in type II cells along with evidence that lipid soluble agents affect normal fetal development following maternal exposure, the type II cell may represent a potential target for THC interactions. The current work addresses possible effects of fetal THC exposure on surfactant synthesis and secretion in isolated fetal type II cells.

Pulmonary surfactant is essential for proper lung functioning at birth. The principal component of surfactant is DSPC and it is the lipid-packing qualities of

this phospholipid that allows surfactant, upon compression, to achieve very low surface tension (King, 1984). Our initial experiments with cultured fetal rabbit type II cells suggested that exposure to THC (10^{-5}-10^{-4}M) over 24 hours resulted in a decrease of [^{3}H]choline incorporation into DSPC. Studies using freshly isolated type II cells indicated time and dose-dependent decreases in intracellular DSPC that were of the same magnitude as those observed in cultured cells. In 1997, Rice et al. observed CB_1 receptor mRNA in isolated fetal rat type II cells. However, they were unable to detect receptor mRNA in cells cultured longer than 24 hours. This suggests that the CB_1 receptor may not directly affect the synthesis of surfactant phospholipid in our current model system, as cell cultures were grown to confluence over a period of 6 days. Thus, the decrease in intracellular DSPC in both cultured and freshly isolated type II cells implies that THC affects surfactant phospholipid synthesis through a mechanism not related to the CB_1 receptor.

In contrast to those studies above, pulse-chase experiments consistently showed increased intracellular levels of [^{3}H]DSPC in cell cultures exposed to THC. Although the reason for the discrepancy between these results and the previous results is not clear, it may be due to different methods that were employed in each of the experiments. In our initial experiments with cultured cells and those with freshly isolated type II cells, [^{3}H]choline and THC (10^{-7}-10^{-4}M) were added together prior to isolation of intracellular [^{3}H]DSPC. However, in the pulse-chase studies, cell cultures (grown to confluence over 7 days) were initially pre-labeled with choline for 1 hour and exposed to THC over 30 minutes,

1, 3, 9, 20 or 48 hours. Intracellular incorporation of [^{3}H]choline into DSPC was then determined. Thus, it appears that THC, when introduced to cell cultures in conjunction with [^{3}H]choline, may affect the cellular uptake of this DSPC precursor. Uptake of choline into lung epithelium appears to be mediated by two mechanisms: passive diffusion and active transport (Fisher, 1992). Passive diffusion appears to play only a minor role in choline uptake by type II cells as intracellular choline levels may be as much as 8 times greater than extracellular levels (Fisher, 1992). Results by Fisher et al. (1989) suggest that the majority of choline is internalized by an active transport mechanism. Active uptake of choline in type II cells is energy dependent and operates in both a Na^+-dependent and –independent manner (Fisher, 1992). As choline is an important precursor in DSPC synthesis, decreases in [^{3}H]choline uptake from medium in the presence of THC would be reflected in decreased intracellular levels available for synthesis of [^{3}H]DSPC. THC has been shown to produce membrane perturbations thereby affecting membrane-associated proteins (Tahir, 1991). These disruptions may account for the inhibition of active uptake of choline by fetal type II cells. Since cannabinoids have been shown to alter cellular uptake and distribution of macromolecular precursors (Mon, 1981), this possibility does seem likely. However, cannabinoids have also been shown to inhibit ATPase activity in several tissues (Martin, 1986). By inhibiting ATPases, the energy dependence of active choline uptake may be jeopardized. In either case, availability of choline may be rate-limiting for DSPC synthesis in those samples exposed concurrently to THC and the radioactive precursor. Decreases in intracellular [^{3}H]choline levels

would be reflected in decreased synthesis of [^{3}H]DSPC. Further investigations into the effects of THC on choline uptake by isolated fetal type II cells are required to elucidate the discrepancy between these results. Nevertheless, increases or decreases in DSPC synthesis in developing fetal type II cells would have severe repercussions related to the synthesis of surfactant, thereby posing life-threatening conditions to the neonate.

Within type II cells, newly synthesized PC is in one of two forms based on the configuration of fatty acids esterified to the glycerol backbone. When cholinephosphotransferase incorporates CDP-choline with dipalmitoylglycerol, DSPC is produced directly (Ide, 1982). However, when DAG is not in the disaturated state, 1-saturated-2-unsaturated PC is formed. A deacylation-reacylation process, catalyzed by phospholipase A_2 (PLA_2) and lysoPC acyltransferase, replaces the unsaturated fatty acid with a saturated species (Post, 1983). Recently, it has been suggested that cannabinoids and THC increase the activity of some phospholipases (Evans, 1987; Audette, 1991). Evans et al. (1987) have shown that cannabinoids incubated with PLA_2 in a cell-free system induce a 50% increase in enzyme activity. As PLA_2 is responsible for converting PC into DSPC in type II cells, stimulation may result in increased synthesis of DSPC. Thus, increased accumulation of [^{3}H]choline in DSPC in our pulse-chase experiments may be a reflection of increases in PLA_2 enzyme activity. Further studies of PLA_2 enzymatic activity are necessary to further examine this hypothesis.

PC is synthesized primarily by the *de novo* pathway in type II cells, which incorporates choline into PC through sequential formation of phosphocholine and CDP-choline. In order to elucidate the mechanism(s) through which THC increases the synthesis of PC and DSPC in fetal type II cell cultures previously labeled with [^{3}H]choline, accumulation of radiolabel within the intermediates of DSPC synthesis were determined. Results from these studies indicate that radiolabel associated with choline was reduced in cultures treated with THC compared to the corresponding control values. Similarly, phosphocholine levels decreased when fetal type II cultures were exposed to THC over 22 and 48 hours. In contrast, radiolabeled CDP-choline levels increased by as much as 40% in cell cultures exposed to THC. Increased levels of PC with corresponding increases in DSPC were also observed. Conversion of phosphocholine to CDP-choline is catalyzed by CPCT (E.C. 2.7.7.15). There is good evidence to suggest that this enzyme catalyzes the rate-limiting step in PC biosynthesis (Zimmerman, 1993). From our pulse-chase studies, equilibrium in phosphocholine-CDP-choline conversion appears to be shifted in the forward direction upon cellular exposure to 10^{-4}M THC. Increases in CPCT specific activity in type II cells by THC may account for this shift in equilibrium.

CPCT exists as both a low-molecular weight (LMW) (inactive) and a high molecular weight (HMW) (active) form. Evidence indicates that the LMW form is predominantly located within cytosols of type II cells whereas the HMW form is associated with membranes (Stern, 1976). The inactive form of the enzyme may be activated by addition of anionic phospholipids, particularly PG (Stern, 1976;

Zimmerman, 1995). Within adult lung cytosols, CPCT predominantly exists in the HMW state and is therefore unable to be further activated by addition of lipids (Feldman, 1978; Mallampalli, 1993). Results from our experiments involving adult rabbit lung cytosols are consistent with those of Mallampalli and colleagues. Addition of PG or 10^{-4}M THC to cell fractions did not change the rate of CDP-choline formation, indicating that CPCT is already in its active state and may not be further stimulated.

In fetal and neonatal lung, Chan et al. (1982) observed high CPCT specific activity in microsomal fractions. However, greater proportions of total lung enzyme activity were associated with high-speed supernates which represent the cytosolic fraction (Chan, 1982). Our results are consistent with those of Chan et al. CPCT specific activity was 26% greater in microsomal than cytosolic fractions obtained from fetal gestational day 24 rabbit lung. Total CPCT activity was observed to be three times greater in cytosolic than corresponding membrane fractions. With increasing gestational age, CPCT activity has been shown to shift from cytosol to microsomal membranes (Zimmerman, 1993) suggesting translocation of the enzyme from cytosol to endoplasmic reticulum. In type II cells this phenomenon produces not only increases in CPCT activity, but also increases in the rate of PC synthesis. Thus, one would assume that fetal lung cytosol acts as an intracellular reservoir of inactivated enzyme which, under appropriate conditions, could translocate to membrane compartments. However, increases in CPCT activity late in gestation are thought to be mediated not only by activation, but also by increased mRNA levels (Zimmerman, 1995). Further

studies in isolated fetal type II cells, following the movement of CPCT from the cytosol to microsomal membranes upon exposure to THC, would provide convincing evidence to support these observations. Experiments utilizing receptor autoradiography techniques are currently in progress to detect CPCT translocation upon cellular exposure to THC.

In fetal lung, CPCT exists predominantly as the LMW form in cytosolic fractions. Conversion from the LMW to the HMW form is dependent upon the addition of phospholipids (Chan, 1982; Mallampalli, 1993). Chan et al. (1982) demonstrated in rabbit fetal lung (27 gestation day) that addition of lyso-PE to isolated lung cytosols results in an 8-fold increase in CPCT specific activity. However, addition of phospholipids to microsomal fractions had little effect on enzyme activity. Similarly, our results from whole fetal rabbit lung fractions (24 days gestation) suggested that cytosolic, but not microsomal CPCT, may be stimulated by PG. PG appeared to increase CPCT specific activity by as much as 25% while microsomal CPCT could not be stimulated by addition of lipids. Although THC was unable to stimulate CPCT in fetal lung microsomes as the enzyme is already activated, cytosolic fractions displayed a 45% increase in enzyme specific activity when incubated with THC. Thus, THC appears to activate fetal cytosolic CPCT but not microsomal enzyme. Addition of THC in combination with PG to cytosolic fractions did not significantly increase enzyme activity beyond that achieved by THC or PG alone suggesting a similar mechanism of activation. The mechanism through which THC increases CPCT activity remains unknown. Although PG has been shown to maximally stimulate

CPCT, the mechanism in which this activation occurs remains unclear. Studies by Arnold et al. (1996) suggest that an initial electrostatic interaction between the anionic phospholipid and enzyme is required. This is followed by a hydrophobic insertion of the protein into the membrane (Arnold, 1996). Thus, interaction between phospholipid and CPCT converts the enzyme from the LMW to the HMW form and this is accompanied by translocation from cytosol to endoplasmic reticulum (Zimmerman, 1993). As THC is highly lipophilic the possibility that it interacts with the PG binding site appears unlikely, as initial binding requires hydrophilic interactions.

Although these results do not clearly indicate how THC affects CPCT activity in fetal type II cells, conclusions may still be drawn. Increased CPCT activity in cytosolic fractions from fetal rabbit lung clearly corresponds to alterations we observed in the pool sizes of choline, phosphocholine and CDP-choline in our pulse-chase studies. Activation of cytosolic CPCT by THC would result in a decreased intracellular pool of phosphocholine while concurrently increasing CDP-choline levels. With increased production of CDP-choline, increases in PC and DSPC over a 22-hour time period would be predicted.

During normal development, synthesis of PC in isolated type II cells increases during late gestation corresponding to increases in CPCT activity (Viscardi, 1994; Zimmerman, 1993). In developing rat lung, Casola et al. (1980) observed increases in CPCT activity that corresponds to increases in enzyme activities involved in PG synthesis. As PG synthesis is known to increase dramatically late in gestation, particularly 2-3 days prior to birth, coordinate

increases in CPCT activity suggest that production of surfactant PC may be regulated by PG. Increases in PG synthesis have also been shown to be accompanied by increased mRNA levels of CPCT. Studies reflecting the effects of THC on CPCT activity and thus PC synthesis throughout various stages of development should be done as fetal exposure to marijuana at different developmental stages may affect normal lung maturation differently.

Release of surfactant-related material from type II cells may be stimulated by a number of different agonists which function through several different receptors (Rice, 1985; Sano, 1987; Scott, 1994; Chander, 1995). Activation of PKC after $P2Y_2$ receptor activation results in a release of surfactant phospholipids in fetal type II cells (Scott, 1994). Although isozymes of PKC have been identified in type II cells (Linke, 1997), fetal levels appear to be lower than those of the adult (Gobran, 1998). Differences in PKC levels between fetal and adult tissues are thought to be responsible for developmental delays in the response of type II cells to surfactant secretagogues (Gobran 1998). Nonetheless, treatment of fetal rabbit day 24 type II cells with the potent PKC activator TPA stimulates surfactant secretion by these cells (Scott, 1994; Samuels, 1999). Collectively, these results suggest an important role for PKC or one of its isoforms in surfactant secretion.

In rat forebrain, THC has been shown to increase the phosphorylating activity of PKC (Hillard, 1994). This enzyme activation is thought to be specific for Ca^{2+}-PS-dependent isoforms of PKC, as THC does not affect enzyme activity in the absence of these cofactors (Hillard, 1994). Furthermore, the increase in

PKC activity appears to be mediated through nonspecific interactions between THC and membrane lipids rather than receptors. Our studies with cultured fetal rabbit type II cells indicate that release of surfactant phospholipid increases upon cellular exposure to THC. Activation of PKC isoenzymes by THC could account for increased surfactant secretion by these cells. Once activated, PKC probably phosphorylates actin, thereby leading to surfactant release. The possibility that these effects are mediated by receptors seems unlikely as CB_1 receptor mRNA is lost in type II cells after several days in culture (Rice, 1997).

In our cultured fetal type II cell model system for examining surfactant phospholipid secretion, release of cellular LDH is considered to be an indicator of cellular damage. As a result, release of phospholipid may occur concurrently with cellular damage, suggesting that the response is simply one of cellular toxicity and damage and not an indicator of surfactant phospholipid release. The fact that THC exposure of cultured type II cells over 9 and 48 hours was not accompanied by a significant increase in LDH activity, is suggestive of a system which is tolerant to 10^{-4}M THC concentrations. Thus, increased release of surfactant DSPC reflects a true response by type II cells in terms of surfactant secretion.

Microtubules and microfilaments also play a prominent role in surfactant secretion. Intracellular movement of lamellar bodies to the cell surface for exocytosis is thought to be mediated by alterations in the cytoskeleton (Tsilibary, 1983). As microtubules and microfilaments have been shown to be disrupted by THC concentrations of 10-30uM (Tahir, 1992), surfactant secretion may inherently be affected. As our studies have focused on maximal THC

concentrations of 10^{-4}M, increased concentrations of THC may disrupt the cytoskeleton of type II cells, thereby inhibiting intracellular movement of lamellar bodies. If this were true, then stimulation of surfactant secretion by several different secretagogues in the presence of THC would also be inhibited.

In order to characterize the effects of THC on secretion of surfactant by isolated type II cells release of DSPC from freshly isolated cells was determined. Our results indicated that THC induces release of surfactant DSPC from freshly isolated type II cells. As this response was similar to that of cultured fetal type II cells, activation of PKC by THC may play an important role in surfactant release by both cultured and freshly isolated cells. However, the fact that secretion may also be induced through cannabinoid receptor activation should not be overlooked.

Cannabinoid CB_1 receptors have been linked to several different cellular effects including inhibition of adenylate cyclase (Condie, 1996; Ameri, 1999) and inhibition of voltage-dependent calcium channels (Mackie, 1992). A strong indication that cannabinoid receptors are negatively coupled to adenylate cyclase through G-proteins arises from the observation that pertussis toxin, a compound which prevents G-protein dissociation, hinders the inhibitory effect of THC on adenylate cyclase activity (Howlett, 1986). In addition to PKC involvement in surfactant secretion, some evidence suggests adenylate cyclase activation may also induce secretion through G-proteins (Mescher, 1983). Stimulation of adenylate cyclase is thought to activate cAMP-dependent protein kinases which phosphorylate actin, leading to the release of surfactant. In type II cells,

activation of cannabinoid receptors would inhibit release of surfactant-related phospholipids by the inhibition of adenylate cyclase. As THC appears to stimulate release of surfactant DSPC from freshly isolated type II cells, the drug may not be acting through its receptor.

CONCLUSIONS

Today, a significant number of pregnant women report marijuana use throughout their pregnancy. THC, the most psychoactive component of marijuana, has been shown to cross the placenta and thereby potentially affect developing fetal tissues. Given that pulmonary surfactant is composed mostly of phospholipids and that THC is highly lipophilic, a strong possibility exists that THC affects normal fetal lung development. The results obtained from this study suggest that THC affects surfactant metabolism in isolated fetal type II cells. In cultured or freshly isolated cells, two different effects can be identified. The first occurs at the level of the cell membranes and depresses the availability of choline for surfactant synthesis. The second is an intracellular effect on the rate-limiting enzyme in PC synthesis. There it induces an increase in CPCT activity. In contrast, release of surfactant from type II cells is stimulated by THC. Rice et al. (1997) have identified cannabinoid receptor mRNA in isolated fetal rat type II cells. However, receptor mRNA was undetectable in cells cultured over 24 hours. Thus, in our current model system, cultured fetal type II cells should not display CB1 receptors on their cell surface whereas freshly isolated cells should display this receptor. In our surfactant metabolism studies, similar responses to THC exposure were observed from both cultured and freshly isolated type II cells. Thus, it does not appear that the effects of THC on the synthesis and secretion of surfactant from fetal type II cells involves the cannabinoid receptor. Rather, these effects appear to be mediated through non-specific membrane perturbations and

enzyme activation. The role of cannabinoid receptors within the lung, specifically in the surfactant-producing type II cells, remains to be determined.

FUTURE DIRECTIONS

The current study demonstrates that THC does in fact affect surfactant phospholipid metabolism in isolated type II cells. However, the mechanism(s) through which THC exerts these effects is not clear. THC has been shown to produce cellular effects not only through non-specific interactions mediated by its highly lipophilic nature, but also through recently identified cannabinoid receptors. Within type II cells, THC may be affecting active choline uptake, microtubule structure as well as enzymatic activity of PLA_2, PKC and adenylate cyclase. Within developing and mature type II cells, these factors mediate surfactant metabolism. Without them, insufficient or altered surfactant levels may result thereby potentially leading to respiratory distress. Until we understand how marijuana exposure affects this system, maternal marijuana use of this drug must be discouraged. We have only just begun to touch the tip of an enormous iceberg in terms of knowing how THC affects normal fetal lung development.

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