EFFECTS OF PLASMA MEMBRANE PERTURBATION ON THE ADENYLATE CYCLASE SYSTEM IN RAT LUNGS. COMPARISON WITH AND FURTHER CHARACTERIZATION OF THE SOLUBLE ADENYLATE CYCLASE ACTIVATOR PROTEINS

A Thesis

Presented to the Faculty of Graduate Studies,
University of Manitoba, in partial fulfillment
of the requirements for the degree of
Master of Science.

by

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ABSTRACT

Cyclic AMP is known to be important in the development, maturation, and maintenance of the lung, thus factors which regulate the cellular levels of cyclic AMP have a crucial role in lung metabolism. In the present study, plasma membrane perturbation with a variety of phospholipases and proteases was used to investigate the importance of the membrane structure to the regulation of the rat lung adenylate cyclase activity, the enzyme responsible for the generation of cAMP. This effect of membrane perturbation was compared with the effects of the cytosolic adenylate cyclase activator proteins on adenylate cyclase, 5'-nucleotidase and total ATPase activities. Treatment of plasma membranes with phospholipase A_2,C, and D; trypsin, chymotrypsin, and the adenylate cyclase activator proteins all resulted in concentration dependent increases in the basal adenylate cyclase activity, but to various degrees. Of the phospholipases, only phospholipase D showed a general perturbing effect by altering the activity of all three membrane enzymes, while phospholipase C treatment could slightly inhibit the ATPase activity. A phosphatidylinositol-specific phospholipase C had no effect on any of the membrane enzymes studied. Further characterization of the soluble adenylate cyclase activating proteins showed specificity for basal adenylate cyclase activity and suggested that the mechanism of action is not via a phospholipase-like membrane perturbation or nonspecific ATP-sparing effect , nor is the stimulation mediated through β-adrenergic, histaminic, or prostaglandin receptors. The results further suggest that phospholipase treatment of membranes may prove to be a valuable tool in studying the regulation of adenylate cyclase activity in the rat lung.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

A. INTRODUCTION

Neonatal respiratory distress syndrome is the leading cause of perinatal mortality and morbidity (1,2). In addition, a number of possible long term consequences of neonatal respiratory distress syndrome have been observed in survivors, most notably in very low birth weight infants. These sequelae include general abnormalities such as growth retardation; neurological disturbances such as mental retardation, learning disabilities, and neuromotor deficits; and prolonged respiratory dysfunction such as bronchopulmonary dysplasia or chronic, pulmonary insufficiency. The consequent abnormalities may be related to acute complications or be associated with the techniques employed in providing the intensive respiratory care (3).

The neonatal respiratory distress syndrome is associated with a deficiency of pulmonary surfactant phospholipids at birth, most notably in premature babies due to inadequate maturation of enzymes involved in phospholipid synthesis (4-7). The finding that various agonists can accelerate fetal lung maturation has made studies of the regulation of the concentrations of physiological agents which may have an important in vivo role in the maturation of fetal lung surfactant phospholipid synthesis of major importance. One such physiological agent, cyclic AMP, has been suggested to be involved in the neonatal development and maturation of the lung as phasic changes in the concentration of the β-adrenergic second messenger have been correlated with a prenatal mobilization of glycogen stores for phospholipid synthesis and a postnatal period of lung cellular differentiation (8).

Thus it would seem that factors which control the relative activities of the adenylate cyclase enzyme responsible for the synthesis of cyclic AMP and the cyclic AMP-phosphodiesterase enzyme which catalyzes the hydrolysis of cyclic AMP to AMP are important in lung development.

B. LITERATURE REVIEW

1. Lung morphology

The primary function of the lung is to provide a means of gas exchange in the living organism allowing the transfer of oxygen from the environment to the bloodstream and carbon dioxide from the blood to the environment (9). The high degree of efficiency of this gas exchange function is facilitated by the unique structural design of the lungs (10.11). The huge internal surface of the lung, [approximately 40-120 m^2,(12-14)], afforded by the branching of the airways into a dense myriad of alveoli in apposition with an extensive intermeshed vascular network separated by an approximately 1 µm thick barrier of two cell layers allows for near optimal gas exchange by diffusion.

The lung also has a number of nonventilatory functions, the majority of which are concerned with the defense and maintenance of the gas exchange apparatus (15). These include the synthesis and maintenance of the connective tissue matrix, the alveolar epithelium , and the bronchoalveolar lining materials. The pulmonary capillary endothelium is the site of the metabolism of a variety of vasoactive substances such as the uptake and degradation of 5-hydroxytryptamine and norepinephrine; the degradation of prostaglandins and adenine nucleotides; and the conversion of angiotensin I to the potent vasoconstrictor, angiotensin II (16). The lung seems to be well suited for this metabolic role as it is the only organ to receive the entire

cardiac output during each circulation. In addition, the lung may act as a blood filter by trapping and clearing particulate matter such as small emboli (17). Water exchange in the lung is also important to prevent the loss of fluid from the blood into the alveoli (18), and when venous return is decreased, the pulmonary blood volume may act as a reservoir of blood for the systemic circulation(18).

2. Lung composition and organization

The lung is comprised of three functional zones consisting of the conducting zone in which gas and blood are transported, the transitional zone which is the region of overlap between the conducting and respiratory zones, and the respiratory zone which is the site of gas exchange (19). The lung can be morphologically divided into nonparenchymal tissue consisting of the conducting airways and blood vessels, and the connective tissue structures such as interlobular septa, and the parenchymal tissue consisting of the alveoli, alveolar ducts and capillaries (9). Since the latter comprises the majority of the lung and is fundamental in the gas exchange process, the major emphasis will be on the composition of the lung parenchyma.

i) Parenchyma

The four major cell types comprising the lung parenchyma are the alveolar type I cells, alveolar type II cells, endothelial and mesenchymal cells (9). In addition, these and the nonparenchymal cell types are structurally and functionally supported by a complex connective tissue matrix composed of collagen, elastic fibres, and an amorphous ground substance of proteoglycans (20-25).

The alveolar type I cell is the least prevalent cell type of the lung parenchyma, comprising roughly only 4 % of the total number of lung cells (9), yet, they cover approximately 96 % of the internal alveolar surface area (26). Type I cells are simple squamous epithelial cells which have a centrally oriented, ovoid nucleus with scant surrounding cytoplasm containing only few organelles such as mitochondria, rough endoplasmic reticulum cisternae, Golgi apparatus, and lysosomes. The remainder of the approximately 50 μm diameter of the type I alveolar cells is comprised of broad cytoplasmic extensions which are spread to a thickness of only 0.2 to 0.5 μm and virtually devoid of organelles save for the numerous pinocytotic vescicles. Thus, it is not surprising that these cells distinctively have very little enzyme activity as studied histochemically (27,28). In addition, the type I alveolar cells have been shown to be joined by tight junctions (29), and to be the major barrier to the diffusion of solutes across the blood-gas exchange surface (30). It would seem that the alveolar type I cells are morphologically specialized to provide a stable yet thin barrier for gas exchange by diffusion, while consuming very little oxygen for its own biochemical maintenance (31, 32).

The alveolar type II cell is the most abundant epithelial cell of the alveolar surface, outnumbering the type I cells by 1.5-2:1, yet they cover relatively little of the alveolar surface area (32). Type II cells are cuboidal with a central nucleus and an approximate diameter of 9 μm. These cells are much more metabolically active and embody more cellular organelles than the type I alveolar cell including

larger mitochondria, more extensive Golgi apparatus and rough endoplasmic reticulum, as well as plentiful free ribosomes and multivescicular bodies. The distinctive organelles of the type II cells are the osmiophilic lamellar bodies which exhibit uniform 4.5 nm centre-centre spacing of the approximately 3.0 to 3.5 nm thick laminae when lungs are perfused with multiple fixatives (33). Type II cells are joined with the type I alveolar cells by occluding junctions and may be partially overlaid by their cytoplasmic extensions. The type II cells are usually located at the junctions of alveolar septa, in recesses of the alveolar wall (34). The apical surface at the airspace lumen has numerous microvilli covered by a 30 to 40 nm thick glycocalyx (35). The major function of the alveolar type II cell is the synthesis and secretion of pulmonary surfactant (36), the material which comprises at least part of the inclusions of the lamellar bodies (37-40). Secretion of pulmonary surfactant and its subsequent lining of the alveolar lumen is prominent in the maintenance of a functional residual lung volume during expiration by the reduction of alveolar surface tension(41). Surfactant may also preclude pulmonary edema (42,43), and aid in the elimination of inhaled particles and ingested bacteria by alveolar macrophages (44,45). A second principal role of the type II cell is that of regenerative repair of the alveolar lining (32,36). As noted previously, the squamous type I alveolar cells cover the vast majority of the alveolar surface and as such, are more subject to damage by inhaled agents. These cells are also relatively deficient of the cellular machinery required to initiate reparation. Type II alveolar cells have been shown to act as the proliferative cells in lung repair following lung

exposure to a variety of noxious agents such as oxygen (46,47), ozone (48), nitrogen dioxide (49,50), and bleomycin (51). The primary hyperplasia of the type II cells following type I cellular necrosis led to differentiation into type I epithelium, except after exposure to bleomycin which showed secondary metaplasia to ciliated cell forms (51). Type II cells have also been shown to give rise to type I cells during the development of lungs in rats (52), which by ^{3}H-thymidine incorporation studies, seem to be fully differentiated end stage cells unable to multiply further (53-55). In addition, type II cells have been suggested to be phagocytic (32), and their limited phagocytic activity in situ (56,57) could be important in the reuptake and repackaging of extracellular surfactant (58,59).

A third alveolar cell type has been described in rats which may comprise up to 5 % of the total alveolar cell population (60). The alveolar brush cell is characterized by blunt microvilli on the alveolar surface which contain fibrils extending into the cell cytoplasm housing a well developed complement of cellular organelles. Most of the cell surface is overlaid by cytoplasmic extensions of the alveolar type I cells in the vicinity, and their cellular function is so far unknown.

The endothelial cells of the lung parenchyma form the capillary layer of the blood-air barrier and account for roughly 33 % of the total lung cells (9). Endothelial cells are similar in shape to the alveolar type I cells with attenuated cytoplasmic extensions. These two cell types are located in close apposition, separated by a shared

basement membrane, producing an exceptionally thin surface for gas diffusion. The endothelium of the pulmonary capillaries is non-fenestrated and complete (32), yet, the intercellular junctions are permeable to small proteins (29). Major cellular organelles are located around the nucleus, although numerous pinocytotic vescicles may be observed on both membrane surfaces of the cytoplasmic extensions (31). The endothelial cells are histochemically devoid of hydrolytic or oxidative enzymes (27,28), but are metabolically active in many of the nonventilatory functions of the lung (32,34).

The mesenchymal cells of the lung parenchyma are comprised of the undifferentiated connective tissue cells which occupy the interstitium between the alveoli and the capillaries. These are the most abundant cell type of the lung accounting for approximately 43 % of the total lung cell population, encompassing the interstitial cell, pericyte and fibroblast. The mesenchymal cells in general, function in the maintenance of the interstitial connective tissue (9).

Another cell which is present in the lung parenchyma is the alveolar macrophage. These cells are derived as monoctyes from the bone marrow (61-63), and spend a portion of their life cycle in the lung interstitium, (62,64), where the monocyte divides and matures into the alveolar macrophage which is characteristically found adherent to the alveolar walls, spread flush over the type I cells. The alveolar macrophages contain a vast assortment of lysosomal hydrolytic enzymes (65-67), which aid in their function of particle engulfment and elimination of microorganisms.

ii) Nonparenchyma

The major cells of the nonparenchymal lung tissue are those which compose the conducting airways and blood vessels. Endothelial cells, smooth muscle cells, and mesenchymal cells make up the large blood vessels of the lung, while the vast majority of the cells composing the conducting airways are a variety of epithelial cells. These cells include the basal, brush, goblet, glandular, and ciliated cells which are the most abundant. In the smaller airways past the ninth generation, Clara cells become apparent and together with the ciliated cells form the major epithelial lining (9). Each of the epithelial cell types have a role in the preservation and protection of the airways from inhaled particles and agents, however, the function of the Clara cell which is believed to be secretory (68), is as yet unknown. There are also some endocrine-like neuroepithelial cells present in the bronchiolar airways (69,70), the function of which is unknown, although polypeptide hormone secretion has been suggested for single cells (70), and a sensory reception function has been postulated for clusters of cells or neuroepithelial bodies which are clearly innervated (71,72). The conducting airways also consist of smooth muscle cells and connective tissue cells such as chondroblasts and mesenchymal cells. The lung also contains cells of the nervous and lymphatic systems which are clearly important in lung function, however, the organization of these systems within the lung are poorly understood (9).

3. Pulmonary surfactant system

As has been noted previously, the major function of the pulmonary surface active material that lines the alveolar epithelium is to maintain a functional residual lung volume during expiration by the reduction of alveolar surface tension (41), thus preventing spontaneous atelectasis (4).

i) Composition

A knowledge of the components of pulmonary surfactant offers insight into how this lining material achieves its physiologic role of reducing surface tension through its physical properties. As expected, a number of studies (73-78) have confirmed that surfactant has a well defined chemical composition which is highly similar with various isolation techniques and animal species. The principal constituents are phospholipids, neutral lipids, and protein. The neutral lipids such as cholesterol, triglycerides, and free fatty acids account for only 5-13 % of the total lipids, the remainder consisting of phospholipids. Cholesterol is the major neutral lipid which on a molar basis represents from 10-25 % of surfactant (79). Dipalmitoylphosphatidylcholine is the major phospholipid species constituting up to 58 % of the total surfactant lipids (79) and 45 % of the surface active material by weight (80). Monoenoic phosphatidylcholines account for a further 11-29 % of the total lipids with significantly less proportions of phosphatidylethanolamine and acidic phospholipids such as phosphatidylglycerol and phosphatidylserine (79).

Dipalmitoylphosphatidylcholine is an unusual phospholipid that although present in most animal tissues, it constitutes a major molecular species of phosphatidylcholine only in the lung (81), and most notably in surfactant. Of the components which have been found in pulmonary surfactant, only this unique phospholipid meets the requirements of lowering surface tension of an air-liquid interface to less than 10 dynes/cm (82). The presence of two fully saturated fatty acid moieties on the glycerol phosphorylcholine backbone allows the phosphatidylcholine to be compressed to extremely closely packed molecular areas (79). Dipalmitoylphosphatidylcholine is also able to maintain surface tensions lower than 10 dynes/cm for longer periods than pulmonary surfactant (82). Addition of other phospholipids or cholesterol to dipalmitoylphosphatidylcholine films leads to faster collapse (83). Although dipalmitoylphosphatidylcholine imparts the surface tension lowering properties to surfactant, it lacks some of the kinetic properties associated with the natural surfactant complex. Pure dipalmitoylphosphatidylcholine forms surface films at an exceedingly slow rate compared to natural surfactant in vitro (75). Adsorption of surfactant to the alveolar surfaces in vivo is also known to be rapid (82), suggesting that the other constituents of natural surfactant have important functions. Studies of the protein components of pulmonary surfactant suggest that a 34,000 Dalton apoprotein may be important in the formation of the natural surfactant complex (84). This apoprotein seems to be specifically associated with the pulmonary surface active material since it cannot be found in serum or plasma (76,85), it can be isolated from extracellular sources excluding the possibility of parenchymal plasma membrane

contamination (76,85), and it can be localized at the alveolar interface by immunological techniques (85,86). An 11,000 Dalton apoprotein has also been found specifically associated with surfactant (79,80,85,86), but metabolic kinetics suggest a precursor-product relationship with the 11,000 Dalton apoprotein being a metabolic fragment of the 34,000 Dalton apoprotein (87). It seems that the apolipoprotein in association with phosphatidylglycerol, is required to form a surfactant complex with the rapid adsorption properties found in natural surfactant (84). The integration of the apoprotein with the lipids is dependent upon calcium ions when the lipids are in a fluid state (84). In addition, phosphatidylglycerol has been implicated in contributing to surface properties of surfactant (88), and the stabilization of the physical structure during secretion into the alveolar space (89).

ii) Synthesis and secretion of dipalmitoylphosphatidylcholine

Type II alveolar cells are generally considered to be the cells responsible for the synthesis and secretion of pulmonary surfactant (36-40), although the bronchiolar epithelial Clara cells were once postulated for this role (90,91). Surfactant phospholipids are synthesized in the endoplasmic reticulum of the type II cells and transferred to the lamellar bodies for storage, possibly as a lipoprotein, via the Golgi apparatus (92,93).

Phospholipid synthesis in the lung requires sn-glycerol-3-phosphate or dihydroxyacetonephosphate as well as fatty acids as precursors. The majority of the sn-glycerol-3-phosphate backbone of

phospholipids is formed from dihydroxyacetonephosphate (94), a glycolytic intermediate, although glycerol is also a source (95). The principal origin of fatty acids for pulmonary phospholipid synthesis is through the uptake of free fatty acids from the circulation (94). Alternative sources of fatty acids include circulating lipoproteins as lipoprotein lipase has been found in the lung (96), and the de novo synthesis of fatty acids in general (97), and palmitate in particular (98), from acetate.

The de novo synthesis of phosphatidylcholine in the lung involves two main pathways, both dependent upon the double acylation of sn-glycerol-3-phosphate or dihydroxyacetonephosphate to form phosphatidic acid. Phosphatidic acid is then dephosphorylated by phosphatidate phosphohydrolase to form 1,2-diacylglycerol (94), which can react with cytidine diphosphate choline or cytidine diphosphate ethanolamine to form phosphatidylcholine or phosphatidylethanolamine respectively (99). Phosphatidylcholine may be formed from phosphatidylethanolamine by sequential N-methylation (100). The CDP-choline pathway has been shown to be the major route of de novo synthesis of phosphatidylcholine in the lung (101,102). The de novo synthesis of dipalmitoylphosphatidylcholine seems to be limited since the molecular composition of phosphatidylcholines and diglycerides are different with respect to the amounts of disaturated species, and dipalmitoylphosphatidylethanolamine has not been detected in rat lung (81,94). In addition, incorporation of labelled glycerol has been shown to occur rapidly into unsaturated phosphatidylcholine followed by a time-dependent decrease with a concomitant rise in dipalmitoylphosphatidyl-

choline incorporation (103). This suggests the presence of accessory pathways for the synthesis of dipalmitoylphosphatidylcholine which could include a deacylation-reacylation process (104) and a deacylation-transacylation process (105). Phospholipase A_2 activity is essential for both processes and has been found in the lung in the 105,000xg supernatant (106), in microsomes (107,108), and in lysosomes and lamellar bodies (109,110). The actual site of the phosphatidylcholine remodelling is disputed, but the microsomal phospholipase A_2 has been found to be specific for endogenous phosphatidylcholines with unsaturated fatty acids in the sn-2 position (107,108). The 1-acyl-lysophosphatidylcholine thus produced, which is mainly 1-palmitoyl-lysophosphatidylcholine, is primarily reacylated with palmitoyl-CoA by the microsomal acyl-CoA:lysophosphatidylcholine acyltransferase (111, 112), since the lysophospholipase-transacylase enzyme in lung shows predominantly lysophospholipase rather than transacylase activity, both in vitro and embedded in liposomes (112). However, the trans-acylation pathway may be important in the developing lung (113). In addition, recent studies suggest that dipalmitoylphosphatidylcholine present in the alveolar spaces as surfactant, may be reutilized by reuptake into the type II cells (58,59,114).

Surfactant phospholipids are released into the alveolar spaces by exocytosis (115), under the control of both adrenergic (116) and cholinergic mechanisms (117), although the cholinergic effect may be indirect via stimulation of catecholamine release from the adrenal medulla (117). Secretion of surfactant phospholipids has also been shown to be enhanced by the mechanical distension at the onset of

ventilation in the newborn (118).

4. Development and maturation of the lung

i) Development

The epithelium of the lung develops as an endodermal outpouching of the ventral wall of the foregut, the caudal aspect of which elongates rapidly to separate the future intestinal tract and the primary lung bud. The primitive esophago-trachea, anterior to the primary lung bud divides by fusion of the lateral ridges while the lung bud divides into right and left secondary buds. The endodermal outpouchings carry a mass of mesenchymal tissue as they grow which later gives rise to the pulmonary circulation, lymphatics, and supporting elements such as cartilage, smooth muscle, and connective tissue. The lung buds divide to form the main bronchial pattern, growing into the pleuroperitoneal space rapidly (119).

Intrauterine development of the lung is divided into four stages (120). The embryonic period comprises the earliest phases of lung development. The pseudoglandular period is the phase of bronchial branching whereby the columnar epithelial lined airways appear to be glands. The canalicular period is characterized by mesenchymal proliferation with vascularization and flattening of the airway epithelium. The terminal sac period exhibits progressive epithelial thinning with development of capillaries in close proximity so that respiration may be maintained, however, alveoli are not yet present. This period begins at approximately the twenty-fourth to twenty-sixth week of gestation in the human, while birth occurs during this period

in some species such as the rat.

Postnatal development of the lung may be divided into three phases using the rat lung as an example. The phase of lung expansion occurs from days one to four with very little increase in lung tissue. During this period, the respiratory units are comprised of primary saccules which are larger and different in shape than adult lung alveoli. The phase of tissue proliferation is characterized by the formation of definitive alveoli with a rapid growth and increase in the alveolar surface area. This phase lasts approxiamately from day four to day thirteen after which the phase of equilibrated growth begins as the increase in lung volume is gradualized. Maturation of the interstitium occurs and alveoli develop continuously at a slower rate. In most species a phase of simple expansion begins when the alveolar multiplication ceases, however, it is not clear whether the alveoli cease to increase in the rat as the lung grows continuously with somatic growth in this species (120).

At birth with the onset of air breathing, it is essential that the lung have a functionally mature surfactant system. A deficiency in the pulmonary surface active material is associated with the neonatal respiratory distress syndrome (4-7), especially in those infants who are born prematurely or to diabetic mothers (1). Respiratory distress syndrome is the principal cause of perinatal mortality and morbidity, and is characterized by generalized atelectasis, early respiratory failure, pulmonary edema,and deposition of eosinophilic hyaline membranes in the terminal airspaces (1,2).

Primary lung immaturity with inadequate surfactant production may occur by the interruption of gestational development prior to natural promotion of lung maturation in utero during premature delivery, or by the delay of lung maturation due to various maternal disorders (1).

ii) Involvement of cyclic AMP and other physiological agents in lung maturation

A number of agents have been found to induce fetal lung maturation such as cyclic AMP, aminophylline, β-adrenergic agonists, glucocorticoids, thyroid hormones, thyrotropin-releasing hormone, estradiol, and prolactin (121). The influence of cyclic AMP on fetal lung maturation has been shown by enhancement of phospholipid synthesis in fetal lung with exposure to either cAMP or aminophylline, a cAMP-phosphodiesterase inhibitor (124-127). Cyclic AMP may act to mobilize glycogen stores for phospholipid synthesis in the fetal lung since a prenatal fall in lung glycogen content coincides with high cAMP concentrations (8), epithelial cell differentiation, an increase in pulmonary phospholipid content, and the appearance of lamellar bodies in type II alveolar cells (37,127). In addition, β-adrenergic agonists which act to produce cAMP, stimulate the release of surfactant in the perfused lung (128) and in isolated type II cells (116,128). Glucocorticoid treatment appears to enhance normal fetal lung maturation by increasing the activity of key enzymes involved in phospholipid synthesis such as cholinephosphate cytidyltransferase in the CDP-choline incorporation pathway (122,123), and phosphatidate phosphohydrolase which produces diacylglycerol (122). The effect of glucocorticoids on other phospholipid synthesizing enzymes in lung are

disputed, however, it seems likely that one or both of the accessory remodelling pathways for synthesis of dipalmitoylphosphatidylcholine from unsaturated species may also be affected (121). At present, it is uncertain whether glucocorticoids have a direct effect on fetal lung maturation or an indirect effect through the enhancement of some second messenger (121), as glucocorticoids have been shown to elevate cAMP concentrations (124). Other possible contributors to the physiological induction of fetal lung maturation and surfactant production are thyroid hormones and 17-β-estradiol since the administration of thyroid hormones to pregnant rats (129), or 17-β-estradiol to pregnant rabbits (130,131), has been shown to enhance the fetal lung synthesis and storage of surfactant. In addition, low plasma concentrations of triiodothyronine and thyroxine (132), and estradiol (133) have been found in infants with respiratory distress syndrome. It is not clear whether thyroid hormones induce fetal lung maturation directly or indirectly (121), but the estrogen effect appears to be by a direct stimulation of cholinephosphate cytidyltransferase activity in the fetus (131), which is similar to the effect of maternal glucocorticoid administration (122,123).

5. Cyclic AMP metabolism

Cellular cyclic AMP levels are controlled by alterations in the relative activities of the enzymes which catalyze the production (adenylate cyclase) and degradation (cAMP-phosphodiesterase) of cAMP.

i) Adenylate cyclase: general properties and regulation.

Adenylate cyclase is an ubiquitous membrane-bound enzyme that

mediates the effects of a number of hormones on their target tissues by catalyzing the production of the second messenger, cyclic AMP. When a hormone-stimulatable adenylate cyclase was first demonstrated, it was believed that a single protein enzyme spanned the bilayer with a hormone receptor site located on the outer surface of the plasma membrane which would undergo a conformational change upon the binding of a hormone, resulting in increased cyclic AMP production by the catalytic site located on the inner plasma membrane surface (134). Evidence that hormone receptors are distinct proteins from the adenylate cyclase catalytic moiety first came from kinetic studies of adipocyte adenylate cyclase activity by Rodbell and associates, where the binding of multiple hormones to different receptor sites appear to compete for a limited number of adenylate cyclase catalytic molecules (135). Orly and Schramm (136) provided direct evidence for a mobile receptor by cell fusion of Friend erythroleukemia cells which lack β-adrenergic receptors, but contain adenylate cyclase catalytic activity; with turkey erythrocytes which had been treated with N-ethylmaleimide to inactivate the catalytic activity without affecting the β-adrenergic binding capacity. Plasma membranes isolated from the resultant heterokaryon exhibited catecholamine-stimulatable adenylate cyclase activity. The presence of a third component of the adenylate cyclase system was proposed by Rodbell and associates (137) to explain the enhancement of adenylate cyclase by concentrations of GTP on the order of 10^{-7} M, with synergistic activation in the presence of hormone. It was later shown that some ATP preparations contain GTP in sufficient concentrations for hormone activation and that if highly purified preparations of ATP were used for substrate,

hormonal stimulation of adenylate cyclase activity was dependent upon the addition of GTP (138). These findings suggested that the guanyl nucleotide binding site has an important role in the regulation of hormone stimulation of adenylate cyclase activity. Analogues of GTP which are resistant to hydrolysis, such as guanylyl-5'-imidodiphosphate (GppNHp), have been shown to irreversibly stimulate adenylate cyclase activity as compared to GTP, independent of hormones after a considerable lag phase (139). This lag phase in activation could be reduced or eliminated by the inclusion of hormone in increasing concentrations (139). Turkey erythrocyte plasma membranes contain a specific guanosine triphosphatase (GTPase) activity which is stimulated by catecholamines (140). In addition, this GTPase activity is inhibited by cholera toxin in the presence of nicotinamide adenine dinucleotide (NAD), which also activates adenylate cyclase irreversibly if GTP is included in the assay medium (141). The effect of hormone-receptor binding seems to be to allow the exchange of bound GDP produced by the GTPase enzyme with free GTP so that a fresh cycle of adenylate cyclase stimulation can begin (142). Thus, regulation of the catalytic activity of adenylate cyclase appears to be associated with the functional state of the guanine nucleotide binding protein as summarized in Figure 1. Binding of GTP to this protein (denoted G/F protein) in the presence of hormone promotes an increase in the catalytic activity of the adenylate cyclase and GTPase moieties, as well as decreasing the receptor affinity for hormone (143). The resulting hydrolysis of GTP to GDP leads to deactivation of the adenylate cyclase catalytic site with a concomitant increase in the receptor affinity for agonist.

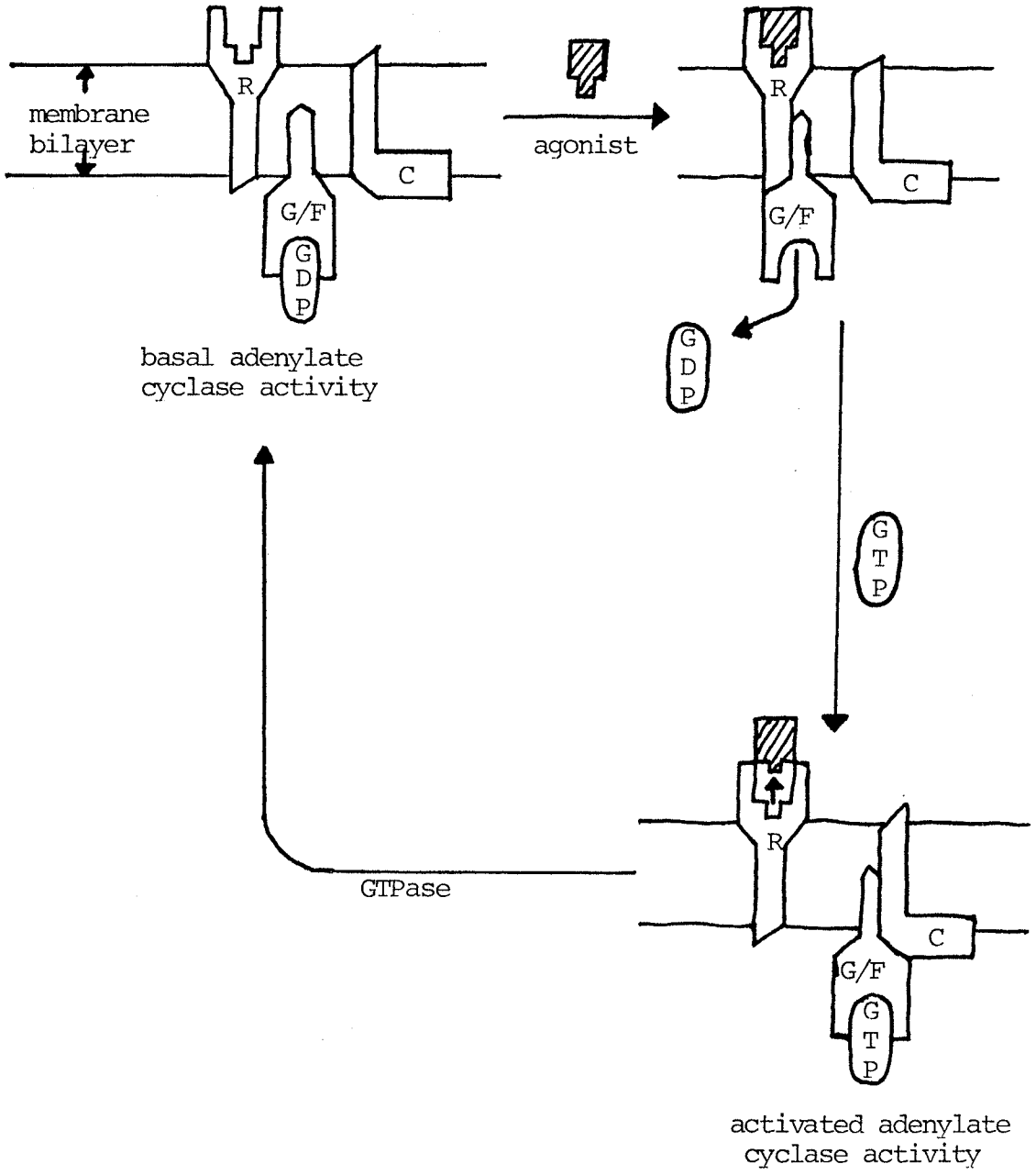


Figure 1 Regulation of adenylate cyclase activity by hormones and guanine nucleotides. [After Limbird (143)].
R - receptor, G/F - guanine nucleotide binding regulatory protein, C - catalytic component.

The guanine nucleotide binding site was first shown to be a distinct protein by Pfeuffer (144) using chromatography of detergent extracted pigeon erythrocyte plasma membranes on GTP-agarose. Adenylate cyclase activity did not bind to the GTP-agarose, however, the enzyme had decreased responsiveness to GppNHp or fluoride stimulation which could be partially restored by the addition of column fractions eluted with GTP or GppNHp. Ross and Gilman (145,146) also demonstrated the existence of separate proteins for guanine nucleotide binding and catalytic activity by reconstituting hormone, GppNHp, and fluoride stimulatable activity in S49 lymphoma cell mutants (cyc^-), which have β-adrenergic receptors but no guanine nucleotide binding regulatory protein or assayable adenylate cyclase activity, with solubilized functional adenylate cyclase enzyme which had been thermally denatured to destroy the adenylate cyclase activity. The cyc^- S49 lymphoma cells appear to have a thermolabile catalytic unit which becomes functional when recombined with a more thermostable guanine nucleotide binding moiety. These findings also provided reconstitutive assay procedures so that the various components of the adenylate cyclase system could be monitored during fractionation.

The guanine nucleotide regulatory and catalytic proteins have now been resolved from a number of tissues such as bovine brain (147), rabbit liver (148,149), turkey erythrocytes (150), and human erythrocytes (151). Characterization of the catalytic unit of adenylate cyclase has been limited by the extreme lability of the protein to thermal denaturation at 30°C, when resolved from the guanine nucleotide protein. However, preliminary studies show that the separated

catalytic protein has an approxiamate molecular weight of 190,000 Daltons, is unresponsive to GppNHp and fluoride, and has minimal activity when assayed with Mg^{2+}-ATP, but is stimulated by Mn^{2+} (147, 152). At least two sulfhydryl residues which are susceptible to N-ethylmaleimide alkylation are present in the catalytic protein, the most reactive of which is required for interaction with the G/F protein, but not for catalytic activity (152). It is not known whether the catalytic moiety is composed of subunits, as this and studies of the regulation of the catalytic protein require new methods to increase its stability so that purification can be achieved.

In contrast, the guanine nucleotide binding regulatory protein is relatively stable (152) and as such is better characterized. Purified preparations of the G/F moiety consist of two principal polypeptides with molecular weights of approximately 35,000 and 42,000 to 45,000 Daltons, but a third polypeptide of 52,000 Daltons is present in rabbit liver preparations (148-151). The 45,000 Dalton protein appears to be a proteolytic product of the 52,000 Dalton peptide as they yield similar peptide maps, behave functionally similar (153), and the purified native G/F protein functions as an 80,000 Dalton complex (149,150). The 45,000 and 52,000 Dalton proteins contain the site for ADP-ribosylation by cholera toxin (148,154), suggesting that these proteins may contain the GTPase moiety. Northrup et al. (155,156) have recently resolved the 45,000 Dalton α-subunit and the 35,000 Dalton β-subunit from rabbit liver G/F protein into separate entities. The resolved α-subunit contains a high affinity guanine nucleotide binding site, is sufficient to

activate resolved catalytic moieties, and is only separable from the β-subunit when activated by ligands such as GppNHp or fluoride in the presence of Al^{3+} and Mg^{2+} (155). The β-subunit appears to facilitate the reversal of the activated state of the α-subunit which is stable when resolved (155,156).

The activity of adenylate cyclase is highly dependent upon ions. The true substrate of the regulatory protein stimulated enzyme is Mg^{2+}-ATP, although the resolved catalytic units may only be able to utilize Mn^{2+}-ATP (153). Reconstitution of stimulatable adenylate cyclase in cyc^- S49 lymphoma cells requires Mg^{2+} to form an activated G/F protein with guanyl nucleotides, as well as an excess of Mg^{2+} concentrations over that required for formation of the Mg^{2+}-ATP substrate (157). It was suggested that to fully reconstitute stimulated adenylate cyclase activity, Mg^{2+} occupancy of an allosteric divalent cation binding site on the catalytic moiety is required (157). Fluoride ions stimulate eukaryotic adenylate cyclase enzymes ubiquitously (158) in the presence of Al^{3+} and Mg^{2+}, by promoting the dissociation of the β-subunit of the G/F protein and interaction of the α-subunit with the catalytic moiety (155,156). In addition, a variety of inorganic salts appear to activate liver adenylate cyclase qualitatively different from fluoride ions in which both anions and cations contribute to the effect (159). These salt effects are also greater in the presence of glucagon and fluoride ions.

The regulation of adenylate cyclase activity may also be affected by intracellular modulators such as the Ca^{2+}-binding protein,

calmodulin, which has been shown to stimulate the adenylate cyclase activity in the presence of Ca^{2+} in brain (160), pancreatic islets (161), and most recently in lung tissues after EGTA washings (162). A number of other endogenous factors which can be found soluble in the supernatants of a variety of tissue homogenates, and which appear to act in a different mode from Ca^{2+}-calmodulin, have been described. Rat liver cytosol contains a heat stable, nondialyzable, protease sensitive factor which gives a similar adenylate cyclase response pattern as GTP stimulation (163-166), suggesting that the soluble factors may act at the G/F protein. These cytosolic factors were also able to restore β-adrenergic stimulatable adenylate cyclase activity(164-166). Cytosolic factors with similar properties have been reported in erythroid cells (167,168), rat osteogenic sarcoma and cultured bone cells (169,170), as well as in heart and other tissues (171). Cytosolic factors from rabbit liver and rat liver, heart, and skeletal muscle homogenate preparations have properties similar to resolved G/F proteins and are able to reconstitute stimulatable activity in cyc^- S49 lymphoma cells (172). These findings suggest that the guanine nucleotide regulatory complex or a component thereof, may be a loosely associated peripheral membrane protein which is dissociated during the homogenization and fractionation procedures.

Nijjar (173) has reported the presence of an age-dependent cytosolic activator of basal adenylate cyclase activity in rat lungs. This factor seems to be protein in nature as it is trypsin sensitive, heat labile and nondialyzable. The factor becomes apparent around the twentieth postnatal day, concurrent with a decreased rate of lung

growth and an increase in cellular differentiation. It doesn't appear to act via a GTP or calmodulin effect on the adenylate cyclase activity as these factors had no effect upon the basal activity under similar experimental conditions. This factor has also been shown to have increased activity in the cytosols which are isolated from the lungs of rats with streptozotocin-induced diabetes, however, the basal adenylate cyclase activity was depressed in the corresponding particulate fraction (174). The lung cytoplasmic activator of adenylate cyclase has now been partially purified by DEAE-cellulose chromatography into two less active but cooperative peaks with approxiamate molecular weights of 15,000 and 65,000 Daltons (175). Preliminary studies using these two partially purified peaks showed a characteristic lag phase of about 10 minutes, after which the adenylate cyclase enzyme was activated by an increase in the maximum reaction velocity and an increase in the affinity for the ATP substrate. In addition, these two peaks did not stimulate cyclic AMP-dependent phosphodiesterase activity (175).

The activity of the adenylate cyclase enzyme is also profoundly affected by the membrane structure of which it is a part (176). Plasma membrane perturbation by detergents, organic solvent, and phospholipases has been used to study the importance of the membrane structure for various stimulated and basal adenylate cyclase activities (158). Decreased basal, epinephrine, and glucagon stimulated adenylate cyclase activities have been reported after treatment of liver plasma membranes with phospholipase A_2 (177-179), and phospholipase C (177, 180). Re-addition of acidic phospholipids such as phosphatidylserine

and phosphatidylinositol could partially restore the adenylate cyclase activities, with the greatest effect on the restoration of glucagon stimulation (177,178,180). Treatment of rat liver plasma membranes with phospholipase C from Clostridium welchii, which has a specificity for neutral phospholipids, has been shown to stimulate basal and inhibit the isoproterenol-stimulated adenylate cyclase activities (181). Phospholipase A_2 treatment of rat brain caudate nucleus has been reported to increase basal and fluoride stimulated adenylate cyclase activities by the depletion of membrane lipid, rather than the generation of lysophospholipids (182). Treatment of rat heart sarcolemma with a phosphatidylinositol-specific phospholipase C followed by ultracentrifugation resulted in a loss of basal adenylate cyclase activity in the membranes which could be restored by the recombination of the treatment supernatant, suggesting that phosphatidylinositol may anchor some component responsible for the expression of basal adenylate cyclase activity in myocardial cells (183). Attempts to determine specific phospholipid requirements for the functional reconstitution of resolved adenylate cyclase components into liposomes suggest that phosphatidylcholine may be important for deoxycholate solubilized adenylate cyclase activity (184), and for a productive regulatory interaction of the catalytic unit and the G/F complex (185). However, these studies did not exclude the possibility that a very small amount of tightly associated lipid is also solubilized and may be important for expression of adenylate cyclase activity after phosphatidylcholine displacement of detergent, since the addition of nonionic detergents also leads to functional reconstitution (184). Thus, the lipid bilayer may exert specific

effects by the chemical nature of the surrounding annular lipids or influence the lateral migration and internal motion of the protein components by regulation of membrane fluidity (176).

ii) Cyclic AMP-dependent phosphodiesterase

Cyclic AMP phosphodiesterase terminates the action of cAMP by catalyzing its hydrolysis to 5'-AMP. At least three distinct forms of cyclic nucleotide phosphodiesterase have been found in most mammalian tissues which differ in chromatographic properties, substrate specificity, and kinetic and regulatory properties (186). The high K_m form is active with both cAMP and cGMP substrates, however, the hydrolysis of each cyclic nucleotide is competitively inhibited by the other substrate. The affinity for cGMP is higher than cAMP, but the enzyme has a greater V_{max} for cAMP, and is sensitive to Ca^{2+} - calmodulin stimulation (187). The low K_m form is characterized by a relative specificity for cAMP as substrate with negatively cooperative interaction (186). This low K_m form is insulin sensitive, Ca^{2+} - calmodulin insensitive (186), and may be stimulated by phosphatidyl-serine and phosphatidylglycerol in adipocyte microsomes (188). A third type of phosphodiesterase hydrolyzes both cGMP and cAMP with equal maximum velocities, however, the hydrolysis of cyclic AMP in low concentrations is allosterically activated by low concentrations of cGMP. In addition, a variety of other phosphodiesterase forms may also be present in mammalian tissues (186). The mammalian lung is relatively complex with regard to the number of soluble forms of cyclic nucleotide phosphodiesterase, as at least four chromatographic-ally distinct forms have been found in human lung including a high

affinity cAMP phosphodiesterase which is insensitive to Ca^{2+} - calmodulin and a cGMP stimulated form which is specific for cAMP substrate (189). Rat lungs (190), and bovine lungs (191), also contain multiple forms of phosphodiesterase including the cGMP stimulated enzyme. The functions of the individual forms of cyclic nucleotide phosphodiesterase and their interrelationships within the same tissue are not presently known, but the existence of multiple forms may reflect the complex regulation of eukaryotic cellular functions (192).

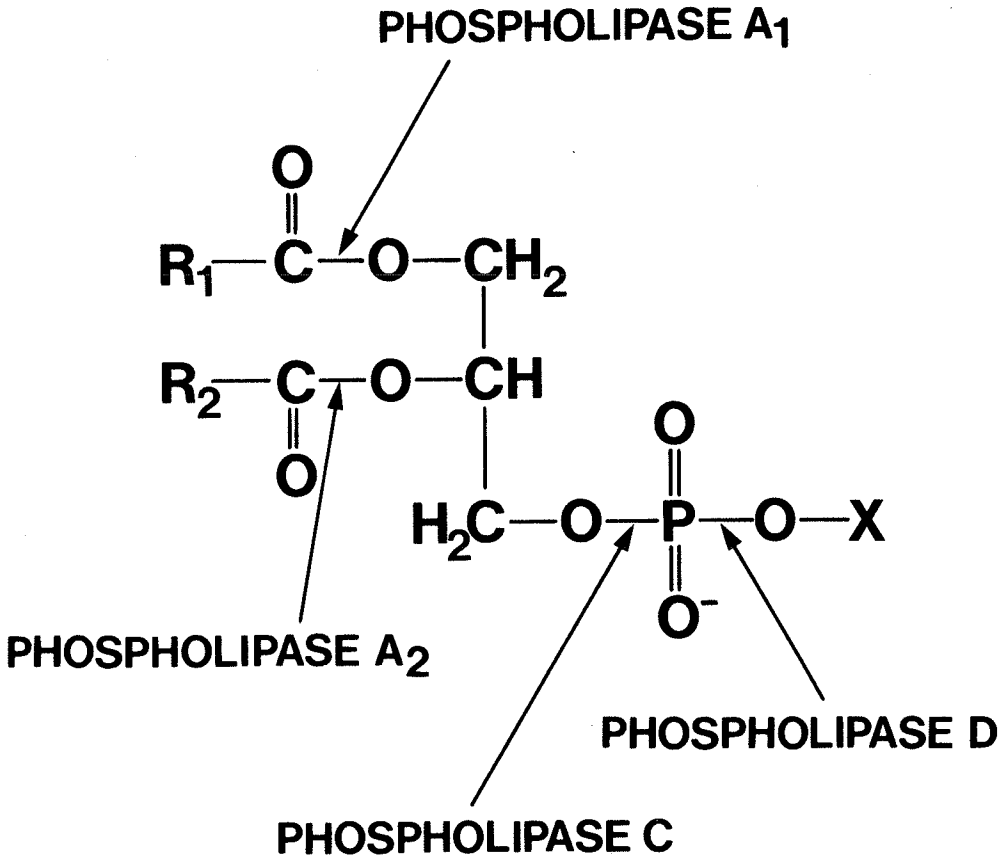
C. PRESENT STUDY

In view of the suggested involvement of cyclic AMP in the enhancement of the neonatal development and maturation of the lung so that the risk of respiratory distress syndrome at birth is reduced, the study of factors which regulate the cellular concentrations of cAMP are of major importance. Adenylate cyclase, which catalyzes the production of cAMP, appears to play a major role in the control of cAMP levels since the activity of this enzyme may be stimulated by β-adrenergic agonists and is profoundly affected by the soluble activator proteins prepared from the supernatant obtained after ultra-centrifugation of lung homogenate. This enzyme complex is part of an intricate membrane structure which is best described by the Singer-Nicholson fluid mosaic model (223) in which the membrane proteins are relatively freely floating in a fluid bilayer of phospholipids which are oriented with the polar bases at the aqueous surfaces and the hydrophobic fatty acid moities projecting into the centre of the bilayer.

Thus, the objectives of the present study were to investigate the influence of the plasma membrane environment upon the adenylate cyclase system in rat lungs. Perturbation of the plasma membrane structure with various phospholipases and proteases were used to study some aspects of the activation of the adenylate cyclase enzyme in rat lung by various stimulatory agents such as epinephrine, NaF, GppNHp, and the cytosolic adenylate cyclase activator proteins. In addition, the effect of these perturbing agents and the adenylate cyclase

activator proteins on other rat lung membrane-bound enzymes such as 5'-nucleotidase and sodium, potassium-dependent adenosine triphosphatase were studied in order to see if these treatments have a general effect on membrane enzymic activities. Finally, the endogenous phospholipase activities in rat lung homogenate fractions were assayed to determine if the cytosolic adenylate cyclase activator proteins exert their stimulatory effect by modification of the plasma membrane environment. The phospholipases were chosen for their ability to cleave membrane phospholipids at specific molecular sites (Figure 2). In addition, the effects of these phospholipases (177-183) and proteases (216-219) on the adenylate cyclase activities of tissues other than the lung have been reported. Throughout these studies, lungs from 35 to 55 day old rats were used since this is a period of peak adenylate cyclase activator activity in the lung cytosol (173).

Figure 2 Molecular sites of action of various phospholipases upon phospholipids. R_1 and R_2 are faty acyl moities, X is the polar phospholipid base.


PHOSPHOLIPASE A1
R1—C—O—CH2
R2—C—O—CH
H2C—O—P—O—X
O⁻
PHOSPHOLIPASE A2
PHOSPHOLIPASE D
PHOSPHOLIPASE C

D. MATERIALS AND METHODS

1. Materials

Proteose peptone, ammonium lactate, and Staphylococcus aureus were generous gifts of Dr. G. Wiseman, Microbiology Department. Millipore screens were a gift of Dr. F. W. Orr, Department of Pathology, and metiamide was donated by Dr. E. Kroeger, Department of Physiology. Amberlite CG-50 and silica gel 60-F254 plates were purchased from Terochem (Edmonton). Preadsorbent silica gel 60 plates were purchased from Mandel Scientific (Quebec), and Kodak No-screen X-ray film was obtained from Treck Photographic (Winnipeg). L-α- (dipalmitoyl-1-^{14}C-)phosphatidylcholine and (8-^{14}C-)ATP were purchased from New England Nuclear (Boston). All other biochemicals and reagents were purchased from Sigma Chemical Company (St. Louis), or from Fisher Scientific locally.

2. Animals

Male Sprague-Dawley strain rats between 35 and 55 days of age were obtained from the Faculty of Dentistry, University of Manitoba, or from the Canadian Breeding Laboratories, Charles River, Quebec. A minimum of 3 animals were used in each preparation of the various lung tissue fractions.

3. Preparation of a phosphatidylinositol-specific phospholipase C

A phosphatidylinositol-specific phospholipase C was prepared from Staphylococcus aureus (Newman strain) culture supernatants, essentially as described by Low and Finean (193).

i) Preparation of Staphylococcus aureus culture supernatants

Culture media was prepared by dissolving 22 g of proteose peptone in 50 ml of distilled water and dialyzed for 24 hours in 500 ml of distilled water. The pH of the diffusate was adjusted to 7.4 with 1N NaOH after the addition of 5 g ammonium lactate, 1 g KH_2PO_4, 1 g K_2HPO_4, and 0.4 g $MgSO_4$-$7H_2O$; and the final volume made to 1 litre with water. The culture media was distributed between two 1 L flasks and autoclaved at 115°C for 15 minutes. After cooling and temperature equilibration, each flask was inoculated with 5 ml of an overnight culture of the Newman strain of S. aureus and incubated in a horizontal shaker, (100 rpm), for 24 hours at 37°C in a controlled CO_2/O_2 environment. The bacteria were removed by centrifugation at 10,000xg for 30 minutes followed by passage through a millipore sieve to further sterilize the supernatant. All further procedures were carried out at 4°C.

ii) Chromatography on Amberlite CG-50 and Sephadex G-75

An Amberlite CG-50 column (1.5 cm X 15 cm) was prepared by washing the resin in 2 X 25 volumes of water, 10 volumes of 0.1 N NaOH, 2 X 25 volumes of water, and finally with 2 X 10 volumes of equilibrating buffer consisting of 0.15 M NaCl in 0.05 M sodium

acetate-acetic acid, pH 5.5, before packing the column. NaCl (2.5 g), was added to the supernatant and the pH adjusted to 5.5 with acetic acid before loading on the pre-equilibrated Amberlite column at a flow rate of 2.5 ml/minute. The column was washed with 10 bed volumes of the equilibrating buffer and eluted by a linear gradient formed with 3 bed volumes each of the equilibrating buffer and 1 M trisodium citrate at a flow rate of 0.5 ml/minute. Fractions containing more than 3.0 units/ml of phosphatidylinositol-specific phospholipase C were pooled and concentrated with a PM 10 membrane in an Amicon ultrafiltration cell to about 4.5 ml. The concentrate was then loaded onto a Sephadex G-75 column (1.4 cm X 35 cm) previously equilibrated in 50 mM Tris-HCl buffer, pH 7.4, and eluted at a flow rate of about 8 ml/hour. Fractions of about 4 ml were collected and assayed for phospholipase C activity as outlined below. Those fractions containing more than 1.0 units/ml were pooled and concentrated by ultrafiltration with a PM 10 membrane.

iii) Assay of phosphatidylinositol-specific phospholipase C.

Phosphatidylinositol-specific phospholipase C was assayed as described by Low and Finean (1964), using soybean phosphatidylcholine (Type IV-S), which by lipid extraction and analysis as described later, was found to contain approxiamately 5 % phosphatidylinositol. This soybean lipid fraction was homogenized in a blender, (20 mg/ml), and dialyzed for 4 hours against 20 volumes of water and stored at -20^{o}C until use. Immediately prior to assay, aliquots of the homogenized soybean lipid were sonicated for use as a phosphatidylinositol substrate. In a 1.0 ml final volume, the assay mixture contained

0.5 ml sonicated soybean lipid, 0.2 ml of 100 mM hydroxyethyl piperazine-N'-2-ethanesulfonic acid (HEPES)- NaOH buffer, pH 7.0, and 0.3 ml of enzyme source. Final concentration of the phosphatidyl-inositol in the assay mixture was 0.5 mM. The mixture was incubated for 2 hours at 37°C and the reaction terminated by the addition of 8 ml of chloroform:methanol:Conc. HCl, (100:100:0.6, v/v/v), followed by 1.7 ml of 1N HCl to separate the phases (195). Aliquots of the upper phase were taken for the determination of organic phosphate by the method of Bartlett (196), as described later. A unit of phospho-lipase C activity was defined as the amount of activity releasing 0.1 umol of organic phosphorus into the upper phase in 2 hours at 37°C. For the assay of phospholipase C activity in the culture supernatants, the samples were dialyzed overnight at 4°C against 750 volumes of water to remove the phosphate buffers. Amberlite column fractions were dialyzed overnight at 4°C in order to remove NaCl which is inhibitory at the concentrations present (194). These dialyzates were lyophilized and reconstituted in the original aliquot volume of water before the assay for phospholipase C activity.

4. Preparation of lung tissue fractions

The various rat lung tissue fractions used in this study were prepared by a modification of the method of Nijjar and Ho (197). The animals were sacrificed by decapitation and the lungs were rapidly excised and washed in cold homogenizing buffer (0.27 M sucrose/10 mM Tris-HCl, pH 7.4). Subsequent fractionation procedures were performed at 2-4°C. The trachea and hilar regions containing the major bronchi

and blood vessels were dissected away, and the remaining lung portions were sliced with a sharp razor blade before homogenization in a Potter-Elvehjem homogenizer by 15 hand-driven strokes with a loose fitting teflon pestle. The whole homogenate was filtered through 4 layers of cheesecloth to remove connective tissue and cellular debris before adjusting the final volume to a 20 % (w/v) solution. After removal of a small aliquot, the homogenate was centrifuged at 900xg for 10 minutes, the supernatant (S1) withdrawn, and centrifuged at 6,000xg for 10 minutes. The supernatant (S2) was centrifuged at 8,000xg for 10 minutes to yield a supernatant (S3) for layering onto a discontinous sucrose gradient consisting of, from bottom to top, 7.0 ml of 35 % sucrose, 7.5 ml of 25 % sucrose, and 7.5 ml of S3. The gradient was ultracentrifuged in a Beckman Ti60 rotor, at 105,000xg (37,000 rpm) in a Beckman model L5-65 ultracentrifuge for 90 minutes. After ultracentrifugation, fractions were carefully withdrawn from the top; 4.6 ml (F1), and 7.8 ml (F2); and the same fractions from different tubes were pooled. The F1 fraction or 105,000xg supernatant was saved for the subsequent preparation of the adenylate cyclase activator proteins. The plasma membrane enriched fraction, F2, was diluted to 10 % sucrose with cold distilled water and ultracentrifuged at 144,000xg (45,000 rpm) for 60 minutes. All the pellets produced;900xg, 6,000xg, 8,000xg and 144,000xg; were washed by resuspension and respun at their respective speeds before final suspension in a small volume of the homogenizing buffer. The samples were stored at -20^{o}C until the various enzyme assays were performed, usually within one month. The fractions isolated by this procedure were previously characterized by enzyme marker assays (197).

5. Preparation of rat lung cytoplasmic activator proteins of adenylate cyclase activity

A cytoplasmic activator of rat lung adenylate cyclase activity (173), was prepared by modifications of the method of Nijjar et al. (174), from the 105,000xg supernatant obtained during the preparation of a plasma membrane enriched fraction of rat lung homogenate. The supernatant was made to pH 5.0 with 0.5 N acetic acid and centrifuged at 15,000xg for 15 minutes to remove precipitated proteins. The pellet was discarded and the pH of the resultant supernatant was brought back to pH 7.4 with 1.0N NaOH before its application at a 1 ml/minute flow rate on a DEAE-52 cellulose column, (2.2 cm X 40 cm), previously equilibrated in 10 mM Tris-HCl buffer, pH 7.4. The column was washed with one bed volume, (150 ml), of the equilibrating buffer, and bound proteins were eluted with a linear gradient formed by mixing 375 ml each of 10 mM and 300 mM Tris-HCl, pH 7.4. The absorbance at 280 nm was measured and the protein contents and abilities to enhance adenylate cyclase activity in membranes were assayed for in the resulting fractions as described in later sections. The two resulting peaks of adenylate cyclase activator activity from the 105,000xg supernatant were further resolved separately. Subsequent adenylate cyclase assays on column fractions were carried out in the presence of a small amount of the corresponding activator protein peak.

Peak 1 activator activity protein containing fractions were pooled, diluted to the same conductivity as the equilibrating buffer and loaded onto a second DEAE-cellulose column, (1.6 cm X 15 cm, 30 ml

bed volume), previously equilibrated in 10 mM Tris-HCl buffer, pH 7.4. The column was washed with 2.5 bed volumes of 20 mM Tris-HCl, pH 7.4 and eluted by a linear gradient formed from 90 ml each of 20 mM and 150 mM Tris-HCl, pH 7.4, at a flow rate of 0.75 ml/minute. The fractions obtained were analyzed as above with a small amount of peak 2 included in the adenylate cyclase assay. Those fractions containing activator activity were pooled and concentrated in an ultrafiltration cell with a PM 10 membrane and saved for various experiments.

Peak 2 activator activity protein containing fractions from the first DEAE-cellulose column were pooled, diluted 4-fold, and loaded onto another DEAE-cellulose column, (1.6 cm X 15 cm), previously equilibrated as above. The column was washed with one bed volume of 20 mM Tris-HCl, pH 7.4, and eluted with a linear gradient formed from 100 ml each of 50 mM and 200 mM Tris-HCl, pH 7.4, followed by 50 ml of 250 mM Tris-HCl and 50 ml of a 1 mM imidazole buffer, pH 7.0, containing 20 mM Tris-HCl, 1 mM magnesium acetate, 10 mM $CaCl_2$, and 0.65 M NaCl, titrated to pH 8.0 with 1N NaOH. The post-gradient buffers were required to remove the peak 2 proteins with activator activity which were tightly bound to the column. Those fractions containing peak 2 activator activity, as assayed in the presence of a small amount of peak 1, were pooled, concentrated in an ultrafiltration cell with a PM 10 membrane and passed through a Sepharose 6B column. Fractions were analyzed as above and those fractions containing peak 2 adenylate cyclase activator activity, were pooled and saved for various experiments.

6. Treatment of rat lung plasma membranes or 6,000xg pellet with phospholipases, proteases, and the adenylate cyclase activator peaks 1 and 2.

Unless otherwise specified in the table or figure legends, plasma membranes or 6,000xg pellets were treated with the phospholipases, proteases, and activator peaks at 37°C for 15 minutes in the presence of the various enzyme assay media, prior to the addition of substrate. Chymotrypsin, trypsin, and phospholipases A_2 (Naja naja), C (Clostridium perfringens), and D were dissolved in 10 mM Tris-HCl, pH 7.4. Phospholipase A_2 (Vipera russelli) in a 50 % glycerol suspension, was diluted to the appropriate concentrations with 10 mM Tris-HCl, pH 7.4. Phosphatidylinositol-specific phospholipase C and the adenylate cyclase activator peaks were added directly from the pooled column fractions.

7. Estimation of protein

Protein was determined by the method of Lowry et al. (198), using bovine serum albumin as the standard. The formation of a protein-copper complex in alkali and the subsequent reduction of a phosphomolybdic-phosphotungstic reagent by this complex is the basis of the color reaction. An aliquot of tissue fraction was solubilized in 0.2 ml of 1N NaOH and the volume made up to 0.4 ml with distilled water. The color reagent containing 0.01 % (w/v), cupric sulfate, 0.02 % (w/v), sodium-potassium tartrate, and 0.05 % (w/v) of 1N NaOH in 2.0 ml of 2 % (w/v) sodium carbonate, was added and the mixture

allowed to stand for 10 minutes before the addition of 0.2 ml of 1N Folin's reagent. The color reaction was allowed to continue at room temperature for 30 minutes before reading the absorbance at 750 nm. When whole homogenate protein contents were estimated, an aliquot was dissolved in 3.0 ml of 1N NaOH overnight, of which, 0.2 ml was used for the assay as above.

Protein content in column fractions obtained during the preparation of the cytoplasmic activator proteins of rat lung adenylate cyclase activity was assayed by the micro-method of Bradford (199). Aliquots of column fractions were made to 0.1 ml with 10 mM Tris-HCl, pH 7.4, and 1.0 ml of color reagent containing 0.01 % (w/v), Coomassie Brilliant Blue G-250, 4.7 % (w/v), ethanol, and 8.5 % (w/v), phosphoric acid, was added before measuring the absorbance at 595 nm.

8. Assay of adenylate cyclase activity

Adenylate cyclase activity was assayed as described by Drummond and Duncan (200), using (8-^{14}C-)ATP as substrate. The assay medium contained in 100 µl final volume, 50 mM Tris-maleate buffer, pH 7.4, 25 mM caffeine, 5.5 mM KCl, 15 mM $MgCl_2$, 2 mM cyclic AMP, 20 mM phosphoenol pyruvate, 15 u/ml pyruvate kinase, 0.56 mM (8-^{14}C-)ATP, (0.2 µCi), and 30 to 50 µg of plasma membrane protein, or 75 to 100 µg of the lower speed pellets. The assay mixture was preincubated at 37^{o}C for 3 minutes in the standard assay,or for various time intervals in the presence of the activator proteins, phospholipases, or proteases

as described earlier. The reaction was started by the addition of ^{14}C-ATP and allowed to continue for 15 minutes before termination by placing the tubes in a boiling water bath for 3 minutes. Control tubes were treated as above, except that they were boiled prior to the addition of the ^{14}C-ATP. Denatured proteins were sedimented by centrifugation in a clinical centrifuge at full speed for 15 minutes. An aliquot, 75 ul, of the clear supernatant was spotted on Whatman 3MM chromatography paper and subjected to descending chromatography for 18 hours at room temperature in a solvent consisting of 1 M ammonium acetate:95 % ethanol, (3:7, v/v). The papers were air dryed and the cyclic AMP spot was identified under ultraviolet light by the comigration of a cyclic AMP standard spot applied to each paper. This area containing cyclic AMP was cut out and counted in plastic vials with 18 ml of scintillation fluid containing 4.0 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per litre of toluene. The radioactivity was measured in a Searle Mark III or an LKB Rack Beta liquid scintillation counter, and the amount of cyclic AMP was calculated from the specific activity of the ^{14}C-ATP substrate after correction for the DPM's found in the cyclic AMP spot of the boiled control samples.

9. Assay of 5'-nucleotidase activity

The 5'-nucleotidase activity of phospholipase treated and control membranes was assayed by the method of Michell and Hawthorne (201). The assay medium contained 50 mM Tris-HCl, pH 7.4, 100 mM KCl, 10 mM $MgCl_2$, 10 mM sodium-potassium tartrate, 2 mM 5'-AMP, and between

50 to 75 µg of membrane protein in a final volume of 0.5 ml. After treatment of the plasma membranes for 30 minutes, aliquots were removed and added to the assay medium. The reaction was initiated by the addition of 5'-AMP after a 3 minute preincubation at 37°C, and stopped after 15 minutes by the addition of 0.5 ml of ice-cold 10 % trichloroacetic acid. Denatured proteins were sedimented by centrifugation in a clinical centrifuge at full speed for 15 minutes, and an aliquot of the clear supernatant was analyzed for inorganic phosphate using the method of Fiske and Subbarow (202), as modified by Butcher and Sutherland (203). To 0.5 ml of the TCA supernatant, 0.5 ml of 0.55 % ammonium molybdate in 1.1 N H_2SO_4 was added. The phosphomolybdic complex thus formed was reduced by the addition of 50 µl of ANSA reagent containing 6 g sodium metabisulfite, 0.6 g sodium sulfite, and 0.125 g 1-amino-2-napthol-4-sulfonic acid per 50 ml. The absorbance was read at 660 nm after 7 minutes. Endogenous and phospholipase produced P_i was corrected for by the addition of 5'-AMP to control tubes after protein precipitation by TCA.

10. Assay of sodium-potassium dependent and total adenosine triphosphatase activities

Sodium-potassium dependent and total magnesium dependent adenosine triphosphatase (ATPase) activity was assayed in rat lung plasma membranes by the method of Matsui and Schwartz (204). The assay medium contained approximately 50 µg of plasma membrane protein, 100 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA (ethylene diaminotetraacetic acid), 50 mM Tris-HCl buffer, pH 7.4, and 5 mM

Tris-ATP in a final volume of 0.5 ml. After a 15 minute preincubation at 37°C with the phospholipases, activator protein peaks, or buffer in controls, the reaction was initiated by the addition of ATP and allowed to continue for 15 minutes before termination by the addition of 0.5 ml of 10 % TCA. Precipitated proteins were spun down at full speed in a clinical centrifuge for 15 minutes and the supernatants were analyzed for inorganic phosphate as described earlier. To determine the sodium-potassium dependent ATPase, the assay medium was prepared with 1 mM ouabain and/or without NaCl and KCl. ATPase activity found with this media represents the sodium-potassium independent activity while the complete assay medium would yield the total magnesium dependent ATPase activity.

11. Estimation of phospholipid content

i) Extraction of lipids

Membrane lipids were extracted by the method of Folch et al. (205) as modified by Lefebvre et al. (195). About 600 to 750 µg of plasma membrane protein was incubated with the activator proteins or phospholipases for 30 minutes and the treatment terminated by the addition of 8 volumes of chloroform:methanol:Conc. HCl, (100:100:0.6, v/v/v). After extraction of the lipids for at least 30 minutes at room temperature, the mixture was separated into two phases by the addition of 0.2 volumes of 1N HCl, lightly vortexed at low speed, and centrifuged in a clinical centrifuge at full speed for 5 minutes. The upper aqueous phase and the denatured protein interphase were carefully removed with a Pasteur pipette and the lower chloroform phase

containing the extracted lipids was washed twice with a volume of synthetic upper phase, (chloroform:methanol:0.01N HCl, 3:48:47, v/v/v) equal to the original aqueous phase volume. A few drops of methanol and two drops of concentrated NH_4OH were added to make the lower phase homogenous and neutral. Aliquots of the lower, chloroform phase were dried under a stream of nitrogen and analyzed for total lipid phosphorus, while the remainder was used for isolation of the various phospholipids by thin layer chromatography.

ii) Separation of phospholipids by thin layer chromatography

The total lipid extracts were dried under a stream of nitrogen at 37°C in a water bath. The lipid residues were redissolved in a small volume of chloroform:methanol:water, (75:25:2, v/v/v), and spotted onto 0.25 mm thick precoated silica gel 60-F254 thin layer chromatography plates which had been previously activated at 110°C for 1 hour and cooled in a desiccator. The various phospholipid species were separated by the two dimensional chromatography method described by Pumphrey (206). The plates were run in the ascending direction in the first solvent system, (chloroform:methanol:7 M NH_4OH, 12:7:1, v/v/v), and thoroughly dried using a hair dryer. The plates were then developed at right angles in the second dimension solvent, chloroform:methanol:acetic acid:water, (80:40:7.4:1.2, by volume). Following thorough air drying, the phospholipid spots were visualized under ultraviolet light after the plates were sprayed with 5 % H_2SO_4, (w/v) and charred in an oven at 160°C for 15 minutes.

iii) Estimation of lipid phosphorus

Phospholipid spots were scraped, placed into individual tubes and analyzed for lipid phosphorus by the method of Bartlett (196), using 0.7 ml of 70 % perchloric acid, (w/v), for digestion at 160^{o}C for 3 hours until clear (207). After cooling the digests, liberated lipid phosphorus was measured by the addition of 4 ml of distilled water followed by 0.2 ml of 5 % ammonium molybdate, (w/v), and 0.2 ml of ANSA reducing agent containing 15 g sodium metabisulfite, 0.5 g sodium sulfite, and 0.25 g of 1-amino-2-napthol-4-sulfonic acid per 100 ml of distilled water. The mixture was heated in a boiling water bath for 15 minutes, cooled, and the silica gel was sedimented by centrifugation in a clinical centrifuge at full speed for 5 minutes. Absorbance of the supernatants was measured at 830 nm against blanks prepared by scraping silica gel from regions on the plate containing no phospholipid spots and like the phosphate standards, were treated as above.

12. Assay of phospholipase A activity

Phospholipase A activity in rat lung homogenate fractions was assayed by a modification of the method of Garcia et al. (107). The assay media contained 500 µM L-α-(dipalmitoyl-1-^{14}C-)phosphatidyl-choline, (0.5 µCi/µmol), as an ultrasonicated suspension in 10 mM $CaCl_2$- 100 mM Tris-HCl buffer, pH 8.9, and approximately 50 µg of plasma membrane protein or up to 200 µg protein of the lower speed fractions, in a final volume of 0.2 ml. Following a 3 minute preincubation at 37^{o}C, the reaction was started by the addition of

the various homogenate fractions and allowed to continue for 30 minutes. The reaction was terminated by the addition of 0.75 ml of chloroform:methanol, (1:2, v/v), and the lipids were extracted by the method of Bligh and Dyer (208). The precipitated proteins were removed by centrifugation for 10 minutes in a clinical centrifuge at full speed. The supernatants were carefully withdrawn and the pellets were washed with 0.475 ml of chloroform:methanol:water, (1:2:0.8, v/v/v), and resedimented. This supernatant was withdrawn and pooled with the original before making the solution to 2:2:1.8 by volume of chloroform:methanol:water by the addition of 0.375 ml of chloroform followed by 0.375 ml of distilled water and centrifugation for 10 minutes to separate the phases. The upper aqueous phase was carefully removed as much as possible and the remainder was made homogenous with the lower chloroform phase by the addition of a few drops of methanol. About 50 nmoles each of carrier dipalmitoyl-glyceride and palmitate were added before evaporating the chloroform extracts to dryness under a stream of nitrogen in a 37°C water bath. The lipids were redissolved in a small volume of chloroform:methanol, (2:1, v/v), and spotted on preadsorbent silica gel 60 thin layer chromatography plates which were previously activated at 110°C for 1 hour and cooled. Neutral lipids were separated by the method of Skipski et al. (209), using a two step development of the chromatograms. The first solvent consisting of isopropyl ether:acetic acid, (96:4, v/v), was allowed to develop to approximately 13 cm from the bottom of the plates. The plates were thoroughly dried by cool air from a hair dryer, and developed in a second solvent system containing petroleum ether:diethyl ether:acetic acid, (90:10:1, v/v/v), to 0.5 cm

below the top of the chromatography plates. Lipid spots were visualized by autoradiography for 1 week using Kodak No-screen X-ray film and/or by spraying with 5 % H_2SO_4 and charring at 160°C. Spots corresponding to palmitate were scraped into scintillation vials and counted using 10 ml of Scintiverse as the cocktail. Aliquots of the upper aqueous phase were also counted in 10 ml of Scintiverse to monitor the lipid loss into the upper phase during extraction.

13. Assay of phospholipase C activity

Phospholipase C activity in rat lung homogenate fractions was assayed by a modification of the method of Matsuzawa and Hostetler (210). The assay medium contained 50 μM L-α-(dipalmitoyl-1-^{14}C-) phosphatidylcholine, (10 μCi/μmol), as an ultrasonicated suspension in 2 mM $CaCl_2$ - 50 mM sodium acetate buffer, pH 4.5, and up to 200 μg of homogenate fraction in a final volume of 0.2 ml. After a 3 minute preincubation at 37°C, the assay was initiated by the addition of membrane protein or homogenate fraction, and allowed to continue for 60 minutes. The reaction was terminated by the addition of 0.75 ml of chloroform:methanol, (1:2, v/v), and the lipids were extracted by the procedure of Bligh and Dyer (208), and separated as described in the phospholipase A assay except that the spots corresponding to dipalmitoylglyceride were scraped and counted. Phospholipase C was also assayed in neutral pH as described above except that the ^{14}C-phosphatidylcholine was dispersed in a 50 mM final concentration of Tris-HCl buffer, pH 7.5, instead of the sodium acetate buffer. All other assay conditions and analytical procedures were kept the same.

14. Assay of phospholipase D activity

Phospholipase D activity in rat lung homogenate fractions was assayed by a modification of the method of Chalifour and Kanfer (211). The assay medium contained 5 mM L-α-(dipalmitoyl-1-^{14}C-)phosphatidylcholine, (1 mCi/mmol), as an ultrasonicated suspension in 5 mM EDTA-6 mM sodium deoxycholate - 50 mM HEPES buffer, pH 6.5, in a final reaction volume of 0.1 ml. After a 3 minute preincubation at 30^{o}C, the reaction was initiated by the addition of up to 200 µg of lung homogenate fraction protein and allowed to continue for 30 minutes. The reaction was terminated by the addition of 0.375 ml of chloroform: methanol, (1:2, v/v), and the lipids were extracted by the method of Bligh and Dyer (208), using one-half the volumes described earlier. Carrier phosphatidic acid was added to the lower chloroform phase and the lipid extracts were dried under a stream of nitrogen in a 37^{o}C water bath. The lipids were redissolved in a small volume of chloroform:methanol, (2:1, v/v), and spotted onto preadsorbent silica gel 60 thin layer chromatography plates which were previously activated at 110^{o}C for 1 hour and cooled. The chromatography plates were developed twice in the ascending direction using diethyl ether: acetic acid, (100:1, v/v), as the first solvent followed by the second solvent system containing chloroform:acetone:acetic acid:methanol: water, (50:20:10:10:5, by volume). The plates were thoroughly dryed between solvents using cool air from a hair dryer. Lipids were visualized by autoradiography for 1 week using Kodak No-screen X-ray film and/ or by exposure to iodine vapours. Spots corresponding to phosphatidic acid were scraped into scintillation vials containing

10 ml of Scintiverse and counted. Aliquots of the upper phase were also counted to monitor lipid loss during extraction procedures.

E. RESULTS

Analysis of the commercial phosphatidylcholine Type IV-S for phospholipid content revealed a number of phospholipids other than phosphatidylcholine, as well as a considerable amount of neutral lipids. Phosphatidylcholine and phosphatidylethanolamine were the major phospholipid species present at 46.5 % and 28.6 % respectively, while phosphatidylinositol comprised 4.96 % of the total phospholipid content, (Table I). The soybean lipid suspension was found to contain approximately 1.0 µmol of lipid phosphorus per mg resulting in a final assay concentration of 10 mM for total phospholipids and 0.5 mM phosphatidylinositol during the phosphatidylinositol-specific phospholipase C assay.

The culture supernatants prepared from Staphylococcus aureus (Newman) were found to release organic phosphorus into the upper phase of the lipid suspension extracts, presumably as inositolphosphate produced by the hydrolysis of phosphatidylinositol by a phospholipase C mode of action. To check which species of phospholipid was being hydrolyzed, the lipid extracts of culture supernatant treated and control treated lipid suspensions were analyzed by two dimensional thin layer chromatography as described. Table I shows the amount of each phospholipid species remaining after S. aureus culture supernatant treatment, as well as the phospholipid content in percent of the total phospholipids present for the control incubations. Only the phosphatidylinositol content was changed by the culture supernatant treatment, resulting in about 41 % hydrolysis of the total phospha-

TABLE I

Analysis of commercial soybean phosphatidylcholine type IV-S for phospholipid content and the specificity of the Staphylococcus aureus culture supernatant phospholipase C.

Phospholipid species	Control µmol P	S. aureus supernatant treated µmol P	Control Species % of total
Phosphatidylcholine	4.11	4.14	46.5
Phosphatidylethanolamine	2.66	2.67	28.6
Phosphatidic acid	0.69	0.70	7.27
Phosphatidylinositol	0.47	0.28*	4.96
Lysophosphatidylcholine	0.37	0.42	3.29
Phosphatidylglycerol	0.20	0.21	2.14
Lysophosphatidylethanolamine	0.22	0.20	1.94
Origin	0.15	0.16	1.63
Cardiolipin	0.13	0.14	1.38
Unknown	0.11	0.11	1.15
Phosphatidylserine	0.01	0.01	0.13

Values represent the means of duplicate results, or of two experiments performed in duplicate for the control phospholipid species percentage of the total. P - phosphorus.

In a 1.0 ml final volume, 0.5 ml of soybean lipid suspension (20 mg/ml), 0.2 ml of 100 mM HEPES-NaOH buffer, pH 7.0, and 0.3 ml of dialyzed culture supernatant (or water for the controls) were incubated for 2 hours at 37°C. The phospholipids were extracted, isolated, and analyzed for lipid phosphorus as described in the methods.
Significance level (Student's t-test): * - $p < .001$

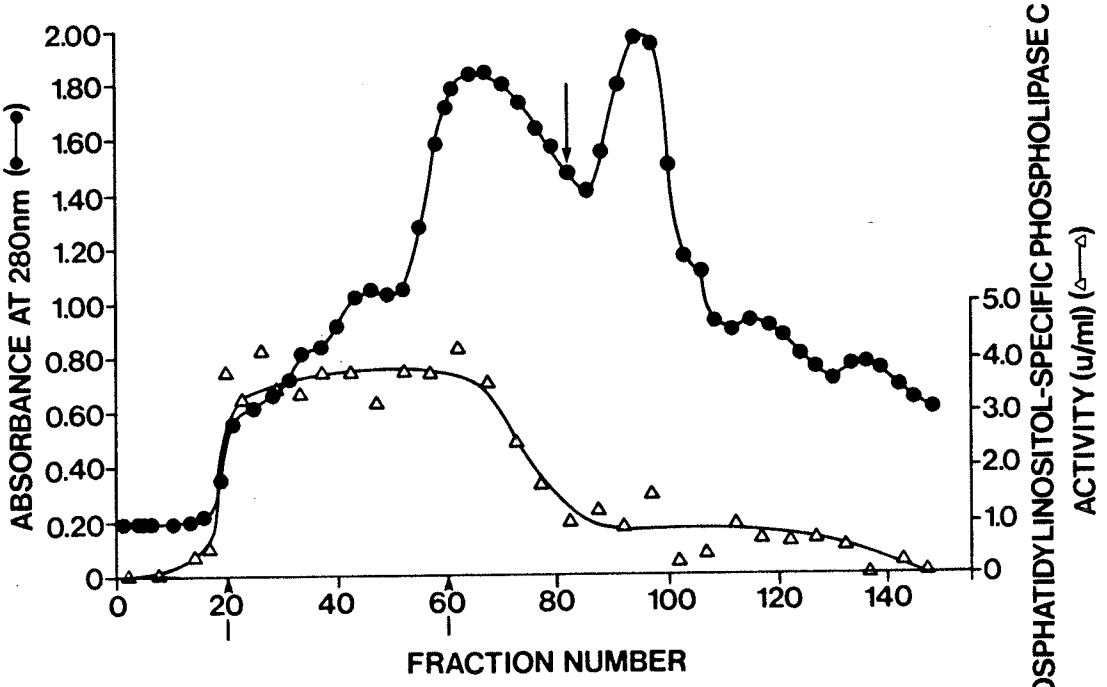
tidylinositol present. Further assays of phosphatidylinositol-specific phospholipase C were carried out by measuring the amount of organic phosphorus released as found in the upper phase of the lipid extracts. By this method, the phosphatidylinositol-specific phospholipase C activity was found to be 7.9 units per ml of culture supernatant. Subsequent preparations contained approximately 4.4 and 1.3 units/ml of culture supernatant.

Passage of the culture supernatants through an Amberlite CG-50 column resulted in binding of the phospholipase C with a substantial loss of total activity which could not be detected in the eluate collected during sample application or in the column washings. Phospholipase C activity was eluted as a wide band during the gradient, (Figure 3). Although substantial protein remained on the column after the gradient finished, further addition of 1 M trisodium citrate, (top arrow) or 5N NaCl (not shown), could elute most of the proteins, but no substantial phosphatidylinositol-specific phospholipase C activity. No phospholipase C activity could be detected if the column fractions were assayed without dialysis followed by lyophilization and reconstitution. Fractions containing greater than 3.0 units/ml, (Fractions 20-60, Figure 3), were pooled and concentrated by ultrafiltration with no loss of activity.

Gel filtration of the pooled and concentrated Amberlite CG-50 fractions was used to further purify the phosphatidylinositol-specific phospholipase C activity. One major and at least two minor protein peaks were found using a Sephadex G-75 column, (Figure 4). Phospho-

Figure 3 Amberlite CG-50 column of Staphylococcus aureus culture supernatant for the preparation of a phosphatidylinositol-specific phospholipase C. (Arrows indicate the fractions which were pooled for further purification)

Figure 4 Fractionation of pooled and concentrated Amberlite CG-50 column fractions on a Sephadex G-75 column for the preparation of a phosphatidylinositol-specific phospholipase C. (Arrows indicate the fractions which were pooled and concentrated for later use)


ABSORBANCE AT 280nm (●—●)
2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0
0
20
40
60
80
100
120
140
FRACTION NUMBER
PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C ACTIVITY (u/ml) (△—△)
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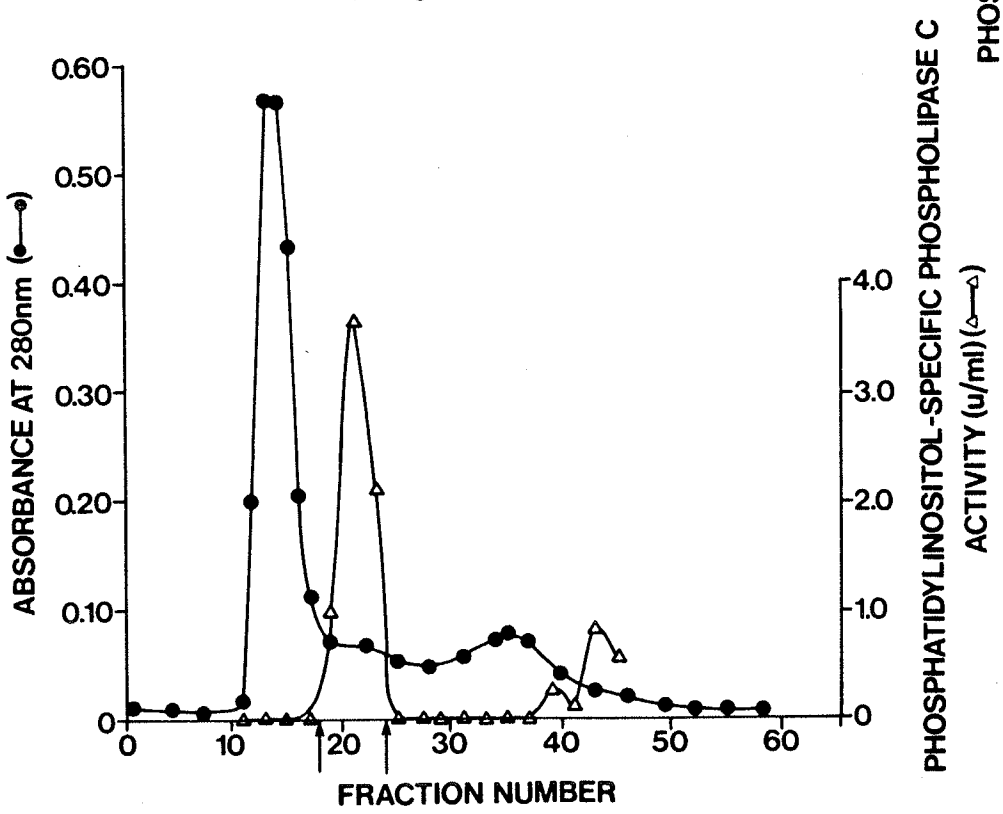
4.0
3.0
2.0
1.0
0


ABSORBANCE AT 280nm (●—●)
0.60
0.50
0.40
0.30
0.20
0.10
0
0
10
20
30
40
50
60
FRACTION NUMBER
PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C ACTIVITY (u/ml) (△—△)
4.0
3.0
2.0
1.0
0

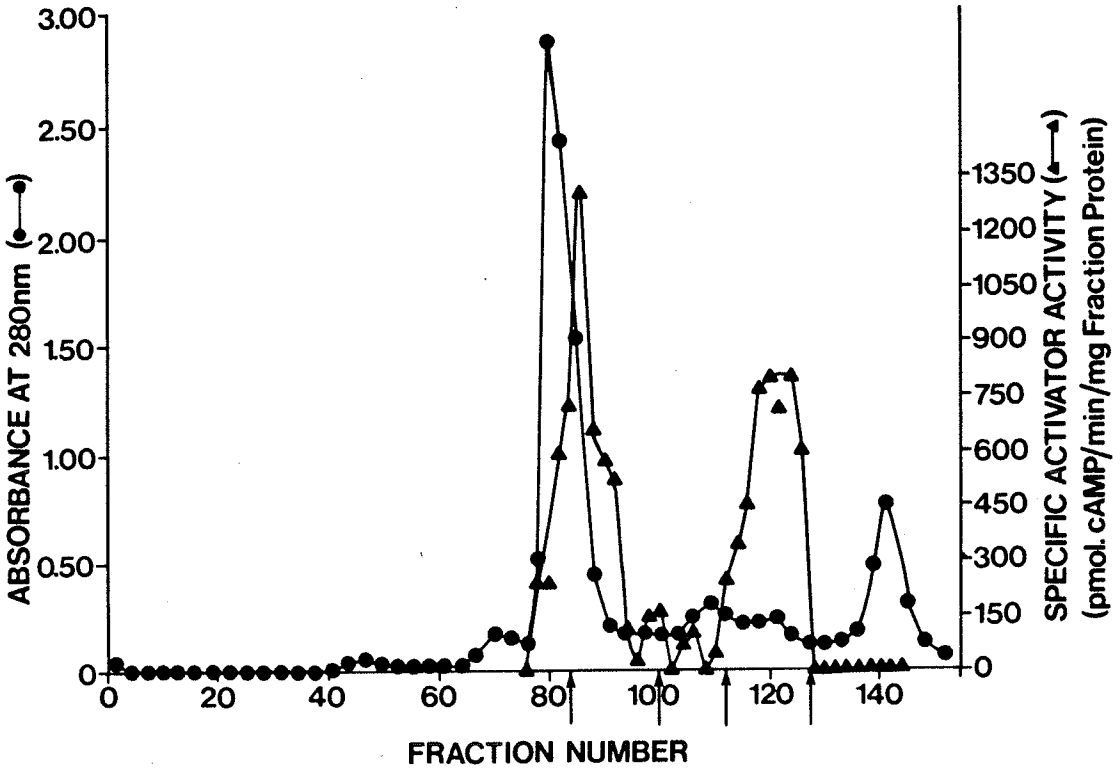
lipase C activity was associated with the minor protein peak in fractions 18 to 24. No activity could be detected in either the major protein peak or the second minor protein peak. Those fractions containing greater than 1.0 units/ml were pooled and concentrated by ultrafiltration to a final concentration of 7.0 units/ml and saved for use in later experiments.

The cytoplasmic activator of rat lung adenylate cyclase activity was prepared by acid precipitation of proteins at pH 5.0, followed by ion exchange chromatography of the supernatant on a DEAE-52 cellulose column. Two peaks of adenylate cyclase activator activity (Figure 5), were pooled separately yielding specific activator activities of 530 and 640 pmol cyclic AMP/minute/mg protein respectively, compared to activator activities in the 105,000xg supernatant of 132 and acid precipitated supernatant of 357 pmol cAMP/minute/mg protein. Peak 1 activator activity is low because the fractions pooled were selected so as to avoid the major protein peak as well as to include as much activity as possible for further purification.

The elution profile of the second DEAE-cellulose column of pooled peak 1 proteins is shown in Figure 6. Adenylate cyclase activator activity was eluted in those fractions between the two major protein peaks, indicating that the peak 1 activator protein is a minor constituent. Additional tightly bound proteins could be eluted with 300 mM Tris-HCl, pH 7.4, but these fractions contained no adenylate cyclase activator activity. Fractions 70 to 82 were pooled to yield a final specific activity of about 24,000pmol cAMP/minute/mg peak 1

Figure 5 Fractionation of the supernatant after acid precipitation of the 105,000xg rat lung supernatant on a DEAE 52 cellulose column. (Arrows indicate the fractions containing peak 1 and peak 2 adenylate cyclase activator activity which were pooled separately for further purification.)

Figure 6 Fractionation of the pooled peak 1 activator activity containing fractions on a second DEAE-cellulose column. (Arrows indicate the fractions which were pooled and concentrated for later use.)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2.50
2.00
1.50
1.00
0.50
0
ABSORBANCE AT 280nm (●—●)
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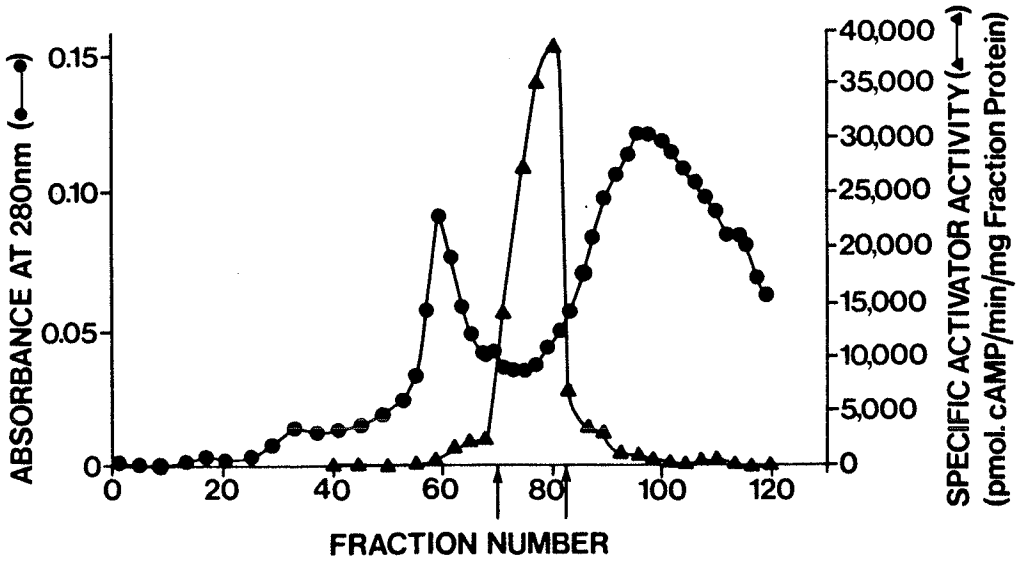
20
40
60
80
100
120
140
FRACTION NUMBER
1350
1200
1050
900
750
600
450
300
150
0
SPECIFIC ACTIVATOR ACTIVITY (▲—▲)
(pmol. cAMP/min/mg Fraction Protein)


0.15
0.10
0.05
0
ABSORBANCE AT 280nm (●—●)
0
20
40
60
80
100
120
FRACTION NUMBER
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35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
SPECIFIC ACTIVATOR ACTIVITY (▲—▲)
(pmol. cAMP/min/mg Fraction Protein)

protein when assayed in the presence of peak 2.

Pooled peak 2 activator proteins from the first DEAE-cellulose column of the supernatant were further resolved as described in the methods to yield a final specific activity of about 1780 pmol cAMP per minute/mg peak 2 protein after the Sepharose 6B column when assayed in the presence of peak 1. When both peak 1 and peak 2 were assayed alone, the activator activity was low but detectable. When either peak was assayed with a very small amount of the other peak included, the activator activity was much more than additive. Recent results suggest that peak 1 and peak 2 in pure form may not contain any detectable activity if assayed separately, (M. S. Nijjar, personal communication). It should be noted that different peak 1 and peak 2 preparations were used in some of the experiments in this study which could contribute to variable degrees of stimulation.

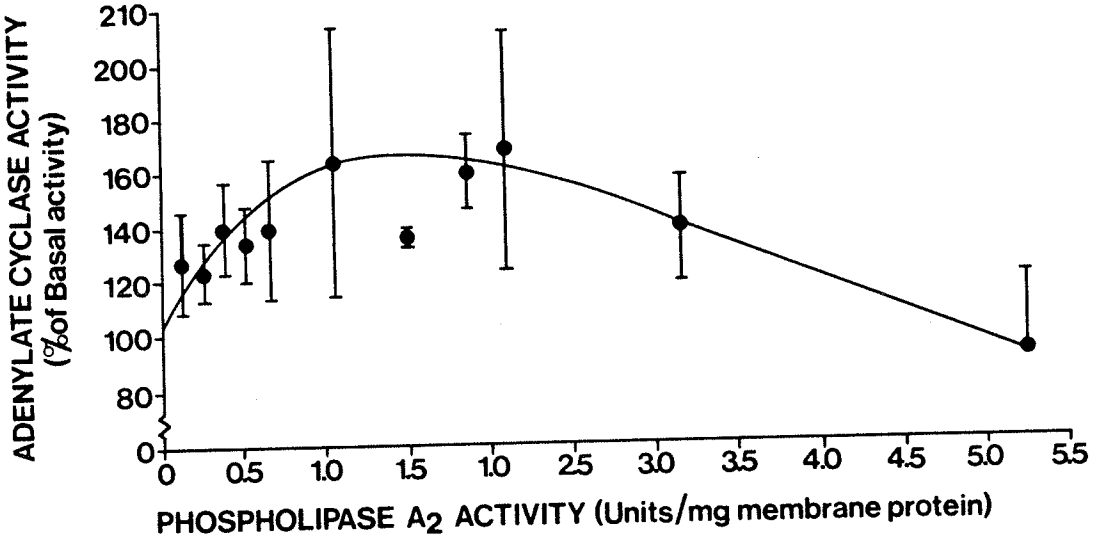
The dose-response curves of adenylate cyclase stimulation by membrane treatment with phospholipase A_2 (Naja naja), phosphatidyl-inositol-specific phospholipase C, general phospholipase C (type I from Clostridium perfringens), and phospholipase D are shown in Figures 7 to 10 respectively. Phospholipase A_2 treatment of plasma membranes gave a highly variable response of adenylate cyclase stimulation, depending upon the source of phospholipase A_2. Treatment with phospholipase A_2 from Naja naja venom showed a maximal stimulation of 1.65 fold of the control activity at approximately 1.0 to 2.0 units per mg of membrane protein, (Figure 7). Greater concentrations of phospholipase A_2 treatment resulted in decreased stimulation and

inhibition to 0.93 fold of control activity at 5.25 u/mg membrane protein. Basal adenylate cyclase activity could be further inhibited to a minimum of 0.29 fold of control membrane activity at approximately 2100 u/mg membrane protein (not shown). When plasma membranes were treated with phospholipase A_2 from Vipera russelli venom (as a 50 % glycerol suspension), the effect on basal adenylate cyclase was highly variable and often showed inhibition at phospholipase concentrations which were stimulatory with the enzyme from N. naja venom (not shown).

Treatment of plasma membranes with the phosphatidylinositol-specific phospholipase C had no appreciable effect on the basal adenylate cyclase activity over the concentrations tested, (Figure 8). In contrast, treatment with phospholipase C from Clostridium perfringens resulted in a 2.65 fold of basal adenylate cyclase activity as compared to control membranes at a 25 u/mg membrane protein concentration (Figure 9). Half maximal stimulation of basal adenylate cyclase activity was apparent at 5 u/mg of phospholipase C and the maximal stimulation was constant up to 100 u/mg membrane protein. Phospholipase D treatment of plasma membranes showed a similar activation profile with a maximal stimulation of 3.7 fold of control activity at 150 u/mg membrane protein which was constant up to 300 u/mg concentrations, (Figure 10). Half maximal stimulation of basal adenylate cyclase activity occurred at approximately 35 u/mg membrane protein. Addition of 1 mM $CaCl_2$ to the phospholipases during the plasma membrane treatment could not significantly alter the effect of any of the phospholipases on the adenylate cyclase activity, however,

Figure 7 Effect of phospholipase A_2, (Naja naja), treatment on the basal adenylate cyclase activity in rat lung plasma membranes. Each point represents the mean percent of control adenylate cyclase activity ± SEM for at least 4 observations.

Figure 8 Effect of phosphatidylinositol-specific phospholipase C treatment on the basal adenylate cyclase activity in rat lung plasma membranes. Each point represents the mean percent of control adenylate cyclase activity ± SEM for 4 observations.


ADENYLATE CYCLASE ACTIVITY
(%of Basal activity)
210
200
180
160
140
120
100
80
0
0
0.5
1.0
1.5
1.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
PHOSPHOLIPASE A2 ACTIVITY (Units/mg membrane protein)

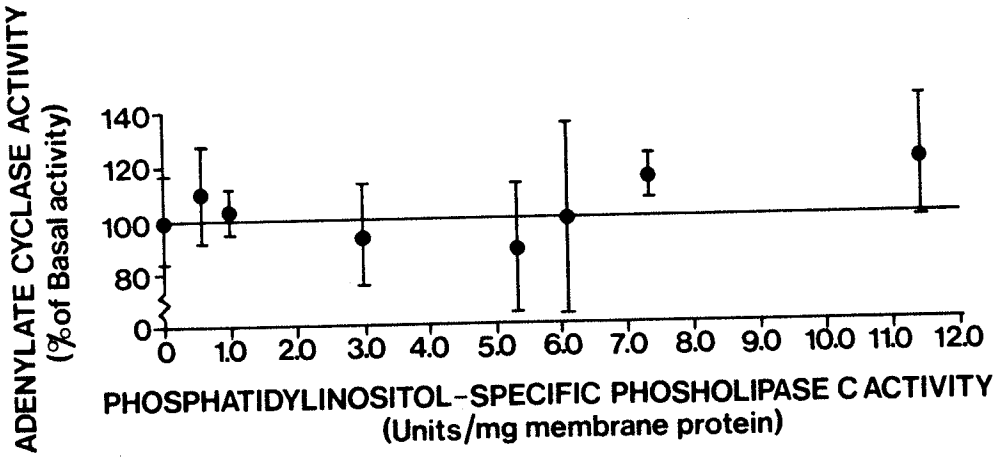
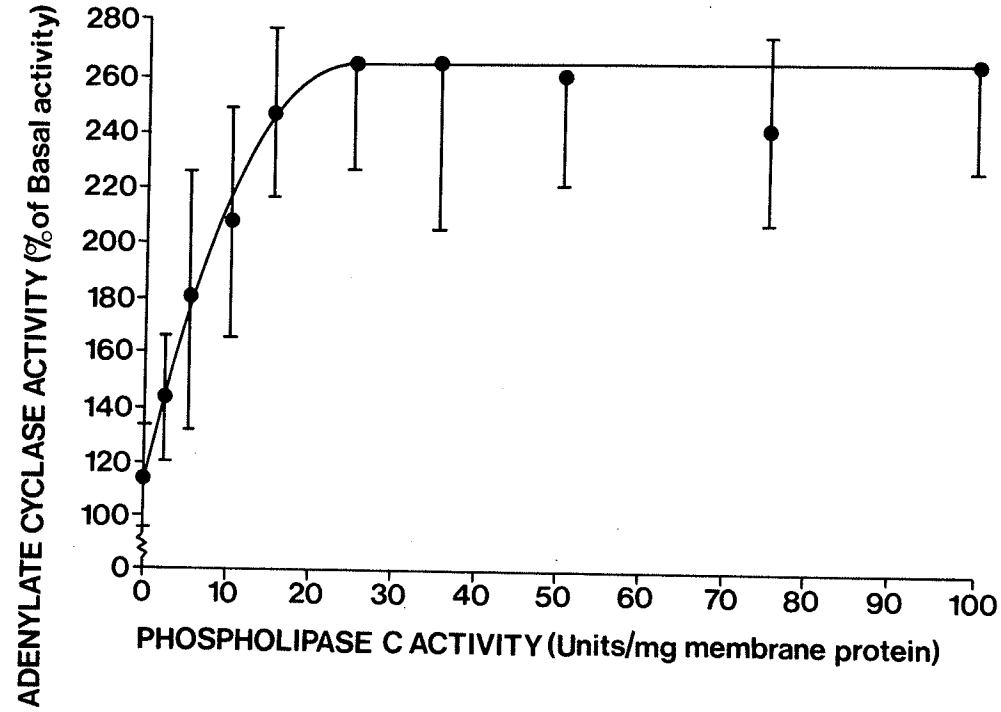
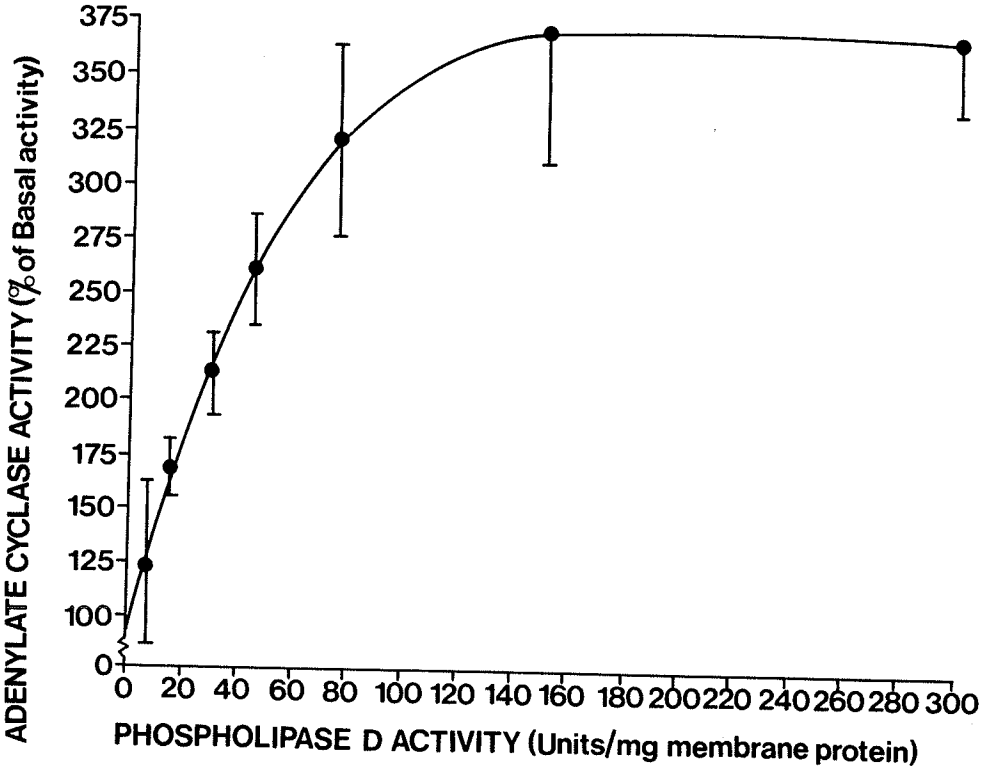

ADENYLATE CYCLASE ACTIVITY
(%of Basal activity)
140
120
100
80
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
11.0
12.0
PHOSPHATIDYLINOSITOL-SPECIFIC PHOSHOLIPASE C ACTIVITY
(Units/mg membrane protein)

Figure 9 Effect of phospholipase C, (Clostridium perfringens), treatment on the basal adenylate cyclase activity in rat lung plasma membranes. Each point represents the mean percent of control adenylate cyclase activity ± SEM of at least 4 observations.

Figure 10 Effect of phospholipase D treatment on the basal adenylate cyclase activity in rat lung plasma membranes. Each point represents the mean percent of control adenylate cyclase activity ± SEM of 4 observations.


ADENYLATE CYCLASE ACTIVITY (% of Basal activity)
280
260
240
220
200
180
160
140
120
100
0
0 10 20 30 40 50 60 70 80 90 100
PHOSPHOLIPASE C ACTIVITY (Units/mg membrane protein)


ADENYLATE CYCLASE ACTIVITY (% of Basal activity)
375
350
325
300
275
250
225
200
175
150
125
100
0
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300
PHOSPHOLIPASE D ACTIVITY (Units/mg membrane protein)

the basal adenylate cyclase activity was inhibited by approximately 20 % of the control level (not shown).

The time course profiles of adenylate cyclase activation by the phospholipases A_2, C, and D; and the activator proteins are seen in Figure 11. The accumulation of cyclic AMP was linear for 30 minutes in control incubated membranes. Phospholipase A_2 and phospholipase D treated membranes also showed a linear cyclic AMP accumulation over 30 minutes, but with greater slopes. Phospholipase C treated membranes exhibited a two phase activation profile with a linear cAMP accumulation slope for 10 minutes similar to the phospholipase D profile, after which the accumulation increased to a slightly steeper slope for the remainder of the 30 minute incubation. In contrast, the adenylate cyclase activator proteins exhibited a lag phase of approxiamately 7.5 minutes with no apparent stimulation before a rapid and constant accumulation of cAMP over the remainder of the incubation. This lag phase could not be abolished by up to 15 minutes of preincubation of the plasma membranes with the activator proteins.

The effects of phospholipases A_2, C, and D; and the activator proteins on the ATP substrate kinetics of adenylate cyclase activity are shown in Table II. Incubated control plasma membranes had an apparent K_m of 0.24 mM ATP and V_{max} of 220 pmol cAMP/minute/mg plasma membrane protein. All treatments resulted in an increased maximum velocity of the adenylate cyclase activity up to about 1.4 fold of control membranes with 50.0 u/mg of phospholipase D and with 2.8 µg

Figure 11 Time course of cAMP accumulation in phospholipase A_2, C, and D; and the activator peaks treated membranes. Each point represents the mean cAMP accumulation expressed in 10^3 pmol/mg membrane protein $\pm$ SEM of two experiments performed in duplicate.

o - Control plasma membranes.

□ - Phospholipase A_2 treated plasma membranes (1.0 u/mg membrane protein, N. naja).

Δ - Phospholipase C treated plasma membranes (25.0 u/mg membrane protein).

● - Phospholipase D treated plasma membranes (50.0 u/mg membrane protein).

▼ - Activator treated plasma membranes (16.8 µg peak 1, 8.0 µg peak 2).

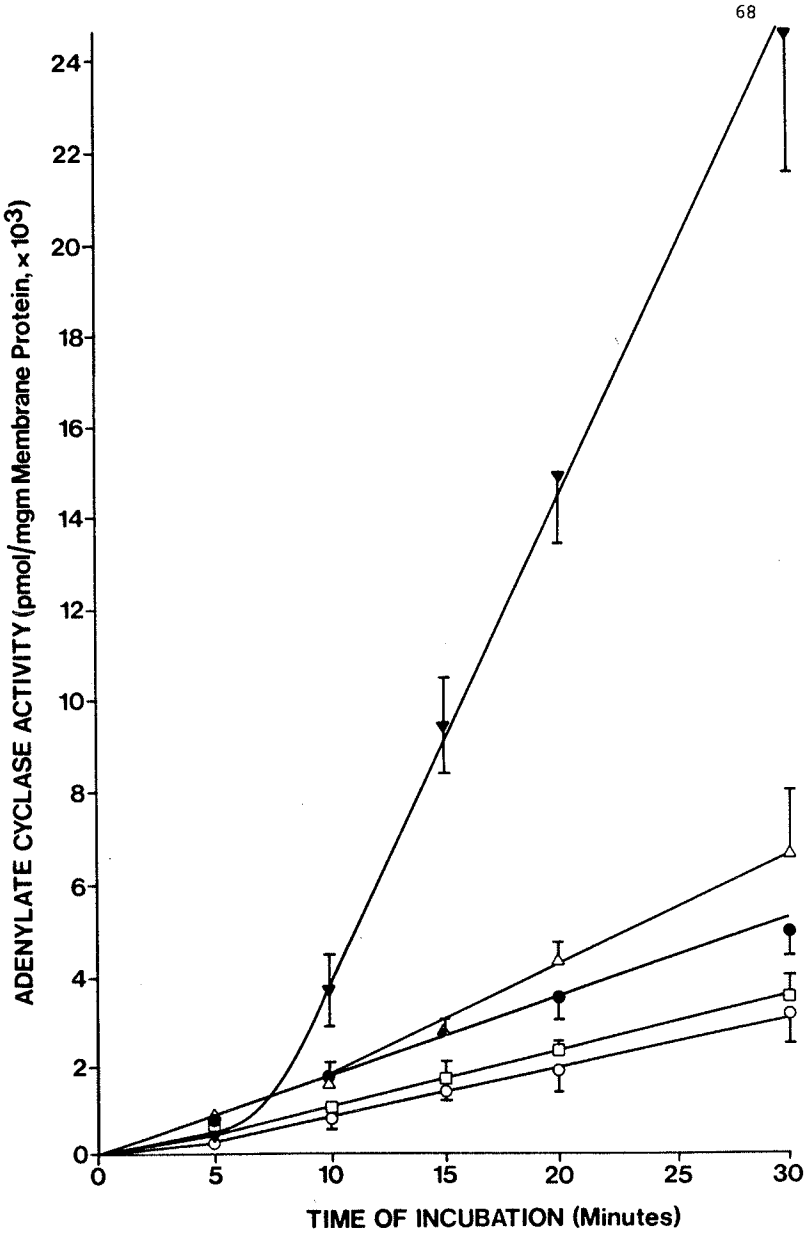

ADENYLATE CYCLASE ACTIVITY (pmol/mgm Membrane Protein, × 10³)
0
2
4
6
8
10
12
14
16
18
20
22
24
0
5
10
15
20
25
30
TIME OF INCUBATION (Minutes)

TABLE II

Effects of phospholipases A_2, C, and D; and the cytosolic activator protein peaks 1 and 2 on the ATP substrate kinetics of the adenylate cyclase activity in rat lung plasma membranes.

Treatment	Apparent K_m (mM ATP)	V_{max} (pmol cyclic AMP/min per mg PM protein)
Control plasma membranes	0.240	220
Phospholipase A_2 (*N. naja*) (1.0 u/mg membrane protein)	0.240	233
Phospholipase C (25.0 u/mg membrane protein)	0.125	253
Phospholipase D (50.0 u/mg membrane protein)	0.273	302
Activator (2.8 µg peak 1, 1.5 µg peak 2)	0.367	301

Plasma membranes were incubated at 37°C for 15 minutes in the presence of the phospholipases or the activator proteins before the addition of varying concentrations of ATP ranging from 0.05 to 1.0 mM, to the assay medium and the adenylate cyclase assay was performed as described in the methods. Control plasma membranes were preincubated in an equal volume of 10 mM Tris-HCl buffer, pH 7.4. The data represent the means of two experiments performed in duplicate and calculated by linear regression analysis of a Lineweaver-Burke plot of the reciprocals of the ATP concentrations and the adenylate cyclase activities.

of peak 1 and 1.5 µg of peak 2 activator proteins, at 302 and 301 pmol cAMP/minute/mg membrane protein respectively. Both of these treatments also resulted in higher apparent K_m's for ATP of the adenylate cyclase activity. Phospholipase C treated membranes showed a slight increase in maximal adenylate cyclase velocity and the apparent K_m for ATP was decreased to 0.125 mM. Phospholipase A_2, (N. naja), treatment of plasma membranes resulted in no change in the affinity for ATP and very little increase in the adenylate cyclase maximum velocity.

The effect of ultracentrifugation on the stimulation of rat lung plasma membrane adenylate cyclase activity by phospholipases and the activator peaks can be seen in Table III. Control membranes incubated for 30 minutes in 10 mM Tris-HCl buffer showed a specific activity of 68.4 pmol cAMP/minute/mg membrane protein. When these incubated control membranes were ultracentrifuged twice at 105,000xg for 60 minutes and resuspended in an equal volume of homogenizing buffer, the specific adenylate cyclase activity was virtually unchanged. Treatment of plasma membranes with 1.0 u/mg membrane protein of phospholipase A_2 from V. russelli for 30 minutes inhibited the basal adenylate cyclase activity to 0.59 fold of control activity which was further decreased to 0.47 fold of control activity by ultracentrifugation and resuspension. Phosphatidylinositol-specific phospholipase C treatment of plasma membranes at 1.0 u/mg membrane protein, had no effect on the adenylate cyclase activity when incubated only, and no change in the specific activity could be detected when treated and ultracentrifuged membranes were assayed. Treatment of

plasma membranes with 25.0 u/mg membrane protein of general phospholipase C and with 50.0 u/mg membrane protein of phospholipase D resulted in stimulation of the basal adenylate cyclase activity to 3.40 and 1.98 fold of control activity respectively. The stimulation of the adenylate cyclase activity by phospholipase C or D treatment could be completely reversed to control levels by the removal of the phospholipases by ultracentrifugation. Incubation of plasma membranes with the activator protein peaks (3.12 µg peak 1, 1.8 µg peak 2 per 50 µg membrane protein) resulted in stimulation to 2.47 fold of control adenylate cyclase activity which could be reversed to only 1.24 fold of control activity when ultracentrifuged before the assay. No adenylate cyclase activity could be detected in the supernatants from ultracentrifugation of any of the treated or control membranes. Readdition of the supernatants to the adenylate cyclase assay of the phospholipase C, D, and activator treated and centrifuged pellets resulted in restimulation of the basal adenylate cyclase activity, but to a slightly lesser degree (not shown). Readdition of the control, phospholipase A_2, or phosphatidylinositol-specific phospholipase C treated supernatants to the assay of their respective pellets resulted in no significant change in the adenylate cyclase activity.

If membranes were treated with phospholipase C or D and spun down at 25,000xg, the adenylate cyclase activity was affected to variable degrees, depending upon the recovery of protein in the resultant pellets (not shown). When the recovery of protein in the phospholipase treated membranes was greater than in control membranes, the increase in the adenylate cyclase activity was still apparent.

TABLE III

The effects of ultracentrifugation on the stimulation of rat lung plasma membrane adenylate cyclase activity by phospholipases and the activator protein peaks 1 and 2.

Treatment	Incubated only	Incubated and ultracentrifuged
Control *	68.4 ± 16.3 (n=7)	68.6 ± 6.0 (n=5)
Phospholipase A_2 (1.0 u/mg PM protein)	0.59 ± 0.06[a] (n=3)	0.47 ± 0.10[a] (n=6)
Phosphatidylinositol-specific phospholipase C (1.0 u/mg)	0.99 ± 0.04 (n=4)	0.97 ± 0.22 (n=7)
Phospholipase C (25.0 u/mg PM protein)	3.40 ± 0.44[a] (n=4)	1.02 ± 0.07 (n=4)
Phospholipase D (50.0 u/mg PM protein)	1.98 ± 0.23[b] (n=4)	1.05 ± 0.18 (n=7)
Activator (3.12 µg peak 1, 1.8 µg peak 2 per 50 µg PM protein)	2.47 ± 0.08[a] (n=3)	1.24 ± 0.22[c] (n=3)

Values represent the mean fold of control adenylate cyclase activity (* - values are pmol cAMP/minute/mg membrane protein) ± SEM of the number of observations in brackets.

Significance levels (Student's t-test): [a - $p<.001$; b - $p<.005$; c - $p<.05$]

Plasma membranes (750 µg) were incubated for 30 minutes at 37°C in the centrifuge tubes containing the indicated amount of phospholipase or activator protein peaks. Control tubes contained an equal volume of 10 mM Tris-HCl buffer, pH 7.4. Aliquots containing 50 µg of membrane protein were taken from each sample and the remainder spun down at 105,000xg for 60 minutes. The supernatants were withdrawn and saved, and the pellets were washed by resuspension and resedimenting at 105,000xg for 60 minutes. The final pellets were resuspended in an equal volume of 0.27 M sucrose in 10 mM Tris-HCl buffer, pH 7.4, before assay of adenylate cyclase activity as described in the methods.

The degree of stimulation retained was dependent upon the difference in the amount of protein recovered between the treated and the control pellets, suggesting that the phospholipases bind tightly to the membranes.

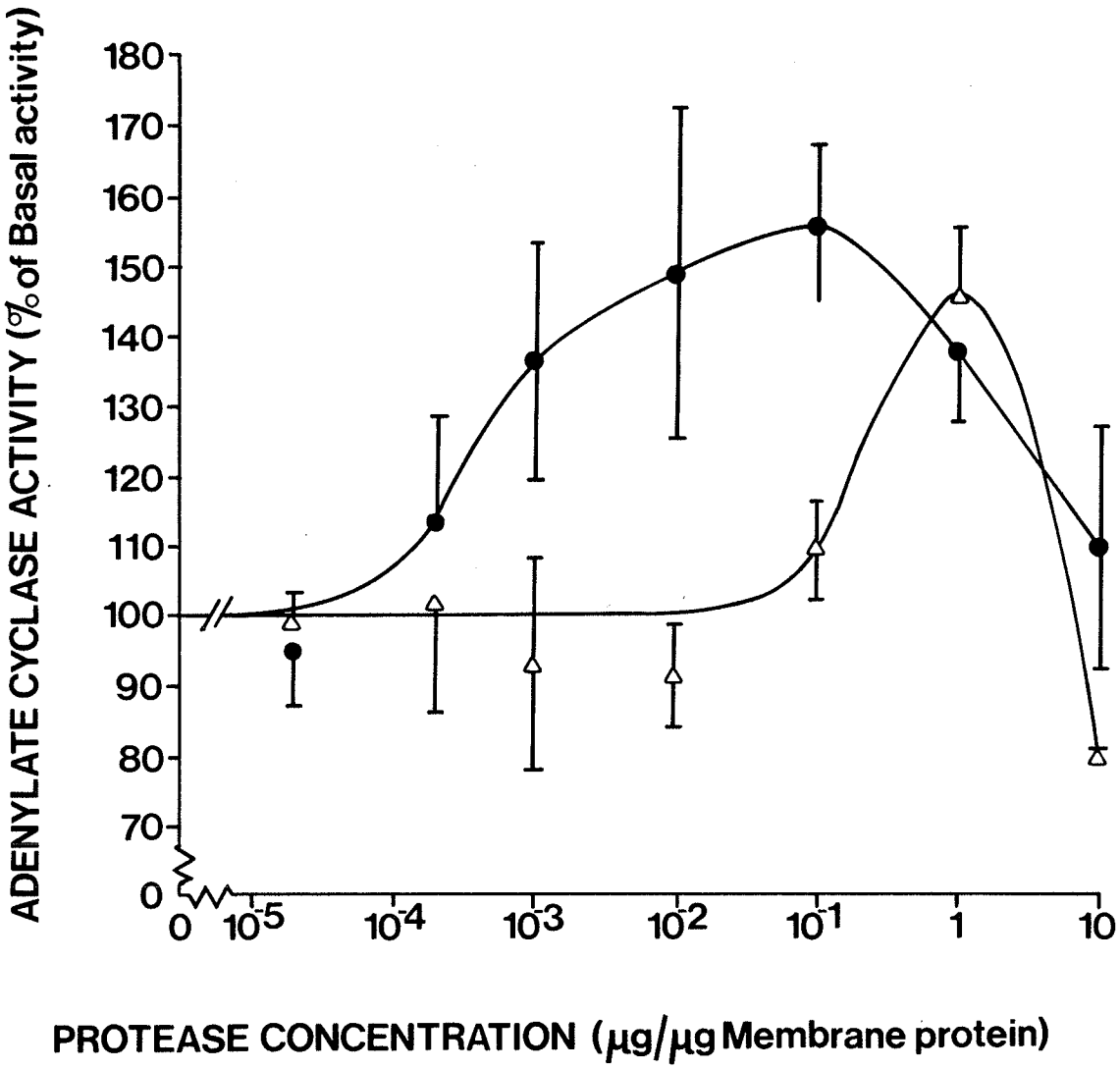
Plasma membranes were also treated with various concentrations of trypsin and chymotrypsin in order to study the effect of limited proteolysis on the control of the adenylate cyclase system. Figure 12 shows the dose-response curves for the two proteases' effect on basal adenylate cyclase activity. Trypsin treatment of plasma membranes showed a narrow activation range of adenylate cyclase activity with a maximum of 1.45 fold of control activity at a concentration of 1.0 µg/µg membrane protein, above which strong inhibition occurred. At concentrations of less than 0.1 µg/µg membrane protein, trypsin had no effect on basal adenylate cyclase activity. In contrast, chymotrypsin treatment of plasma membranes exhibited a much broader adenylate cyclase activation range with a maximum of 1.55 fold of control activity at 0.1 µg/µg membrane protein. Concentrations of less than 1×10^{-4} µg of chymotrypsin per µg of membrane protein had no effect on the basal adenylate cyclase activity, while the stimulation decreased to 1.10 fold of control activity at 10 µg/µg. Higher concentrations were not tested.

In order to assess the effects of the membrane perturbing agents upon various stimulated adenylate cyclase activities, it was necessary to determine a fraction from rat lung homogenate which contained stimulatable adenylate cyclase activity. Table IV shows

Figure 12 Effect of trypsin and chymotrypsin treatment on the basal adenylate cyclase activity in rat lung plasma membranes. Each point represents the mean percent of control adenylate cyclase activity ± SEM of two experiments performed in duplicate.

● - Chymotrypsin treatment.

Δ - Trypsin treatment.


ADENYLATE CYCLASE ACTIVITY (% of Basal activity)
180
170
160
150
140
130
120
110
100
90
80
70
0
0
10-5
10-4
10-3
10-2
10-1
1
10
PROTEASE CONCENTRATION (µg/µg Membrane protein)

the response of adenylate cyclase activity in 900xg, 6,000xg, 8,000xg, and plasma membranes to stimulation with NaF, GppNHp (5'-guanylyl-imidodiphosphate), epinephrine bitartrate, and the activator proteins. Basal adenylate cyclase activity was found to rise with increasing g force in centrifugation to 79.5 pmol cAMP/minute/mg protein in plasma membranes. In contrast, enhancement of adenylate cyclase activity with the various stimulating agents was found to generally decrease in pellets with increasing g force in centrifugation. Stimulation with 15 mM NaF and with 100 μM GppNHp showed the highest response in adenylate cyclase activation in the 900xg pellet with 9.16 and 8.21 fold of control activity respectively. When the adenylate cyclase activity of the various pellets was assayed in the presence of 2.6 μg of peak 1 and 1.5 μg of peak 2 activator proteins, the highest response was found in the 900xg and 6,000xg pellets with 4.75 fold activity relative to the control levels. Assay in the presence of 500 μM epinephrine bitartrate resulted in a maximum stimulation of only 1.54 fold of control activity in the 900xg pellet. Plasma membranes showed no response to GppNHp or epinephrine and only 1.38 fold of control activity in the presence of NaF. Inclusion of the activator peaks in the assay of adenylate cyclase in the plasma membranes yielded 2.80 fold of control activity.

From the data in Table IV it was decided that the 6,000xg pellet would be suitable for studying the effect of phospholipases and proteases on the various stimulated adenylate cyclase activities since this homogenate fraction contained relatively good response to NaF, GppNHp, epinephrine, and the activator peaks, as well as a

TABLE IV

Response of adenylate cyclase activity in various rat lung homogenate fractions to stimulation by NaF, GppNHp, epinephrine, and the activator peaks 1 and 2.

Pellet	Basal adenylate cyclase activity[a]	NaF (15 mM)[b]	GppNHp (100 μM)[b]	Epinephrine bitartrate (500 μM)[b]	Activator (2.6 μg peak 1, 1.5 μg peak 2)[b]
900xg	8.75 ± 1.99 (n=6)	9.16 ± 0.78[a] (n=4)	8.21 ± 0.55[a] (n=4)	1.54 ± 0.23[d] (n=3)	4.75 ± 0.73[a] (n=4)
6,000xg	22.4 ± 10.4 (n=15)	4.24 ± 1.26[a] (n=8)	3.54 ± 1.13[a] (n=8)	1.31 ± 0.20[e] (n=10)	4.75 ± 1.48[a] (n=12)
8,000xg	46.1 ± 14.9 (n=4)	2.85 ± 0.14[a] (n=4)	1.79 ± 0.25[b] (n=4)	1.31 ± 0.29 (n=4)	4.37 ± 1.07[a] (n=4)
Plasma membranes	79.5 ± 26.0 (n=10)	1.38 ± 0.05[c] (n=4)	0.98 ± 0.10 (n=10)	1.02 ± 0.09 (n=5)	2.80 ± 0.45[a] (n=10)

a - Values represent mean pmol cAMP/minute/mg protein ± SEM of the number of observations in brackets.

b - Values represent mean fold of control adenylate cyclase activity ± SEM of the number of observations in brackets.

Significance levels (Student's t-test): [a - $p<.001$; b - $p<.005$; c - $p<.025$; d - $p<.01$; e - $p<.05$]

reasonable basal adenylate cyclase activity. Treatment of the 6,000xg pellet with phospholipase A_2 (V. russelli), C, and D shown in Table V, resulted in slightly different response in adenylate cyclase activity as compared to the similarly treated plasma membranes (Figures 7,9,10). Phospholipase A_2 inhibited the basal adenylate cyclase activity to 0.52 fold of control levels while phospholipase C and D treatment stimulated to 5.56 and 11.5 fold of control activity respectively, (Table V). Trypsin treatment of the 6,000xg pellet yielded only 1.14 fold of control activity while chymotrypsin treatment resulted in 1.54 fold of control adenylate cyclase activity. Addition of 100 μM GTP to the assay medium, stimulated the adenylate cyclase to 1.64 fold of control activity while the nonhydrolyzable analogue, GppNHp, resulted in stimulation to 4.0 fold of control activity at the same concentration. Addition of 500 μM epinephrine bitartrate yielded only 1.26 fold of control adenylate cyclase activity which showed only additive stimulation in the presence of 100 μM GTP (not shown).

Phospholipase A_2 treatment of 6,000xg pellet showed inhibition of all of the stimulated adenylate cyclase activities relative to control levels (Table V). If the adenylate cyclase activities were calculated relative to the phospholipase A_2 treated membranes alone, the NaF and epinephrine enhanced activities are slightly increased to 6.48 versus 5.57 fold and 1.63 versus 1.26 fold of the relative control activities for NaF and epinephrine stimulation respectively. Stimulation of phospholipase A_2 treated membranes with GppNHp and with the activator protein peaks gave slightly less relative fold activity than in the similarly treated control membranes, while the

GTP enhancement of adenylate cyclase activity was completely abolished.

In phospholipase C treated 6,000xg pellets, the predominant stimulatory effect upon the adenylate cyclase activity appeared to be the phospholipase as further stimulation with GppNHp gave only a slight increase in fold activity of controls, while epinephrine had no further stimulation. Addition of NaF or GTP resulted in a slight inhibition of the phospholipase C stimulated activity, while addition of the activator peaks 1 and 2 resulted in slight cooperativity of stimulation of adenylate cyclase up to 11.9 fold of control activity. Similarly, in the phospholipase D treated 6,000xg pellet, the further addition of epinephrine had no effect. Addition of the activator peaks resulted in a slight further increase in phospholipase C stimulated adenylate cyclase activity, which wasn't fully additive to the stimulation found by activator and phospholipase D enhancement alone. Addition of GTP gave an additive effect, while the addition of NaF or GppNHp inhibited the phospholipase D stimulation slightly.

Trypsin treatment almost completely abolished all further stimulation by NaF, GppNHp, GTP, epinephrine, or the activator peaks. In contrast, chymotrypsin treatment of the 6,000xg pellet showed only slight inhibition of further stimulation with the activator peaks and relatively little additional enhancement upon the addition of NaF or epinephrine. Addition of GppNHp or GTP resulted in slight inhibition of the chymotrypsin stimulated adenylate cyclase activity.

Activator peak stimulated adenylate cyclase activity could

TABLE V

Effect of phospholipase and protease membrane treatment on the response of adenylate cyclase activity in 6,000xg pellet to NaF, GppNHp, GTP, epinephrine, and the activator peaks 1 and 2.

Treatment	None	NaF (15 mM)	GppNHp (100 μM)	GTP (100 μM)	Epinephrine bitartrate (500 μM)	Activator (2.6 μg peak 1, 1.5 μg peak 2)
Control	20.6 ± 10.0*	5.57 ± 1.07[a]	4.00 ± 0.38[a]	1.64 ± 0.22[d]	1.26 ± 0.17	4.96 ± 0.28[a]
Phospholipase A_2 (*V. russelli*) (0.5 u/mg)	0.52 ± 0.17[e]	3.37 ± 0.06[a]	1.92 ± 0.02[b]	0.54 ± 0.16[f]	0.85 ± 0.10	1.97 ± 0.17[b]
Phospholipase C (25 u/mg)	5.56 ± 0.10[a]	4.68 ± 0.03[a]	6.46 ± 0.74[a]	4.04 ± 0.20[a]	5.56 ± 0.42[a]	11.9 ± 0.6[a]
Phospholipase D (45 u/mg)	11.5 ± 1.20[a]	7.40 ± 1.49[a]	10.3 ± 0.2[a]	12.8 ± 0.4[a]	11.4 ± 1.10[a]	13.7 ± 1.1[a]
Trypsin (1.0 μg/μg)	1.14 ± 0.14	1.01 ± 0.46	1.21 ± 0.28	1.13 ± 0.08	1.02 ± 0.14	0.92 ± 0.04
Chymotrypsin (0.1 μg/μg)	1.54 ± 0.20[e]	1.76 ± 0.17[c]	1.45 ± 0.23[f]	1.16 ± 0.01	1.70 ± 0.27[d]	4.22 ± 0.58[a]
Activator (2.6 μg peak 1, 1.5 μg peak 2)	4.96 ± 0.28[a]	5.07 ± 0.08[a]	7.31 ± 0.01[a]	3.23 ± 0.56[a]	4.09 ± 0.15[a]	----------

Values represent mean relative fold activity of control (except * - basal adenylate cyclase activity expressed in pmol cAMP/minute/mg pellet protein) ± SEM of two experiments performed in duplicate.
Significance levels (Student's t-test): [a - $p<.001$; b - $p<.005$; c - $p<.01$; d - $p<.025$; e - $p<.05$; f - $p<.10$]
Membranes were treated with phospholipases or proteases as described in the methods and assayed for adenylate cyclase activity in the presence of NaF, GppNHp, GTP, epinephrine, or the activator protein peaks.

only be further enhanced by the addition of GppNHp, while inclusion of GTP or epinephrine resulted in slightly reduced stimulation.

The 6,000xg pellet was also used for testing the effect of various receptor blockers upon the stimulation of adenylate cyclase activity by the activator peaks 1 and 2. Table VI shows that the stimulation of adenylate cyclase activity by the activator peaks 1 and 2 could not be blocked by the β-adrenergic blocker, propanolol; by the prostaglandin blocker, indomethacin; or by the histaminic H_2 blocker, metiamide. Prostaglandin E_1 resulted in adenylate cyclase inhibition to 0.63 fold of control activity which could be partially reversed by indomethacin. Both histamine and the H_2 blocker, metiamide, resulted in slight inhibition of the basal adenylate cyclase activity, while the effect of epinephrine stimulation to 1.33 fold of control activity could be almost completely blocked by propanolol to the level of adenylate cyclase activity found in the presence of propanolol alone.

As the phospholipase C treatment and the activator peaks had a more than additive stimulation of the adenylate cyclase activity in the 6,000xg pellet, it was decided to study their cooperative effect on plasma membranes (Table VII). Treatment of plasma membranes with 5.0 u/mg membrane protein of phospholipase C followed by 1.82 µg peak 1 and 0.75 µg peak 2, resulted in an additive stimulation effect to 2.79 fold of control activity. Treatment of plasma membranes with a maximally stimulating phospholipase C concentration, (35 u/mg membrane protein), followed by addition of 10.4 µg peak 1 and 4.28 µg peak 2, resulted in a highly cooperative stimulation to 18.5 fold of the

TABLE VI

Effect of receptor blockers on the stimulation of adenylate cyclase activity in rat lung 6,000xg pellet by the activator peaks 1 and 2.

Agonist or blocker	Fold of control adenylate cyclase activity	Plus receptor blocker	Plus activator (2.6 µg peak 1, 1.5 µg peak 2)
Epinephrine bitartrate (50 µM)	1.33 ± 0.12[b]	0.87 ± 0.14	------
Propanolol (50 µM)	0.74 ± 0.14[c]	------	3.46 ± 0.10[a]
Prostaglandin E_1 (7 µM)	0.63 ± 0.23[b]	0.88 ± 0.21	------
Indomethacin (10 µM)	1.02 ± 0.21	------	3.81 ± 1.11[a]
Histamine (100 µM)	0.82 ± 0.20	0.83 ± 0.10	------
Metiamide (100 µM)	0.70 ± 0.07[b]	------	3.56 ± 0.37[a]
None	25.9 ± 6.6 *	------	3.53 ± 0.21[a]

Values are mean fold of control adenylate cyclase activity except for (* - basal adenylate cyclase activity of the 6,000xg pellet expressed in pmol cAMP/minute/mg protein) ± SEM of two experiments performed in duplicate.

The 6,000xg pellet was assayed for adenylate cyclase activity in the presence or absence of the various agonists and their respective blockers, and the activator proteins as described in the methods. Significance levels (Student's t-test): [a - $p<.001$; b - $p<.05$; c - $p<.10$]

TABLE VII

Effect of phospholipase C treatment on the stimulation of adenylate cyclase activity in rat lung plasma membranes by the activator protein peaks 1 and 2.

Activator peaks	Phospholipase C treatment		
	None	5.0 u/mg membrane protein	35.0 u/mg membrane protein
None	79.3 ± 12.4*	1.91 ± 0.35[a]	2.65 ± 0.61[a]
1.82 µg peak 1, 0.75 µg peak 2	1.72 ± 0.38[b]	2.79 ± 0.41[a]	NT
10.4 µg peak 1, 4.28 µg peak 2	6.24 ± 1.30[a]	NT	18.5 ± 1.5[a]

Values represent mean fold activity of basal adenylate cyclase activity (* - values are pmol cAMP/minute/mg membrane protein) ± SEM of two experiments performed in triplicate.

NT - not tested

Plasma membranes were incubated for 15 minutes at 37°C in the presence of the phospholipase C or 10 mM Tris-HCl buffer, pH 7.4, before assay of adenylate cyclase activity in the presence or absence of the activator peaks 1 and 2 as described in the methods.

Significance levels (student's t-test): [a - $p < .001$; b - $p < .005$]

control adenylate cyclase activity.

The effect of plasma membrane treatment with the phospholipases and the activator peaks on the phospholipid content is shown in Table VIII. The choline containing phospholipids predominate with phosphatidylcholine accounting for approximately 67.3 % of the total lipid phosphorus. Lysophosphatidylcholine and sphingomyelin accounted for a further 4.8 % and 5.2 % of the total phospholipids respectively. Phosphatidylethanolamine and phosphatidylglycerol content was almost equal at 4.9 % and 4.8 % respectively, while phosphatidylserine and phosphatidylinositol comprised 3.8 % and 2.4 % of the phospholipid content respectively. Only 1.1 % of the total lipid phosphorus could be found remaining at the origin, consisting of inorganic phosphate and polyphosphoinositides (206), while cardiolipin (diphosphatidylglycerol) could account for only 1.7 % of the total. A further 7.1 % of the lipid phosphorus could be detected in an unidentified phospholipid spot.

It can be seen from Table VIII that treatment of plasma membranes with phosphatidylinositol-specific phospholipase C, phospholipase D, or the activator peaks, did not significantly alter the total phospholipid content from the control value of 1125 pmol lipid phosphorus /mg membrane protein. The content of the individual phospholipid species were also unchanged. In contrast, treatment of plasma membranes with phospholipase A_2 (N. naja), or with general phospholipase C (Clostridium perfringens), resulted in a reduction of total lipid phosphorus content by 33 % and 84 % respectively.

Phospholipase A_2 treatment resulted in hydrolysis of 33 % of the phosphatidylcholine with a concomitant rise in the lysophosphatidylcholine content. Phosphatidylserine, sphingomyelin, and cardiolipin were untouched by phospholipase A_2, while the remaining phospholipid species were hydrolysed to various degrees with a maximum of 75 % hydrolysis of phosphatidylglycerol. When plasma membranes were treated with the phospholipase A_2 from V. russelli, the total phospholipid content was reduced by 42 % with 91 % hydrolysis of phosphatidylcholine to lysophosphatidylcholine (not shown). The individual species of phospholipid hydrolysed were the same, however, the degree of hydrolysis of each was increased. Treatment of plasma membranes with phospholipase C resulted in approximately 85 % or greater hydrolysis of the contents of phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, sphingomyelin, and the unidentified phospholipid. Phosphatidylglycerol, cardiolipin, and phosphatidylserine moieties were hydrolysed to a lesser extent, while the phosphatidylinositol content was virtually unchanged.

The effect of phospholipase treatment on other plasma membrane marker enzymes such as 5'-nucleotidase and Na^+,K^+-dependent ATPase, (212), were also tested to see if the membrane perturbation has a general effect on membrane bound enzymes. Table IX shows the effect of phospholipase treatment on the 5'-nucleotidase activity in rat lung plasma membranes. Incubated control membranes showed a specific 5'-nucleotidase activity of 43.7 nmol P_i/minute/mg protein. Phosphatidylinositol-specific phospholipase C treatment of plasma membranes had no effect on 5'-nucleotidase activity, while phospholipase A_2 and

TABLE VIII

Effects of phospholipases and the adenylate cyclase activator peaks upon the phospholipid composition in rat lung plasma membranes.

Phospholipid Species	Treatment					
	Control *	PI-specific Phospholipase C (1.0 u/mg)	Phospholipase D (50 u/mg)	Activator peaks (32.5 µg peak 1, 18.0 µg peak 2)	Phospholipase A_2 (1.0 u/mg)	Phospholipase C (25 u/mg)
Total lipid P	1125 ± 92	1117 ± 117	1166 ± 106	1127 ± 65	868 ± 179[c]	184.7 ± 38.4[a]
Origin	12.2 ± 6.6	8.0 ± 5.2	33.7 ± 1.2[a]	7.3 ± 1.7	20.7 ± 10.6	16.5 ± 4.0
LysoPC	54.2 ± 10.1	63.7 ± 45.6	59.8 ± 35.6	62.3 ± 37.0	125.7 ± 20.4[c]	9.64 ± 1.8[a]
Sphingomyelin	58.8 ± 13.9	55.0 ± 13.1	59.9 ± 12.4	57.5 ± 11.9	56.8 ± 16.3	5.96 ± 1.35[a]
PC	757.2 ± 45.7	754.8 ± 28.1	752.1 ± 24.8	792.8 ± 52.6	506.0 ± 106[b]	16.2 ± 2.4 [a]
Unknown	79.5 ± 3.9	76.8 ± 9.0	76.9 ± 4.5	82.9 ± 1.5	52.9 ± 12.6[b]	3.90 ± 1.2 [a]
PS	42.7 ± 10.5	41.8 ± 8.8	49.8 ± 18.5	44.4 ± 4.2	44.7 ± 5.7	30.1 ± 2.5 [d]
PI	26.7 ± 7.5	25.2 ± 1.5	32.4 ± 22.3	21.2 ± 9.0	9.4 ± 1.7[b]	23.2 ± 2.0
PE	55.5 ± 12.3	60.0 ± 16.8	56.3 ± 16.2	53.7 ± 22.8	16.9 ± 5.8[b]	6.83 ± 3.4 [a]
PG	54.5 ± 5.7	60.5 ± 27.2	52.5 ± 4.0	48.4 ± 8.4	13.6 ± 4.6[a]	21.6 ± 2.3 [a]
Cardiolipin	18.8 ± 12.2	22.0 ± 29.2	17.7 ± 7.4	16.7 ± 6.3	19.6 ± 11.2	9.23 ± 3.67

Values represent mean pmol lipid phosphorus/mg membrane protein ± SEM of 3 observations. (* - control n=7).

Plasma membranes, (750 µg), were incubated for 30 minutes at 37°C in the presence of the various phospholipases, the activator peaks and 10 mM Tris-HCl, pH 7.4, for control membranes; after which the lipids were extracted, isolated, and analyzed for phospholipid phosphorus as described in the methods.
Significance levels (Student's t-test): [a - $p<.001$; b - $p<.05$; c - $p<.025$; d - $p<.05$]
LysoPC - lysophosphatidylcholine; PC - phosphatidylcholine; PS - phosphatidylserine; PI - phosphatidylinositol; PE - phosphatidylethanolamine; PG - phosphatidylglycerol.

C treatment resulted in slight inhibition to 86 % and 88 % of the control activity respectively. Phospholipase D treatment of plasma membranes inhibited the 5'-nucleotidase activity to 64 % of the control activity when still present in the assay medium. Ultracentrifugation of the treated membranes and assay of the 105,000xg pellets and their respective supernatants resulted in approximately 80 % recovery of the original 5'-nucleotidase activity in the control membranes. Phospholipase A_2 and phosphatidylinositol-specific phospholipase C treated and centrifuged membranes exhibited similar recoveries with 76.8 % and 85.7 % of the control added 5'-nucleotidase activity, however, the distribution between pellet and supernatant was altered with almost 2 fold of the activity found in supernatant of similarly treated control membranes. The recovery of 5'-nucleotidase activity in the phospholipase C treated and centrifuged pellet was similar to the control pellet recovery, while the pellet obtained from centrifuging the phospholipase D treated membranes showed a 113 % recovery of the control added activity. Since both phospholipase C and D contained and/or produced inorganic phosphorus during treatment and assay incubations, the relative percent recovery of 5'-nucleotidase activity in the 105,000xg supernatants from the ultracentrifugation could not be estimated as proper controls for these treatments were unavailable.

The effects of phospholipase treatment and the adenylate cyclase activator peaks were studied on the Na^+,K^+-dependent ATPase activity in plasma membranes, however, no significant difference could be found in the total magnesium-dependent activity when the membranes were assayed in the presence of 1.0 mM ouabain or in an

TABLE IX

Effects of phospholipase treatment on the 5'-nucleotidase activity in rat lung plasma membranes.

Treatment *	Incubated only (nmol P_i per minute per mg protein)	Incubated and ultracentrifuged 105,000xg Pellet (% of Control incubated activity recovered	105,000xg Supernatant (% of Control incubated activity recovered)
Control (10 mM Tris-HCl, pH 7.4)	43.7 ± 2.5	64.0 ± 9.0	16.4 ± 1.0
Phospholipase A_2 (*V. russelli*, 1.0 u/mg)	37.6 ± 4.0 [c]	45.1 ± 4.8 [b]	31.7 ± 4.0 [a]
Phosphatidylinositol-specific phospholipase C (1.0 u/mg)	46.8 ± 1.6 [d]	58.5 ± 9.6	27.2 ± 3.3 [a]
Phospholipase C (25.0 u/mg)	38.6 ± 3.1 [c]	62.4 ± 1.3	NA
Phospholipase D (50.0 u/mg)	27.9 ± 2.7 [a]	113 ± 14 [b]	NA

Significance levels (Student's t-test): [a - $p<.001$; b - $p<.005$; c - $p<.025$; d - $p<.05$]

Values represent mean 5'-nucleotidase activity (or % of control incubated activity) ± SEM of two experiments performed in duplicate.

* - Plasma membranes were incubated with phospholipases or buffer in 600 µl final volume at 37°C for 30 minutes in centrifuge tubes. Aliquots were removed for assay of incubated only 5'-nucleotidase activity and the remainder was ultracentrifuged at 105,000xg for 60 minutes. The supernatants were removed and saved for assay and the pellets were resuspended in an equal to precentrifugation volume of 0.27 M sucrose/10 mM Tris-HCl buffer, pH 7.4. The 5'-nucleotidase activity of the treatment aliquots, 105,000xg pellets, and supernatants were assayed as described in the methods.

assay medium deficient in NaCl and KCl. The effect of phospholipase treatment and the adenylate cyclase activator protein peaks upon the total Mg^{2+}-dependent ATPase activity in rat lung plasma membranes is shown in Table X. Treatment with phospholipase A_2, phosphatidyl-inositol-specific phospholipase C and the adenylate cyclase activator protein peaks had no effect on the ATPase activity. Phospholipase C treatment inhibited the total ATPase activity to 83 % of the control level, while treatment with phospholipase D resulted in a slight stimulation to 121 % of control activity.

The rat lung homogenate fractions were assayed for phospholipase A, phospholipase C, and phospholipase D activities in order to see if the adenylate cyclase activator peaks were exerting their effect by the stimulation of an endogenous phospholipase activity. No phospholipase A activity could be detected in any of the rat lung homogenate fractions by the method described. Table XI shows the phospholipase C and D activities found in the various fractions. Phospholipase D activity was found to be the highest in the plasma membrane fraction at 3.72 nmol phosphatidic acid formed/mg protein per hour. Phospholipase C activity was the highest in the 6,000xg pellet in both the acidic and neutral pH, however, in the neutral pH, the activity was reduced to 55 % of that found at pH 4.5. When the 105,000xg supernatant was added to the plasma membranes during the assays, no significant stimulation of phospholipase activity could be found for any of the phospholipase activities tested.

In order to study whether the activator peaks exert their

TABLE X

Effects of phospholipase treatment and the adenylate cyclase activator protein peaks on the total Magnesium-dependent adenosine triphosphatase activity in rat lung plasma membranes.

Treatment	Total Mg^{2+}-dependent ATPase activity (μmol P_i/mg protein/15 minutes)
Control	5.53 ± 0.34
Phospholipase A_2 (*N. naja*) (1.0 u/mg)	5.64 ± 0.36
Phosphatidylinositol-specific phospholipase C (4.6 u/mg)	5.58 ± 0.06
Phospholipase C (25.0 u/mg)	4.59 ± 0.21*
Phospholipase D (50.0 u/mg)	6.70 ± 0.09*
Adenylate cyclase activator (2.8 μg peak 1, 1.5 μg peak2)	5.67 ± 0.10

Values represent mean ATPase activity ± SEM of two experiments performed in duplicate.

Plasma membranes (30-50 μg) were preincubated in 50 mM Tris-HCl buffer, pH 7.4, phospholipase, or the adenylate cyclase activator peaks for 15 minutes at 37°C. The ATPase assay was initiated by the addition of 5 mM Tris-ATP and the assay carried out as described in the methods.

Significance levels (Student's t-test): * - $p < .001$

TABLE XI

Phospholipase C and D activities of rat lung homogenate fractions.

Homogenate fraction	Phospholipase D activity nmol PA/mg/hour	Phospholipase C activity pH 4.5 nmol DG/mg/hour	Phospholipase C activity pH 7.4 nmol DG/mg/hour
Whole Homogenate	0.10	0.095	0.369
900xg Pellet	0.03	0.091	0.310
6,000xg Pellet	1.50	1.23	0.554
8,000xg Pellet	3.25	0.984	0.382
Plasma membranes	3.72	0.134	0.225
105,000xg Supernatant	1.17	0.052	0.150
Plasma membranes + supernatant *	3.47	0.134	0.268

Values are mean phospholipase activities of a representative experiment performed in duplicate.

* - expressed as nmol PA or DG/mg plasma membrane protein/hour.

PA - phosphatidic acid; DG - diacylglyceride.

effect on the adenylate cyclase activity by interaction with the ATP regenerating system, two different concentrations of pyruvate kinase were used in the assay (Table XII). The regular assay system containing 15 u/ml pyruvate kinase resulted in a basal adenylate cyclase activity of 73.8 pmol cAMP/minute/mg membrane protein, with 63.6 % of the original ATP left after assay as calculated by counting the ^{14}C-ATP spot which remains at the origin during the chromatographic separation of cAMP. Addition of a small amount of the activator peaks resulted in a stimulation to 1.30 fold of the control adenylate cyclase activity with a slight increase in the amount of ATP remaining after assay to 69.8 %. Assay of adenylate cyclase activity with 50 u/ml of pyruvate kinase in the assay medium resulted in an increased amount of ATP left after assay to 73.9 %, however, the adenylate cyclase avtivity was inhibited to 0.77 fold of the activity found in the regular assay system. Addition of a small amount of the activator peaks to the assay medium containing 50 u/ml pyruvate kinase resulted in only a slight increase in the amount of ATP remaining with about the same fold increase in the adenylate cyclase activity (1.39), as found in the regular assay medium. It was also noticed that no significant increase in the amount of ATP remaining after the adenylate cyclase assay of any of the phospholipase treated plasma membranes could be observed (not shown).

TABLE XII

Effect of the activator peaks and increased pyruvate kinase concentration upon the ATP regenerating system in the assay of adenylate cyclase activity in rat lung plasma membranes.

Activator peaks	Pyruvate Kinase Concentration			
	15 u/ml		50 u/ml	
	Adenylate cyclase activity *	% of ATP remaining	Adenylate cyclase activity *	% of ATP remaining
None	73.8 ± 4.2	63.6 ± 1.1	56.8 ± 2.8	73.9 ± 3.7
1.8 µg peak 1, 1.5 µg peak 2	95.9 ± 12.2 [b]	69.8 ± 2.5 [a]	79.2 ± 7.1 [c]	76.2 ± 4.1

Values represent mean adenylate cyclase activity in pmol cAMP/minute/mg membrane protein * or mean percentage of added ATP remaining ± SEM of a representative experiment performed in triplicate.

Significance levels (student's t-test): [a - p .01; b - p .025; c - p .05]

F. DISCUSSION

A number of plasma membrane bound enzymes such as alkaline phosphatase and 5'-nucleotidase have been shown to be solubilized and released from rat liver membranes by treatment with a phosphatidylinositol-specific phospholipase C prepared from Staphylococcus aureus culture supernatants (213). Phosphatidylinositol-specific phospholipase C treatment has also been shown to solubilize the 5'-nucleotidase activity (214), and a component required for the basal adenylate cyclase activity (183) of rat heart sarcolemmal preparations. A similarly prepared phosphatidylinositol-specific phospholipase C used in this study was unable to affect the basal adenylate cyclase activity of rat lung plasma membranes when assayed in the presence of the phospholipase (Figure 8), or after ultracentrifugation to remove the phospholipase and any solubilized components (Table III). Similarly, the phosphatidylinositol-specific phospholipase C treatment did not appreciably affect the total Mg^{2+}-ATPase or 5'-nucleotidase activities when assayed in the presence of the phospholipase (Tables IX and X). The ultracentrifugation of phosphatidylinositol-specific phospholipase C treated membranes did not significantly change the 5'-nucleotidase activity recovered in the pellet, however, almost double the percentage of control added activity could be found in this treatment supernatant as compared to the supernatant obtained from a similarly incubated and centrifuged control preparation (Table IX). This finding could be an artifact due to a differential total recovery of 5'-nucleotidase between the control and phosphatidylinositol-specific phospholipase C treated pellets and supernatants. The

absence of an effect of treatment with phosphatidylinositol-specific phospholipase C on the plasma membrane enzymes studied is probably due to this enzyme's inability to alter the rat lung plasma membrane phosphatidylinositol content (Table VIII). This finding could be due to a relatively low membrane phosphatidylinositol content (2.4 % of the total) or to its inaccessibility to the phospholipase C, as has been noted for the majority of the phosphatidylinositol content of rat liver microsomes (194) and of intact erythrocytes (193). The inaccessibility of rat lung plasma membrane phosphatidylinositol is further supported by the observation that aliquots of the phosphatidylinositol-specific phospholipase C used were still able to specifically hydrolyse phosphatidylinositol in the soybean lipid emulsion used for substrate in the isolation and preparation of the enzyme from S. aureus culture supernatants (not shown).

In contrast, treatment with general phospholipase C from Clostridium perfringens resulted in hydrolysis of 84 % of the total plasma membrane phospholipids (Table VIII), and a marked increase in the basal adenylate cyclase activity of both plasma membranes (Figure 9) and 6,000xg pellet (Table V) by increasing both the affinity for the ATP substrate and the maximum reaction velocity (Table II). This is consistent with the recent findings of Nemecz et al. (181), that the basal adenylate cyclase activity in rat liver plasma membranes is stimulated by phospholipase C treatment. These findings may also be in accordance with earlier studies in which treatment with phospholipase C from Bacillus cereus specifically hydrolyzed acidic phospholipids and decreased the basal, fluoride-stimulated, and glucagon-

stimulated liver adenylate cyclase activities which could be partially restored by the addition of phosphatidylserine and phosphatidylinositol (177,180), since both the phospholipase C enzymes used in the present study and that of Nemecz et al. (181) are specific for neutral lipids and had relatively little effect on the phosphatidylserine and phosphatidylinositol content. Since only slight inhibition of both 5'-nucleotidase and total ATPase actitivies was found in the general phospholipase C treated membranes (Tables IX and X), this perturbation effect appears to be relatively specific for influencing adenylate cyclase activity.

Similarly, treatment of plasma membranes with 1.0 u/mg membrane protein of Naja naja venom phospholipase A_2 resulted in 33 % hydrolysis of phosphatidylcholine and reduction in total lipid phosphorus (Table VIII), and a stimulation of basal adenylate cyclase activity to 1.6 fold of the control activity (Figure 7) by slightly increasing the V_{max} with no effect on the enzyme's affinity for ATP (Table II). Treatment of plasma membranes with the same concentration of phospholipase A_2 from Vipera russelli venom in 50 % glycerol suspension resulted in substantially greater hydrolysis of membrane phospholipids to their corresponding lysophospholipids with an even greater reduction in total lipid phosphorus, presumably due to selective loss of lysophospholipids during the lipid extraction procedures (215). The glycerol present in this phospholipase A_2 may allow the enzyme more access to the membrane bilayer and result in greater hydrolysis of the 2-fatty acyl moities of the phospholipids. This increased hydrolytic activity with V. russelli phospholipase A_2 may also explain

the inhibition of adenylate cyclase activity found with concentrations comparable to those of N. naja phospholipase A_2 which are stimulatory, since increased concentrations of the latter enzyme are also inhibitory to basal adenylate cyclase activity (Figure 7). A similar biphasic response to phospholipase A_2 treatment has been noted for hormone and fluoride-stimulated adenylate cyclase activities in rat liver, with high concentrations generally being inhibitory (177-179), whereas the lower concentrations result in slight stimulation of hormone (179) and fluoride-stimulated activities (178,179). In the present study, although basal adenylate cyclase activity was inhibited by the addition of V. russelli phospholipase A_2, the relative stimulation of these treated membranes by NaF and epinephrine was increased slightly compared to similar stimulated activities of control 6,000xg pellet. However, the relative fold stimulation by the addition of the adenylate cyclase activator peaks or GppNHp were slightly reduced (Table V). Phospholipase A_2 treatment had no effect on the plasma membrane total ATPase activity (Table X), and could only slightly inhibit the total 5'-nucleotidase activity with partial solubilization (Table IX). This latter effect is probably due to the production of lysophospholipids in excessive amounts which have well known detergent effects (179). Thus the phospholipase A_2 effect on plasma membranes seems to be specific for the basal adenylate cyclase activity.

Phospholipase D treatment was also found to result in marked stimulation of basal adenylate cyclase activity in the 6,000xg pellet and plasma membranes by an increase in V_{max} (Table II). Unlike the other phospholipases, treatment with phospholipase D also had a

general perturbation effect on membrane enzymes as observed by the substantial inhibition of 5'-nucleotidase activity (Table IX) and slight stimulation of the total Mg^{2+}-ATPase activity (Table X). The finding that the direct incubation of phospholipase D with plasma membranes could not alter membrane phospholipid content (Table VIII), was surprising in view of the effects of this perturbant on the membrane enzymes, however, in those experiments, phospholipase treatments were carried out in the presence of the various enzyme assay reaction mixtures prior to the addition of substrate, suggesting that the phospholipase D may require cations for the expression of activity.

The observation that the addition of stimulatory agents,such as NaF, GppNHp, GTP, and epinephrine which act at or through the G/F regulatory protein (158), to phospholipase C or D treated 6,000xg pellet produced little or no further enhancement of adenylate cyclase activity (Table V), suggests that these agents may sufficiently alter the membrane environment so as to prevent a functional interaction of the G/F protein with the catalytic unit. Thus, the predominant stimulatory effect on adenylate cyclase activity in the treated membranes appears to be that of the phospholipase C or D.

Although the stimulatory effect of phospholipase C and D treatment on adenylate cyclase activity could be reversed by ultra-centrifugation (Table III), it seems unlikely that a contaminant present in these enzymes may be responsible for this effect since the stimulation was dependent upon the concentration of phospholipase

units/mg membrane protein and was not affected by the use of different phospholipase preparations with various specific phospholipase activities. An alternative explanation could be that the hydrolysis of the polar head groups by phospholipase C or D may unmask and solubilize an endogenous membrane component which is stimulatory to the adenylate cyclase activity, similar to the solubilization effect of a component required for basal adenylate cyclase activity in rat heart sarcolemma by phosphatidylinositol-specific phospholipase C (183).

Perturbation of the plasma membrane with trypsin and chymotrypsin was also able to stimulate the basal adenylate cyclase activity (Figure 12), however, further stimulation with a variety of agonist could only be achieved by the addition of the adenylate cyclase activator proteins to chymotrypsin treated 6,000xg pellets (Table V). The concentration dependence of protease treatment shown in Figure 12 is similar to that reported for trypsin and chymotrypsin stimulation of the rat cerebral cortical membrane adenylate cyclase activity (216), however, this stimulatory effect on cerebral cortex adenylate cyclase is much more pronounced and doesn't appear to affect the fluoride or GppNHp stimulated responses as found in the present study. Although proteolytic activation of basal (217,218) and hormonally stimulated (219) adenylate cyclase activities have been reported, the physiological significance of this phenomenon is unknown. The mechanisms involved in the proteolytic activation of adenylate cyclase activity could include the production of an activator protein, the destruction of an inhibitor, or the conversion of an inactive component of the adenylate cyclase system to an active

form (219).

The cytosolic activator of rat lung adenylate cyclase has been previously resolved into two peaks of activity, one of which contains both a 65,000 Dalton protein and a 15,000 Dalton protein as major constituents, while the other contains only the 65,000 Dalton protein as the major constituent (175). These activator peaks were found to stimulate the basal adenylate cyclase activity of plasma membranes after a considerable lag phase by increasing both the affinity for the ATP substrate and the maximum reaction velocity. In the present study, a similar lag phase of activation could be noted (Figure 11) after which a rapid and constant stimulation of adenylate cyclase activity occurred through an increase in the maximum reaction velocity, however, the affinity for ATP was also decreased (Table II). This difference in the effect on the substrate affinity could be due to differences in the preparation of the activator proteins, as the present study employed additional chromatographic procedures in an attempt to further resolve these proteins. The molecular mechanism by which these activator proteins achieve the stimulation of adenylate cyclase activity is as yet unknown, therefore attempts were made in this study to elucidate this mechanism.

Johnson (220) has suggested that the effect of some cytosolic factors on adenylate cyclase activity may be due to interaction with the ATP-regenerating system which is employed during the assay to circumvent membrane enzymes which compete for the ATP substrate, by leading to an ATP-sparing effect. Although a slight increase in the

percentage of ATP remaining after assay could be noted by the addition of a small amount of adenylate cyclase activator peaks to the regular assay medium containing 15 u/ml of pyruvate kinase, this ATP-sparing effect could be almost abolished by increasing the pyruvate kinase concentration to 50 u/ml without altering the relative stimulation of adenylate cyclase activity (Table XII). The decrease in basal adenylate cyclase activity observed by increasing the pyruvate kinase concentration may be due to increased ammonium ion concentration as the pyruvate kinase is a suspension in NH_4OH and the use of ^{14}C-ammonium ATP results in lower basal adenylate cyclase activity than that obtained when ^{14}C-sodium ATP is used as in the regular assay (personal observation). Thus it seems that the adenylate cyclase activator peaks do not act through an ATP-sparing effect.

Similarly, the adenylate cyclase activator proteins don't appear to exert their effect through a receptor mediated mechanism since the stimulatory effect could neither be mimicked by epinephrine, prostaglandin E_1, or histamine, nor blocked by the respective antagonists, propanolol, indomethacin, and metiamide (Table VI).

It also seems unlikely that the activator proteins act by a membrane perturbing effect such as that of a phospholipase either directly or by the activation of an endogenous phospholipase, since the plasma membrane phospholipids were unaltered after a 30 minute incubation with activator proteins (Table VIII). This is also supported by the observations that the time course of cAMP accumulation was vastly different in membranes treated with activator

and any of the phospholipases tested (Figure 11), and that the response of phospholipase treated membranes to the activator proteins was relatively unaffected in phospholipase A_2 and D treated membranes (Table V) and even showed positive cooperativity with a maximally stimulating phospholipase C concentration (Tables V and VII). This latter effect may be due to an increased access of adenylate cyclase components for the activator proteins by hydrolysis of the polar head groups of the phospholipids.

Since the addition of exogenous phospholipases was shown to markedly affect the adenylate cyclase activity in rat lung, the importance of endogenous phospholipases in the stimulation of adenylate cyclase activity by the activator proteins was also studied by directly assaying phospholipase A, C, and D activities in the various rat lung fractions. Phospholipase A_2 activity has been reported in the 105,000xg supernatant (106), in microsomes (107,108), and in lamellar bodies and lysosomes (109,110). This enzyme is thought to be important in the remodelling of unsaturated phosphatidylcholines to the surface active dipalmitoylphosphatidylcholine species of pulmonary surfactant, and since the microsomal enzyme has been shown to be specific for endogenous phosphatidylcholines with unsaturated fatty acids in the sn-2-position (108), the absence of detectable phospholipase A activity in any of the rat lung homogenate fractions may not be totally unexpected using exogenous dipalmitoylphosphatidylcholine as substrate. Phospholipase D activity has been shown to be highly active in rat lung microsomes when assayed in the presence of detergent (211). Similarly, in the present study, phospholipase D

activity was found to be highest in the plasma membranes (Table XI), however, the activity found was much lower than that reported by Chalifour and Kanfer (211). This is possibly due to the use of a different phospholipid substrate, a plasma membrane rather than a microsomal preparation, or the use of deoxycholate rather than taurodeoxycholate as the detergent, since the latter has been shown to yield optimal phospholipase D activity in rat brain (221). Phospholipase C activity was optimal at acidic pH in the 6,000xg and 8,000xg pellets, both of which have been previously shown to contain lysosomes by the presence of acid phosphatase activity (197). This is consistent with the phospholipase C reported in several tissue homogenates including lung (222) which have acidic optimum activities, and with the enzyme isolated from rat liver lysosomes (210). The findings also suggest the existence of another phospholipase C activity present in the rat lung, since the assay at neutral pH showed an increase in the specific activity of those fractions which contain relatively little activity at pH 4.5 and a decrease in the specific phospholipase C activity of the 6,000xg and 8,000xg pellets as compared to that obtained in the acidic pH. The 105,000xg supernatant was found to have relatively low phospholipase activities and its addition to plasma membranes during assay could not enhance the activity of any of the phospholipases tested, further suggesting that the cytosolic adenylate cyclase activator is not a phospholipase and does not exert its stimulatory effect on adenylate cyclase by augmenting the activity of an endogenous phospholipase.

The observation that the response of various agonists which act

at or through the G/F regulatory protein such as NaF, GppNHp, and epinephrine (143,158) is diminished or lost in rat lung homogenate fractions sedimented by increasing g force (Table IV), suggests that some factor which influences the functional activity of the G/F protein or possibly the G/F protein itself may be loosely associated in the membrane such that centrifugation leads to its dissociation. If this were the case, one would expect that this factor would be present in the high speed supernatants and that its readdition would lead to reconstitution of the stimulatable adenylate cyclase activities with no effect on the basal activity. This type of response pattern has been found in a variety of tissues from which cytosolic factors influencing the reconstitution of stimulated adenylate cyclase activities have also been reported (164-172). Unlike these reports, the soluble factors from rat lung in the present study were able to stimulate the basal adenylate cyclase activity with only slightly further stimulation by GppNHp (Table V). This type of effect could be expected if the ATP preparations used as substrate contained sufficient quantities of contaminating GTP so as to activate a G/F complex, as has been reported by Kimura et al. (138). The presence of GTP in the ATP substrate used in this study would also explain the lag phase in adenylate cyclase stimulation if the activator proteins were either a part of the G/F protein or conferred its functional activity, since this lag phase could not be abolished by preincubation with the activator proteins for extended periods suggesting the importance of the substrate to the stimulatory activity. Such a lag phase is also characteristic of GppNHp stimulation of liver adenylate cyclase activity in the absence of

hormone (139) which is probably due to a slow exchange of the bound GDP on the G/F protein for the free GppNHp (139,143). The findings that the adenylate cyclase activator proteins had no effect on the total ATPase activity (Table X) or 5'-nucleotidase activity (M.S. Nijjar, personal communication), suggests that these proteins specifically influence the adenylate cyclase enzyme and would be consistent with a specific reconstitution of the G/F protein. Although the aforementioned is an attractive hypothesis, both the present results and those of Nijjar et al. (175) also contain data contrary to this conjecture. If the ATP substrate preparations used in this study were indeed contaminated with GTP in sufficient concentrations to activate a G/F complex, it is unlikely that additional GTP would cause an increase in the basal adenylate cyclase activity as found in Table V. In addition, although both the G/F protein (149,150) and the adenylate cyclase activator proteins (175) appear to act as an 80,000 Dalton complex of at least two proteins, the reported subunit sizes of the G/F protein of 45,000 and 35,000 Daltons (155,156) is vastly different from the 65,000 and 15,000 Dalton major proteins of the rat lung adenylate cyclase activator (175).

The absence of any detectable sodium, potassium-dependent ATPase activity in the rat lung plasma membranes during the present study may be due to the insensitivity to and rapid reversal of ouabain inhibition or to a high total Mg^{2+}-dependent ATPase activity and the relative insensitivity of the colorimetric assay used in the determination of the released inorganic phosphate. It is possible that the use of a radiometric assay procedure with ATP radiolabelled in the

gamma phosphate would allow the detection of Na^+,K^+-ATPase activity in the rat lung plasma membranes.

From the results in the present study, it can be concluded that although the mechanisms by which the various phospholipases exert their effect remains to be elucidated, the treatment of plasma membranes with these specific phospholipases may provide a valuable tool for studying the regulation of adenylate cyclase activity in rat lungs. The results of this study further suggest that the stimulatory effect of the adenylate cyclase activator proteins isolated from the 105,000xg supernatant is not caused by a phospholipase-like effect, a nonspecific ATP-sparing effect, or mediated through β-adrenergic, histaminic, or prostaglandin receptors. Although other cytosolic factors which enhance stimulated adenylate cyclase activities may be hypothesized to act by reconstituting a functional guanine nucleotide regulatory protein activity, more studies on the rat lung adenylate cyclase activator proteins are required before an explanation of their stimulatory effects may be forwarded. It should be noted that due to the heterogeneity of the lung, it is impossible to tell from which of the cell types the activator proteins originate. Similarly, the plasma membrane perturbants may only be affecting the adenylate cyclase activity from certain of the lung cell types. It is also uncertain whether the membrane environment plays a role in the regulation of adenylate cyclase activity and as such the regulation of cyclic AMP levels in the developing fetal lung, however, the results of the present study suggest that phospholipases may be useful probes in investigating this possibility.

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