ABSTRACT

Taran K. Singh. **ROLE OF ANNEXIN II IN REORGANIZATION OF CYTOSKELETON IN LUNG TYPE II CELLS.** (Under the co-direction of Dr. Lin Liu and Dr. Gerhard Kalmus). Department of Biology, August 1999.

The lung type II cells synthesize and secrete pulmonary surfactant that functions to maintain mechanical stability of alveoli. Secretion of lung surfactant requires the movement of lamellar bodies, the secretory granules of type II cells, to the plasma membranes through a cytoskeletal barrier at the cell cortex. Disassembly of the cytoskeleton is necessary to allow lamellar bodies access to the plasma membrane following stimulation of type II cells. Annexin II tetramer (AIIt) is a member of the Ca^{2+} - dependent phospholipid - binding protein family, and has been shown to bundle or sever actin filament *in vitro*. It was therefore hypothesized that AIIt may facilitate reorganization of the cytoskeleton in type II cells during surfactant secretion. Stimulation of A549 cells, a lung epithelium-derived cell line, with terbutaline caused a transient disassembly of F-actin as determined by the DNase I inhibition assay and staining with Oregon Green 488 Phalloidin. A similar result of also observed in primary culture of lung type II cells. Western blot analysis using anti-actin and anti-annexin II antibodies showed a transient increase of G-actin and annexin II in the soluble fraction of terbutaline-stimulated type II cells. Furthermore, introduction of exogenous AIIt in the presence of Ca^{2+} and MgATP into permeabilized type II cells caused a significant disruption in the cortical actin. Phorbol 12-myristate 13-acetate, an activator of protein kinase C, also decreased cortical fluorescence staining in type II cells by phalloidin. Treatment of type II cells with *N*-ethylmaleimide (NEM), a sulfhydryl reagent, resulted in

a disruption of the cortical actin. *In vitro* experiments demonstrated that incubation of AIIt with NEM inhibited AIIt's F-actin bundling activity without affecting its F-actin binding activity. Therefore, NEM may interfere with AIIt's ability to bundle F-actin and thus lead to disassembly of the cortical F-actin in type II cells. NEM also inhibited liposome aggregation mediated by AIIt, but not by other annexins. The results above suggest that cytoskeleton undergoes reorganization in the stimulated type II cells and AIIt plays a role in this process. Information coming out from this study may be helpful in designing therapies for pulmonary diseases, such as respiratory distress syndrome (RDS) in premature infants and adults.

ROLE OF ANNEXIN II IN REORGANIZATION OF CYTOSKELETON IN LUNG TYPE II CELLS

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by

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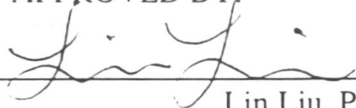
CO-DIRECTOR of THESIS 
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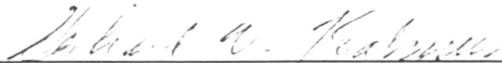
CO-DIRECTOR of THESIS 
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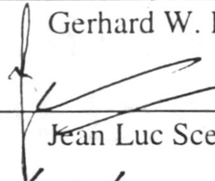
COMMITTEE MEMBER 
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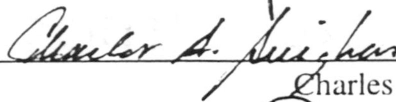
COMMITTEE MEMBER 
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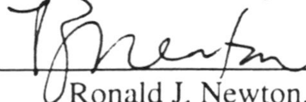
CHAIR of the DEPARTMENT of BIOLOGY 
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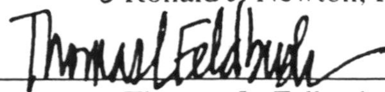
DEAN of the GRADUATE SCHOOL 
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INTRODUCTION

Lung Surfactant

Pulmonary surfactant, a phospholipid-rich lipoprotein complex, lines the lung epithelium and lowers surface tension to prevent collapse at end-expiration. In addition to maintaining low surface tension, lung surfactant is thought to play a role in host defense mechanisms and immune functions of the lung (Tenner et al., 1989; Sherman and Ganz, 1992). Respiratory distress syndrome, caused by lung immaturity or insufficient surfactant secretion, is a major cause of illness in premature infants and can also affect adults (Jobe, 1993; Lewis and Jobe, 1993). Thus, lung surfactant is essential for normal lung function. Surfactant consists of about 90% lipids, 10% proteins and small amounts of carbohydrates. Disaturated phosphatidylcholine is the major lipid and surface-active component of lung surfactant. Four unique surfactant proteins, SP-A, SP-B, SP-C and SP-D, are identified and believed to be involved in the regulation of exocytosis and endocytosis of surfactant, and in transformation of extracellular surfactant. Surfactant is synthesized in epithelia type II cells and stored in lamellar bodies. It is proposed that the newly synthesized surfactant phosphatidylcholine is transported directly from Golgi to immature lamellar bodies and newly synthesized surfactant proteins leave the trans-Golgi and enter multivesicular bodies (mvb). The mvb fuse with the lipid and produce the composite lamellar body. Actin is present in close vicinity of the plasma membrane and lamellar bodies of type II cells (Rooney et al., 1994). The movement and fusion of these lamellar bodies to the plasma membrane is believed to be regulated by cytoskeletal elements.

Lung surfactant secretion

Secretion of surfactant occurs upon fusion of lamellar bodies with plasma membranes (Ryan et al., 1975). The molecular mechanisms of surfactant secretion, especially the factors that participate in and regulate this process, are still poorly understood. The secretion of surfactant can be modulated by various agents such as agonists for β-adrenergic system and purinergic receptors, calcium ionophores, vasopressin, protein kinase C (PKC) activator and arachidonic acid (Chander and Fisher, 1990). They can be categorized into three different signal transduction pathways: those that elevate intracellular cAMP level, those that activate PKC and those that increase calcium influx.

β-adrenergic agonists (terbutaline or isoproterenol) increase surfactant secretion and raise intracellular cAMP levels by activating adenylate cyclase via G protein. A permeable analogue of cAMP, 8-bromoadenosine 3', 5'-cyclic monophosphate, also increases surfactant secretion (Brown and Longmore, 1981). The elevation of cAMP by forskolin (activator of adenylate cyclase) (Rice et al., 1985) and cholera toxin (binding to the stimulatory G protein) (Mescher et al., 1983) is also associated with increased secretion from type II cells. These results indicate a role for cAMP as a second messenger in stimulation of lung surfactant secretion. The increased cAMP activates cAMP-dependent protein kinase (PKA) which phosphorylates the cytosolic actin in lung type II cells (Whitsett et al., 1985). However, other substrates of PKA are undoubtedly involved in secretion from type II cells and need to be delineated. It has been recently

found that stimulation of type II cells by terbutaline resulted in the phosphorylation of two endogenous proteins (Zimmerman et al., 1995).

Stimulation of P_2 purinoceptor with ATP increases lung surfactant secretion from type II cells (Rice and Singleton, 1986; Gilfillan and Rooney, 1988). Adenosine triphosphate also enhances breakdown of phosphotidylinositol-4,5-bisphosphate (PIP_2) by a receptor-coupled phospholipase C and thus leads to the generation of two second messengers: inositol triphosphate (IP_3) and diacylgycerol (DAG), which in turn results in calcium mobilization and activation of PKC, respectively (Rice and Singleton, 1987; Warburton et al., 1989; Rice et al., 1990; Griese et al., 1991b). Direct activators of PKC such as DAG and its analogue, 12-O-tetradecanoylphorbol-13acetate (TPA) also stimulates surfactant secretion (Dobbs and Mason, 1978; Sano et al., 1985). Thus, ATP stimulation of type II cells involves production of IP_3 and DAG, a rise of intracellular calcium, PKC activation and increased surfactant secretion. There is some evidence indicating that cAMP levels also rise in type II cells stimulated with ATP (Griese et al., 1991a; Voyno-Yasenetskaya et al., 1991). Phosphorylation of several proteins with molecular weight of 40-50 kDa was enhanced in ^{32}P-labeled alveolar type II cells stimulated with TPA, suggesting an involvement of PKC in regulation of lung surfactant secretion (Warburton et al., 1991).

Elevation of cellular calcium with calcium ionophores, A23187 or ionomycin, also increases surfactant secretion from type II cells (Sano et al., 1987; Pian et al., 1988). The mechanism of calcium action has not been well established. Since calmodulin has

been suggested to be involved in surfactant secretion from type II cells (Hill et al., 1984), the increased calcium may act as a second messenger to stimulate calcium/calmodulin-dependent protein kinase (CaMK). A recent observation that KN-62, a specific inhibitor of CaMKII, inhibited A23187-stimulated PC secretion supports this notion (Liu, 1998).

Arachidonic acid and its metabolites stimulate surfactant secretion from cultured rat type II cells (Gilfillan and Rooney, 1985; Gilfillan and Rooney, 1986) by an undefined mechanism. Arachidonic acid also increases annexin II - mediated membrane fusion in vitro (Liu et al., 1995a) indicating that arachidonic acid may participate in the fusion of lamellar bodies and plasma membrane. Furthermore, two lipases: cytosolic phospholipase A_2 and diacylglycerol lipase are identified to be responsible for generating arachidonic acid during lung surfactant secretion (Liu, 1999b).

Annexins and exocytosis

Annexin is a large family of cytosolic Ca^{2+}-dependent phospholipid-binding proteins. The name annexin originates from the property of the members of this family to annex phospholipids. At least 13 members have been described. It has two structural domains: a conserved core region of 4 or 8 repeats of about 70 amino acids and a short variable N-terminal tail. The C-terminal core domain contains Ca^{2+} and phospholipid-binding sites. N-termini of annexins are regulatory regions and are subjected to various post-translational modifications such as proteolysis and phosphorylation. The precise physiological function of annexin is unclear, but may relate to various membrane events such as exocytosis, endocytosis, organization of cytoskeleton, signal transduction, cell

proliferation and differentiation, and Ca^{2+} channel activity (Creutz, 1992; Raynal and Pollard, 1994).

Annexin II is widely distributed in various tissues and particularly abundant in lung. It exists in two forms: annexin II monomer and annexin II tetramer. Annexin II tetramer (AIIt) is composed of two copies of a 36-kDa heavy chain, called annexin II monomer (AIIm), and two copies of the 11-kDa light chain, called p11. Annexin II monomer displays a cytoplasmic localization, whereas annexin II tetramer is bound to the membrane in a number of cell types (Zokas and Glenney, Jr., 1987). Annexin II has also been observed to be localized at the contact sites between secretory granules and plasma membranes (Nakata et al., 1990; Senda et al., 1994). Annexin II translocates from the cytosol to a subplasmalemmal region in chromaffin cells and lung type II cells upon stimulation (Chasserot-Golaz et al., 1996; Warburton et al., 1989). Annexin II is able to bind to acidic phospholipid membranes in a calcium-dependent manner (Powell and Glenney, 1987; Khanna et al., 1990). It also causes liposome aggregation and promotes membrane fusion (Glenney, Jr. et al., 1987; Drust and Creutz, 1988). Exogenous annexin II can reconstitute secretion in permeabilized chromaffin cells under certain conditions (Ali et al., 1989; Sarafian et al., 1991). Most recently, AIIt has been shown to be involved in regulated exocytosis in pulmonary artery endothelial cells (Konig et al., 1998). Previous studies have shown that AIIt mediates fusion of lamellar bodies with PS/PE liposomes at μM Ca^{2+} concentration (Liu et al., 1995a; Liu et al., 1997b). It has also been demonstrated that annexin II enhanced Ca^{2+} -triggered secretion from β-escin-

permeabilized alveolar type II cells (Liu et al., 1996). These studies suggest that annexin II may be involved in the fusion of secretory vesicles with plasma membranes during lung surfactant secretion. Other members of the annexins family have also shown to be involved in various aspects of metabolism of lung surfactant (Liu and Zimmerman, 1995; Liu and Chander, 1995; Liu et al., 1995b; Liu et al., 1997a; Cathey and Liu, 1999; Liu, 1999a).

Some annexins have exhibited a Ca^{2+} -dependent binding to F-actin. However, only AIIt causes significant actin filament bundling (Glenney, Jr. et al., 1987; Khanna et al., 1990). This bundling activity requires micromolar Ca^{2+} concentration and is reversed by addition of excess of EGTA (Ikebuchi and Waisman, 1990). A synthetic nonapeptide corresponding to residues 286-294 of annexin II inhibits its Ca^{2+} -dependent activation of actin filament bundling (Jones et al., 1992). It is suggested that annexin II tetramer could rapidly regulate F-actin bundling in a calcium dependent manner at physiologically relevant calcium levels. In addition to the bundling activity, annexin II has also been reported to sever actin filaments (Martin et al., 1988) and has the ability to bind to other cytoskeletal proteins like non-erythroid spectrin (fodrin) (Gerke and Weber, 1984; Cheney and Willard, 1989). These *in vitro* studies suggest that annexin II may be involved in cytoskeletal organization. Much less work has been done in cells regarding interaction between annexin II and the cytoskeleton.

Cytoskeleton and exocytosis

Majority of the eukaryotic cells have some degree of spatial asymmetry arising

from the asymmetrical cell division. Epithelial cells have a highly developed spatial asymmetry. Polarization of epithelial cells is intrinsically linked to their function. One of the functions of polarized epithelial cells is the regulated and asymmetrical transport, which involves the turnover of cytoplasmic vesicles with the plasma membrane through exocytosis and endocytosis. The actin-based cytoskeleton functions not only in determining cell polarity but also participates in polarized secretions by epithelial cells. The cytoskeleton serves several functions in regulating secretory processes, including a barrier to regulate release of secretory vesicles, a track for motor-mediated translocation of secretory vesicles, and a scaffold to buffer the reserve pool and the rapidly releasable pool of secretory vesicles (Yao and Forte, 1996).

Orci and co-workers (1972) were the first to propose that a layer of cortical actin serves as a barrier to granule docking and the exocytic release of these secretory granules. The actin network is regarded as part of the clamping apparatus to regulate release of secretory vesicles. This hypothesis is supported by the observation, that in many secretory cells the actin network underneath the plasma membrane undergoes transient depolymerization during exocytosis (Vitale et al., 1991; Norman et al., 1994). In previous research it has been found that a rapid depolymerization of filamentous actin (F-actin) occurs in toad bladder epithelial cells treated with either ADH or 8-Br-cAMP (Ding et al., 1991). In unstimulated bladder cells, the monomeric globular actin (G-actin) content was 53 μmol/liter, but increased to 77 μmol/liter concomitant with exocytosis and increased water transport (Hays and Lindberg, 1991). The adrenal chromaffin cells also

contain the actin cortical network, which provides a barrier for regulated exocytosis (Perrin et al., 1987; Aunis and Bader, 1988). In stimulated chromaffin cells, catecholamine secretion is preceded by disassembly of cortical actin (Cheek et al., 1989). It is proposed that a layer of actin filaments interferes with secretory vesicle access to the plasma membrane surface until an appropriate signal or stimulation is received.

Another function of the cortical actin network is to serve as a track for motor-mediated translocation of secretory vesicles. One of those motors is myosin. In the intestinal brush border, myosin-I links the actin filaments of the microvilli to the plasma membrane. Previous research has shown that myosin-I associates with vesicles in mature enterocytes, which suggests its potential role in mediating vesicle motility (Drenckhahn and Dermietzel, 1988).

The cytoskeletal network also serves as a scaffold to buffer the interchange between a reserve pool and an immediately releasable pool of transport-containing vesicles. In the adrenal chromaffin cell, translocation of secretory granules to their site of exocytic release is influenced by the cytoskeletal network that involves interaction between actin filaments and proteins associated with the cytoplasmic surface of the granules (Bader and Aunis, 1983; Aunis and Perrin, 1984). It is proposed that chromaffin cells contain one pool of granules immediately subjacent to the plasma membrane, which does not require cytoskeletal reorganization. However, a second pool is entrapped within the actin meshwork, which represents a reserve population to be released when the first pool is exhausted (Aunis and Bader, 1988).

Actin

Actin is a primitive and abundant protein and represents approximately 5% of the total protein in nonmuscle cells. Actin has the ability to self-assemble and disassemble between G-actin and F-actin. Within most cells, the equilibrium for G- and F-actin lies far to the polymerized form. However, almost all cells have mechanisms to control the steady state for interconversion between G-actin and F-actin, so that G-actin frequently makes up 20-60% of the total actin (Yao and Forte, 1996). A variety of actin-binding proteins react with actin monomer or filaments to achieve this steady state. The spatial organization of actin within the cell is largely a function of the distribution of specific actin-binding proteins. The actin-binding proteins can be grouped within three categories, depending on their function (Stossel, 1989). The first category consists of actin-binding proteins that regulate the steady state between polymerization to form F-actin and depolymerization to form G-actin. This group also includes the proteins that sequester, nucleate, sever, and cap the actin forms. Villin is a member of the family of actin-severing proteins that is found in intestinal and kidney proximal tubule microvilli as well as in microvilli that cover the surface of amphibian eggs (Matsudaira, 1991). Actin-binding proteins in the second group crosslink and organize the filaments into tight or loose bundles to promote higher orders of complexity. Some of the actin-binding proteins include annexin, fascin, synapsin I and fimbrin. Fimbrin, a 68 kDa monomeric bundling protein, is found in most cell microvilli, sterocilia and filopodia (Matsudaira, 1991). The last category of actin-binding proteins serves as a link between actin

filaments and membranes, such as the plasma membrane and various organelle surfaces. Spectrin/fodrin are actin-binding proteins that crosslink actin filaments. Through interaction with additional actin-crosslinking proteins, spectrin/fodrin incorporate actin filaments near the membrane into the cytoplasmic actin cytoskeleton (Matsudaira, 1991).

Cysteine residues and protein functions

The sulfhydryl group of cysteine is considered to be one of the most reactive functional group in proteins. It is easy to oxidize, alkylate, acylate, or arylate the thiol side chains in proteins. Thiol groups are very nucleophilic, as is reflected by the reactivity of cysteinyl residues. *N*-ethylmaleimide (NEM) is a commonly used sulfhydryl reagent, which is used to modify cysteine residues. It is used to determine the effect of sulfhydryl-group modification on protein function and to determine the number of sulfhydryl-groups present in various proteins (Han et al., 1987).

Cysteine residues have been shown to be involved in various protein functions. Two highly conserved cysteine residues, cysteines 67 and 95, play an important role in regulating the activity of the human immunodeficiency virus type 1 (HIV-1) protease during viral maturation. Under normal conditions, the cysteines of the HIV-1 protease prevent immature virions from undergoing complete processing (Davis et al., 1999). Another example in which cysteine residues play a role in protein function involves a new type of peroxidase enzyme called thioredoxin peroxidase (TPx). It reduces H_2O_2 with the use of electrons from thioredoxin. Mutation of one of the cysteine residues (Cys47) to serine abolishes the peroxidase activity, suggesting that cysteine is essential

for peroxidase function (Kang et al., 1998). Recent studies have shown that nitric oxide inhibits AIIt-mediated membrane aggregation and fusion. This inhibition is possibly due to S-nitrosylation / formation of disulfide double bonds (Liu et al., 1998), suggesting an important role of cysteine residues in AIIt's function.

Purpose of study

As previously mentioned, pulmonary surfactant that lines the lung epithelium is stored in lamellar bodies of alveolar type II cells and is released by exocytosis of lamellar bodies. The specific mechanisms of surfactant secretion and the factors that participate in and regulate this process are still not well understood. The goal of this project was to elucidate some of the factors that participate in regulation of lung surfactant secretion from alveolar epithelial type II cells. Since deficiency of lung surfactant is the cause of respiratory distress syndrome (RDS) in premature infants, information obtained from this study may give valuable direction to the therapy of these diseases.

The specific aim of the present study was to examine the roles of annexin II in reorganization of the cytoskeleton in alveolar type II cells. The first sub-aim was to determine the redistribution of annexin II and actin between the soluble pool and cytoskeletal pool upon stimulation of type II cells. The second sub-aim was to examine the effects of purified annexin II on actin disassembly in permeabilized type II cells. Lastly, the role of cysteine residues on annexin II was examined by modifying the cysteine residues and observing the effects on cytoskeleton reorganization.

MATERIALS AND METHODS

Materials

Phosphatidylserine (PS) and phosphatidylcholine (PC) were purchased from Avanti Polar Lipids (Alabaster, AL). N-ethylmaleimide (NEM), phenylmethanesulfonyl fluoride (PMSF), DNA, β-escin, rat IgG, anti-actin antibody, bovine serum albumin (BSA), Tween 20, Ponceau S, streptomycin, penicillin, guanidine hydrochloride, DNase I and terbutaline were obtained from Sigma (St. Louis, MO). Actin was a gift from Dr. David Terrian (Department of Anatomy and Cell Biology, East Carolina University) or obtained from Worthington Biochemical Corporation (Lakewood, NJ). A549 cell line was from American Type Culture Collection (Rockville, MD). Oregon Green 488 Phalloidin from Molecular Probes (Eugene, OR). Cell culture dishes were from Corning (Corning, NY). Chamber slides were from Nalge Nunc International (Naperville, IL). Bio-Spin Chromatography Columns, gelatin, Triton X-100, Acrylamide/Bis, TEMED, D_C Protein Assay Kit, HRP Color Development reagent, silver staining reagents and pre-stained molecular mass standards were from Bio-Rad (Melville, NY). Nitrocellulose membrane was from Schleicher and Schell (Keene, NH). Anti-annexin II polyclonal antibody was raised in rabbit and affinity-purified in our laboratory (Liu, 1999a). Elastase was from Worthington Biochemical Co. (Freehold, NJ). Eagle's minimal essential medium (MEM) was from ICN (Costa Mesta, CA). Fetal calf serum (FCS), trypsin-EGTA, L-glutamine, HEPES buffer and Penicillin/Streptomycin were from GIBCO (Grand Island, NY). Enhanced chemiluminescence (ECL) reagents and

Hyperfilm ECL were from Amersham (Arlington Height, IL).

A549 cell culture

A549 cells were grown to confluence in plastic flasks in MEM supplemented with 10% FCS at 37°C in a CO_2 incubator. The cells were harvested with 0.05% trypsin plus 0.02% EDTA and replated in 35 mm plastic cell culture dishes. After two to three days, the cells were used for subsequent experiments. A459 cells are a lung epithelium-derived cell line that displays some characteristics of type II cells and were used as a model for alveolar type II cells.

Stimulation of A549 Cells

A549 cells were washed with MEM and incubated in MEM for 30 min. Then, the cells were stimulated with 10 μM terbutaline for different time spans (0, 0.25, 0.5, 1, 5, 30, and 60 min) in a CO_2 incubator. After the appropriate stimulation time, the medium was removed from the dishes. Cells were lysed by adding 1 ml of lysis buffer (0.1 mM NaF, 50 mM KCl, 1 M sucrose, 2 mM $MgCl_2$, 0.1 mM EGTA, 10 mM sodium phosphate, 0.2 mM 2-mercaptoethanol, 0.5% v/v Triton X-100, and 1 mM PMSF, pH 7.0) and scraped with a rubber policemen. The sample was collected and used for the DNase I inhibition assay.

DNase I Inhibition Assay

The DNase inhibition assay is a simple method for quantitating monomeric and filmentous actin. Muscle G actin and profilactin from nonmuscle cells have been shown to bind rapidly to DNase I and form stable complexes with the enzyme. This 1:1

complex formation inhibits the activity of the enzyme (Blikstad et al., 1978). The DNase inhibiting activity of monomeric actin was used to determine the cellular G-actin and total cell actin contents. Calf thymus DNA was used to prepare substrate for the DNase I inhibition assay. The DNA fibers were cut with a pair of scissors into fine pieces and dissolved in distilled deionized water at a concentration of 450 μg/ml of DNA. The DNA was dissolved by slow stirring at room temperature (23° C) for 24-48 hrs. The solution was then filtered and stored at 4°C. DNase I was dissolved at 1 mg/ml protein in 50 mM Tris-HCl (pH 7.5), 0.01 mM PMSF, and 0.1 mM $CaCl_2$ to improve the stability of the enzyme. DNase I solution was freshly made for every use. To measure G-actin, cell lysates (5 μl) were added to a cuvette containing 100 μl of DNase I and mixed. Then, 900 μl of assay buffer (4.8 mM $MgSO_4$, 2.1 mM $CaCl_2$, and 120 mM Tris-HCl, pH 7.5) was quickly added, followed by 100 μl DNA solution. The cuvette was mixed by inversion. The absorbance at 260 nm was scanned for 120 seconds and the initial reaction rate was calculated. G-actin content was derived from a standard curve, constructed from purified actin (0.05-0.3 μg). To measure total actin in the cell lysate, F-actin was converted to G-actin by incubating samples with 1.5 M guanidine hydrochloride in buffer (1 M sodium acetate, 1 mM $CaCl_2$, 1 mM ATP, and 20 mM Tris-HCl, pH 7.5) for at least 30 min.

Phalloidin staining of F-actin

A549 or type II cells were cultured for 1 or 3 days and overnight, respectively, before being stained with Oregon Green 488 Phalloidin. Cultured cells were washed

twice with MEM and then incubated in MEM for 30 min. Then, the cells were stimulated with 10 μM terbutaline for different time spans (0, 0.25, 0.50, 1, 5, 10, and 30 min) at room temperature. At the end of stimulation, each dish was washed twice with PBS. The cells were fixed for 30 min with 4% formaldehyde in PBS. After two washes with PBS, cells were permeabilized in 1% Triton X-100 for 10 min and incubated with 10% FCS and 1% BSA in PBS for 15 min. Cells were stained with 50 nM Oregon Green 488 Phalloidin for 30 min and washed three times with PBS. Fluorescence was observed with a fluorescent microscope.

Type II cell isolation and culture

Type II cells were isolated from perfused rat lungs. Male Sprague-Dawley rats (180-200 g) were anesthetized intraperitoneally with 0.1 ml of a ketamine (90 mg/ml) and xylazine (10 mg/ml) mixture per 100 g of body weight. The trachea was cannulated and lungs were mechanically ventilated at 40 breaths per minute and 2 ml tidal volume. After the thoracic cavity was exposed surgically, the lungs were perfused via a cannula inserted in the main pulmonary artery with a Solution II (10 mM HEPES, pH 7.4, 2.6 mM Na_2HPO_4 / NaH_2PO_4, 154 mM NaCl, 3.9 mM KCl, 1.7 mM $CaCl_2$, 1.3 mM $MgSO_4$, 10 mM glucose, 100 μg/ml streptomycin and 60 μg/ml penicillin) until lungs were free of blood. Eight ml of Solution I (Ca^{2+}- and Mg^{2+}-free solution II plus 0.16 mM EGTA) was instilled through the trachea with a 10 cc syringe. Then, the lungs were removed and placed in a beaker containing 15 ml of cold Solution II. The rest of the procedure was performed inside the cell culture hood. The lungs were lavaged eight times with 7 ml of

cold Solution I and twice with 7 ml of warm Solution II. The lungs were filled with 6-7 ml of elastase solution (3 units/ml in Solution II) and incubated in a water bath at 37° C for 10-12 minutes. This step was repeated 3 times. Thereafter, the lobes were removed and placed into a beaker containing 3 ml FCS. Each lobe was minced 5-6 times using a tissue chopper and placed in a flask containing 10 ml Solution II and DNase. After mincing all the lobes, the 3 ml of FCS from the beaker was added into the flask. The flask was shaken by hand rapidly for 2 min at 37°C. The minced material was filtered sequentially through 160-, 37-, and 15-μm filters on ice. The resulting cell suspension was centrifuged for at 1100 rpm for 10 min. Cell pellet was resuspended in 10 ml MEM, and placed in an IgG-coated dish (3 mg IgG/5 ml 50 mM Tris-HCl) and incubated for an hour to remove alveolar macrophages. The unattached cells were removed (panning) with a sterile pipette and centrifuged at 1100 rpm for 15 min. The cells were resuspended in MEM containing 10% FCS, counted, and replated onto 35-mm^2 cell culture dishes.

Stimulation of type II cells

Overnight cultured type II cells were washed three times with 2 ml MEM and incubated in 1 ml MEM for 30 min. Then the cells were stimulated with 10 μM terbutaline for different time spans (1, 3, 5, 10, 15, and 30 min). At the end of stimulation the dish was washed twice with 2 ml of cold phosphate-buffered saline (PBS). In order to separate G-actin and F-actin fractions, 400 μl of extraction buffer (50 mM KCl, 0.3 M sucrose, 2 mM $MgCl_2$, 10 mM sodium phosphate, 0.5% Triton X-100, and 1 mM PMSF) was added to each dish. The dish was then incubated for 10 min on

ice. The extract containing G-actin was analyzed for the content of actin and annexin II by western blot.

Immunoblot

The Triton X-100 soluble fraction of type II cells (10-20 μl) was resolved on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The gel was run for 1.5-2 hours at 100 V. Thereafter, the gel was transferred to a nitrocellulose membrane at 100 mA for 2 hours or 25 mA overnight. The blot was transiently stained with Ponceau S to monitor transfer efficiency of proteins and destained in water for 2-3 min. The lanes were marked with indelible ink. The blot was destained for 10 min with Tris-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.5). Then, the blot was blocked with TBS containing 2% gelatin for 1 hour. After blocking, the blot was placed in a plastic bag and incubated with the appropriate primary antibodies (anti-annexin II, 1:1000 dilution or anti-actin, 1:200 dilution) in TTBS (TBS plus 0.05% Tween 20) containing 1% gelatin for 2-3 hours. The blots were removed from the bags and washed three times with TTBS for 5 min each. The washed blots were placed in a new bag and incubated with secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG, 1:5000 dilution) for 1 hour at room temperature. The blots were washed three times and visualized by enhanced chemiluminescence (ECL). The immunoblots were scanned using either the DeskScan or the HP Precision Scan Pro and quantitated using the ImageQuant.

Permeabilizatin of Type II Cells

Type II cells were permeabilized with 40 μM β-escin as previously described (Liu et al., 1996). Overnight cultured cells on chamber slides were washed twice with permeabilization buffer (PB, 139 mM K-glutamate, 1 mM EGTA, 10 mM glucose, 20 mM HEPES, pH 7.0). Then the cells were equilibrated in PB buffer for 30 min at 37° C. 40 μM β-escin was added and incubated for 10 min at room temperature. At the end of incubation, fresh PB buffer containing 0.5 μM Ca^{2+} and 5 mM MgATP and/or 50 μg/ml AIIt were added and the incubation continued for another 10 min. The cells were washed twice with PBS and stained with Oregon Green 488 Phalloidin as described above.

Isolation of lung annexins

Annexins were purified from bovine lung tissue by sequential column chromatography using DEAE-Sepharose CL 6B, Sephacryl S 300 and FPLC Mono S columns according to the method described by (Nakata et al., 1990) with modifications from (Liu et al., 1996). Lung tissue (300 g) was powdered in a blender at slightly above liquid nitrogen temperature and extracted with 1 liter of buffer A (10 mM imidazole, pH 7.4, 150 mM NaCl, 1 mM DTT, 100 μg/ml soybean trypsin inhibitor, 1 mM PMSF, 5 μg/ml leupeptin and 2 mM EGTA). The purification steps were carried out at 4°C except for the fast-protein liquid chromatography (FPLC) which was carried out at room temperature. The lung extract was centrifuged at 650 x g for 10 min. The supernatant fraction was adjusted to 2 mM free Ca^{2+} and centrifuged at 24,000 x g for 40 min. The pellet was washed three times with buffer B (10 mM imidazole, pH 7.4, 150 mM NaCl,

1 mM DTT and 1 mM Ca^{2+}). The final pellet was resuspended in buffer C (buffer B with addition of 5 mM EGTA) and centrifuged at 100,000 x g for 1 hour. The EGTA-extracted fraction was dialyzed against buffer D (10 mM imidazole, pH 7.4, 0.5 mM DTT, and 1 mM EGTA) for 2 days with three changes of the buffer. The dialysate was centrifuged at 100,000 x g for 1 hour, and the supernatant fraction was applied to a DEAE-Sepharose CL 6B column (2.5 x 20 cm) equilibrated with buffer D. After washing with buffer D, the column was developed by a linear salt gradient (0-0.3 M NaCl in buffer D). Three protein peaks (labeled A, B, and C) were separately pooled. Peak A (10-35 mM NaCl) contained annexins I and II, as determined by immunoblot using specific antibodies. Peak B (45-60 mM NaCl) contained annexins III and IV. Peak C (160-190 mM NaCl) contained annexins V and VI.

The proteins in peak A were concentrated on an Amicon concentrator and applied to a Sephacryl S 300 column (1.5 x 150 cm) equilibrated with buffer E (40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5 mM DTT, and 1 mM EGTA). The chromatography further resolved the proteins into two peaks. The lower molecular weight peak containing annexin I (AI) and annexin II monomer (AIIm) was pooled, concentrated and designated as AI and AIIm. This fraction containing ~50% AI and ~50% AII (Fig. 15A). The higher molecular weight peak containing annexin II tetramer (AIIt) was pooled and dialyzed against buffer F [25 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.0, and 0.5 mM DTT], and applied to an FPLC Mono S column at a flow rate of 1 ml/min. The AIIt was eluted with a gradient of 0-0.4 M NaCl I buffer F. Peak B was also concentrated and

chromatographed on a Sephacryl S 300 column. A major peak {containing annexins III (AIII) and IV (AIV)} and a minor peak (containing an unidentified 22-kDa protein) were resolved by this procedure. The major peak was designated as AIII and IV and contains ~50% annexin III and ~50% annexin IV (Fig. 15A). Annexin protein concentration was determined by the method of Bradford using gamma globulin as standard (Bradford 1976).

F-actin Binding and Bundling

Annexin II tetramer (20 μg) was incubated in 100 μl of bundling buffer (2 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM $MgCl_2$, 0.3 mM EGTA, 0.5 mM DTT, 0.1 mM $CaCl_2$, and 0.33 mM ATP) in the absence or presence of 1 mM NEM at room temperature for 10 min. Spin Column Chromatography was used to remove the excess NEM from the AIIt mixture after the incubation. In order to improve the recovery, the buffer in the spin column was changed first with 0.1% bovine serum albumin to reduce the non-specific binding and then three times with the bundling buffer. The sample (AIIt with or without NEM) was added in the spin column and spun at 1,000 x g for 4 min. The AIIt mixture was collected and used for the F-actin binding and bundling assay. G-actin (1 mg/ml) was converted to F-actin by incubating it in bundling buffer overnight. The AIIt mixture was combined with 12 μg F-actin in final volume of 150 μl bundling buffer plus 1 mM free Ca^{2+}. Aliquot (75 μl each) of the reaction mixture was analyzed at low speed (15,600 x g, 10 min) for F-actin bundling and at high-speed (74,800 x g, 70 min) for F-actin binding. The supernatant was removed. The pellet was dissolved in 1x sample buffer at

room temperature overnight and then analyzed on 10% SDS-PAGE. The gel was stained with a silver staining kit.

Preparation of PS Liposomes

Liposomes were prepared by the extrusion method of (Hope et al., 1985). Phosphatidylserine dissolved in chloroform (3.75 mg) was placed in a test tube and dried under a stream of N_2 gas. The lipid film was hydrated with 1.5 ml liposome buffer (40 mM HEPES, pH 7.0, 100 mM KCl). The suspension was vortexed until all lipid film was dissolved, and subsequently passed through a 0.1 μm filter membrane (Nuclepore, Pleasanton, CA) for three times using an Extruder (Lipex Biomembrane, Vancouver, Canada). This procedure produces unilamellar vesicles with diameters in the range of 60-100 nm (Hope et al., 1985).

Preparation of PS/PC Liposomes for Annexin Binding Assay

Phosphatidylserine (1.875 mg) and phosphatidylcholine (1.875 mg) dissolved in chloroform were placed in a test tube and dried under a stream of N_2 gas. The lipid film was hydrated with 1.5 ml liposome buffer and vortexed until all lipid film was dissolved. The suspension (multilamellar vesicles) was spun in a microcentrifuge at 15,600 x g for 10 min at 4°C. The pellet was washed once and resuspended in 1.5 ml liposome buffer.

Liposome Aggregation Assay

Annexin II tetramer or other annexins (5 μg each) were incubated with various concentrations of NEM (1-10 mM) in 50 μl of 0.1 M Tris, pH 7.0 at room temperature for 10 min. Annexin-mediated aggregation of liposomes was determined by following

changes in absorbance at 540 nm. PS liposomes (100 μg) were mixed with 1 ml of Ca^{2+}-EGTA buffer (40 mM HEPES, pH 7.0, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$ and various concentrations of Ca^{2+}). After recording the zero time annexin was added to start the reaction. The absorbance at 540 nm was read after a 30 min incubation. Liposome aggregation activity was expressed as the difference in absorbance between 30 min and zero time.

Binding of Annexin IIt to Liposomes

Annexin II tetramer (10 μg) was preincubated in 0.1 M Tris, pH 7.0 in the presence and absence of 1 mM NEM for 10 min at room temperature. The AIIt mixture was added to eppendorf tubes containing 1 ml of buffer (40 mM HEPES, pH 7.0, 100 mM KCl, 1 mM EGTA) plus or minus 2 mM Ca^{2+}. Multilamellar vesicles (200 μg of lipid, PS:PC; 1:1) were then added to each tube and incubated for 20 min at room temperature. The mixture was spun at 15,600 x g for 10 min. The pellet was suspended in 1x SDS-sample buffer, boiled for 5 min and analyzed on 10% SDS-PAGE. The gel was stained with Coomassie Blue.

RESULTS

Actin disassembly in A549 cells upon stimulation

To test the hypothesis that cytoskeleton serves as a barrier in lung surfactant secretion, A549 cells, a lung epithelium-derived cell line, were used as a model system. A549 cells were stimulated for various times with terbutaline, a well known lung surfactant secretagogue, and cellular monomeric actin (G-actin) was determined by the DNase I inhibition assay (Blikstad et al., 1978). Terbutaline is a β-adrenergic agonist which activates the cAMP-dependent protein kinase (PKA) via the adenylate cyclase pathway (Chander and Fisher, 1990). Treatment of A549 cells with 10 μM terbutaline resulted in a progressive increase of the cellular G-actin content, reaching maximum at 1 min (Fig. 1). A longer stimulation (30-60 min) caused the G-actin content to return to its resting level.

To confirm the results above, filamentous actin (F-actin) staining with Oregon Green 488 Phalloidin was performed in A549 cells. Phalloidin is a phallotoxin derivative isolated from the deadly *Amanita phalloides* mushroom. It binds selectively to F-actin at nanomolar concentrations (Alberts et al., 1999). The staining patterns were first compared under different culture conditions. As seen in Fig. 2 A and B, a diffuse phalloidin staining was observed in 1 day culture of A549 cells (~50% confluence). However, the cells exhibited a continuous cortical fluorescent ring in 3 day culture (~90% confluence) representing cortical F-actin underneath the plasma membrane (Fig. 2 C and D). Next, three day culture of A549 cells were stimulated with 10 μm terbutaline for

Fig.1 G-actin content in A549 cells upon stimulation. A549 cells were exposed to 10 μM terbutaline for different time spans. At the end of incubation, cells were lysed. G-actin in cell lysates was determined by DNase I inhibition assay. Total actin was measure after incubating cell lysate with guanidine hydrochloride. The data were expressed as G-actin / total cell actin x 100%. (n = 1-3)

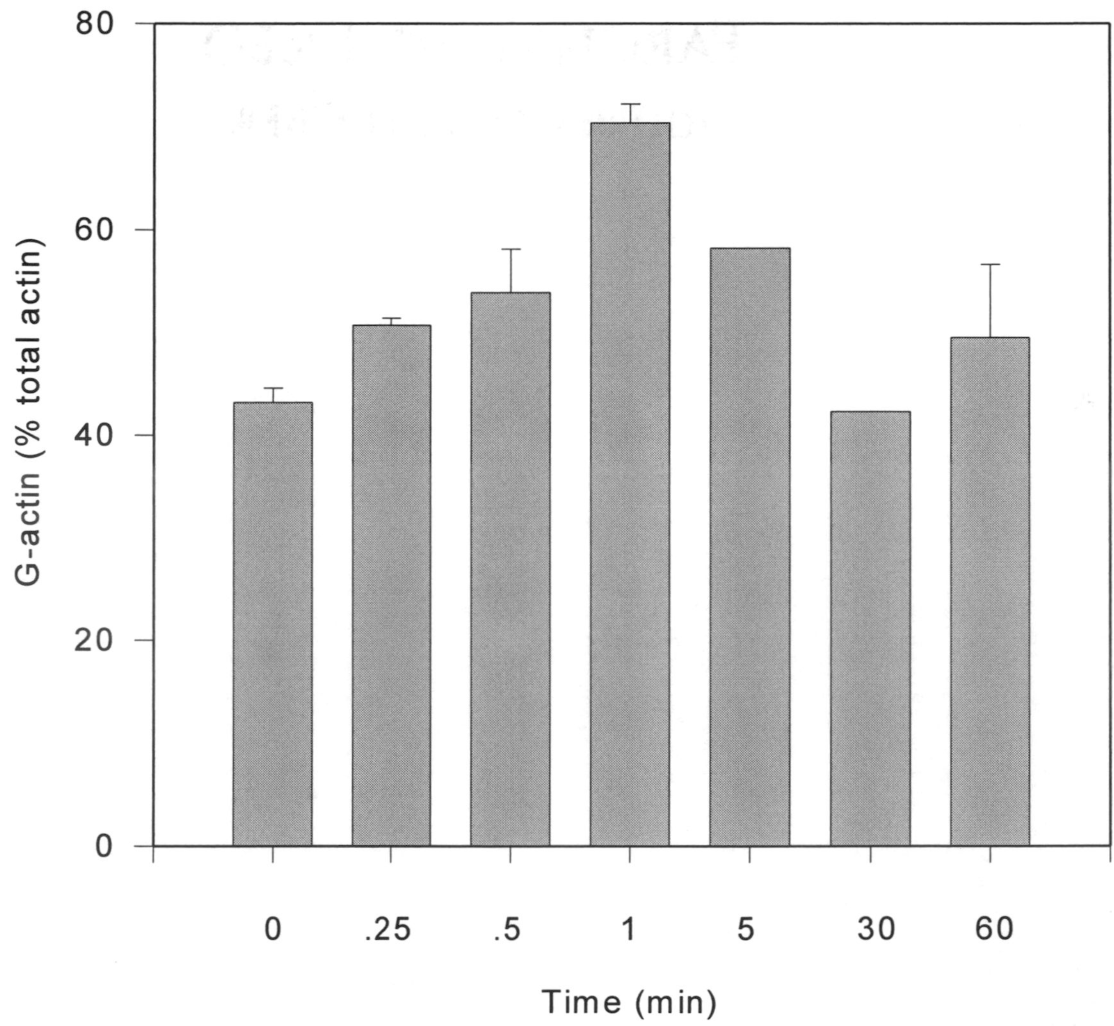
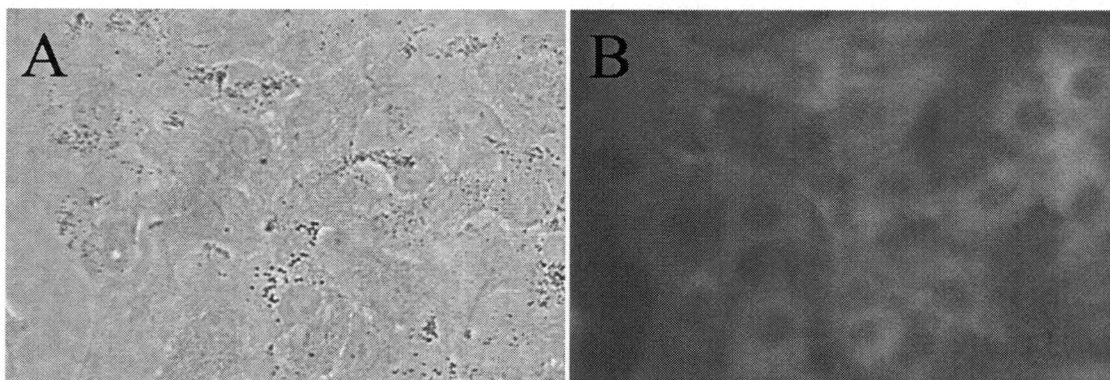
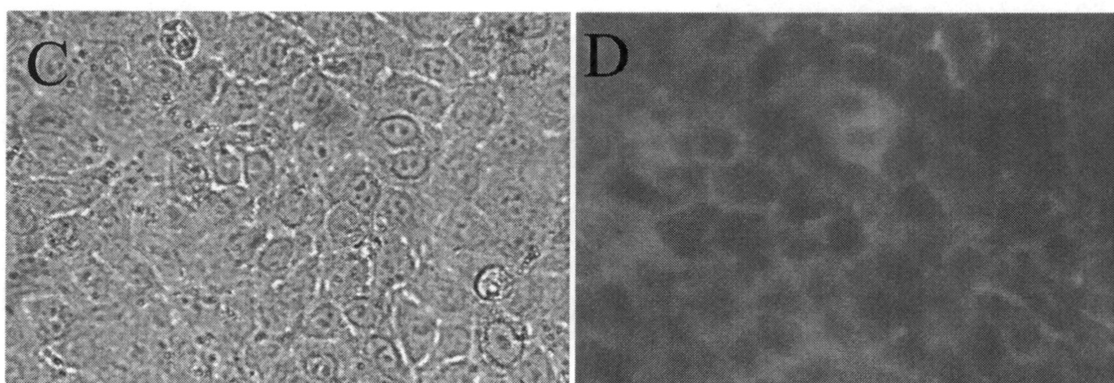

80
60
40
20
0
G-actin (% total actin)
0
.25
.5
1
5
30
60
Time (min)

Fig.2 F-actin staining in 1 and 3 day culture of A549 cells. A549 cells were cultured for 1 day (Panel A,B) and 3-days (Panel C,D). Cells were then fixed with formaldehyde, permeabilized with Triton X-100, and stained with 50 nM Oregon Green Phalloidin. A much clearer fluorescence around the cell periphery is present in the 3-day cultured cells compared to the 1-day cultured cells. Panel A,C: brightfield view; Panel B,D: fluorescence.

1-Day





3-Day

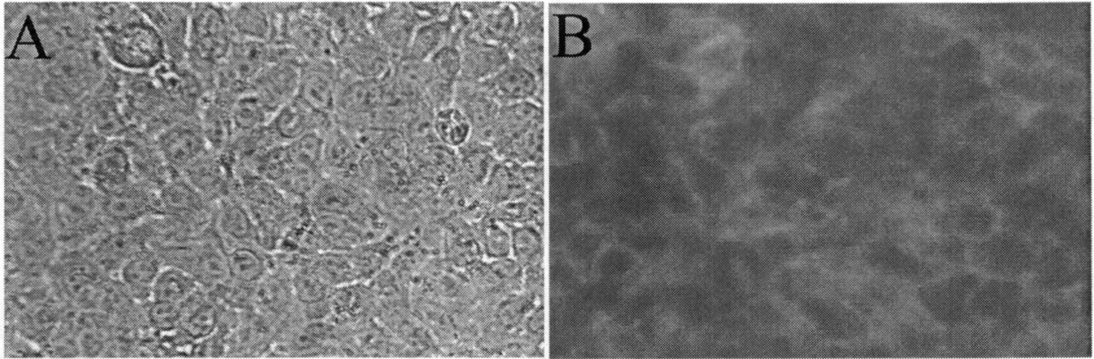
various time intervals (0, 0.25, 0.5, 1, 5, 10 and 30 min). The effect of terbutaline on actin disassembly was assessed by the F-actin staining with phalloidin. Upon 0.25 and 0.5 min stimulation there was no change in the phalloidin staining (data not shown). A disruption in the phalloidin staining around the cortex was observed upon 1 min stimulation (Fig. 3 C and D), indicating depolymerization of F-actin. A longer stimulation (5-30 min) resulted in the reappearance of the cortical fluorescent ring (Fig. E and F). The results from the phalloidin staining and the DNase inhibition suggest that there is a transient disassembly of the cytoskeletal network in A549 cells upon stimulation.

Actin disassembly in lung type II cells upon stimulation

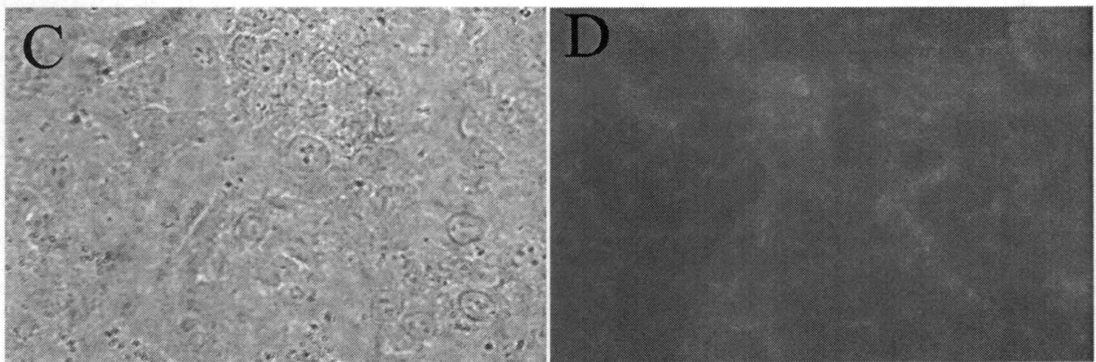
The primary culture of alveolar type II cells were also used to examine the effect of terbutaline on the actin disassembly. Type II cells were stimulated with 10 μM terbutaline for various time spans (0, 1, 3, 5 and 10 min). In resting cells (0 min) phalloidin fluorescence was most intense at the cell cortex (Fig. 4 A and B), indicating that the F-actin was concentrated in that region. However, during 1 and 3 min stimulation there was virtually no binding of phalloidin detected in the cell cortex region (Fig. 4 C and D). Further stimulation (5-10 min) led to the reappearance of the cortical fluorescent ring (Fig. 4 E and F). These results indicate that cortical actin filaments in type II cells are rapidly disassembled and reassembled following terbutaline stimulation.

Fig.3 Actin disassembly in A549 cells upon stimulation. A549 cells, cultured for 3 days, were stimulated with 10 μM terbutaline for 0 (Panel A,B), 1 min (Panel C,D), and 10 min (Panel E,F). The cells were fixed, permeabilized and stained with 50 nM Oregon Green Phalloidin. Stimulation of the cells resulted in a transient disruption of the phalloidin staining (right panels B, D, F). Panels on the left (A, C, E) provide the brightfield view of the corresponding cells.

0 min



1 min



10 min

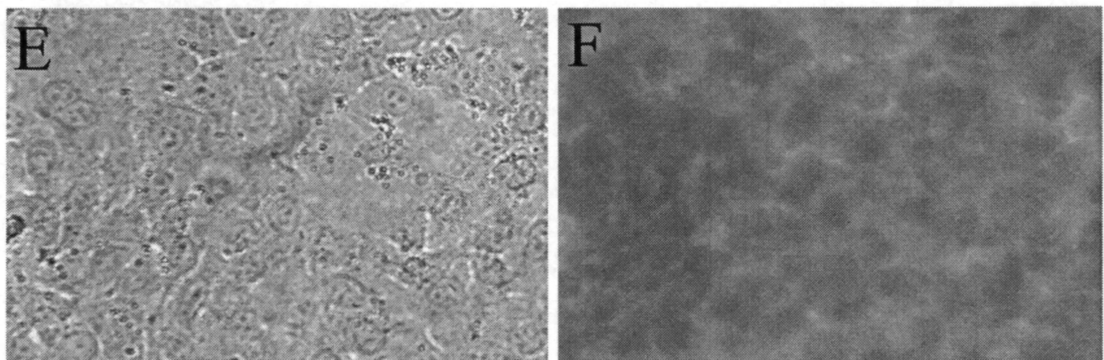
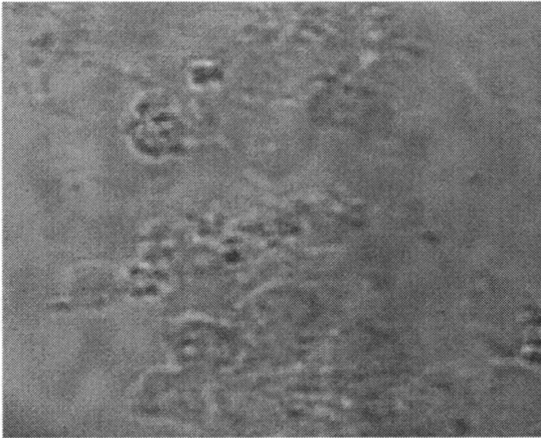
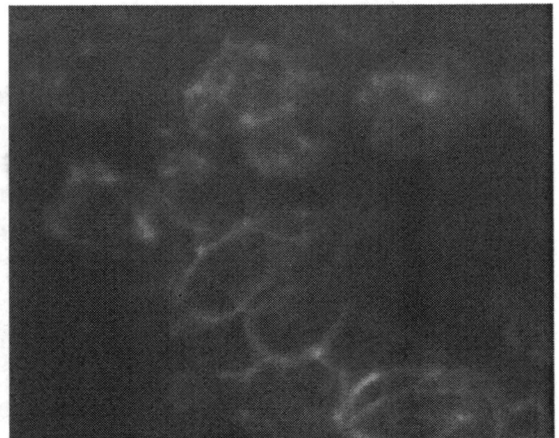
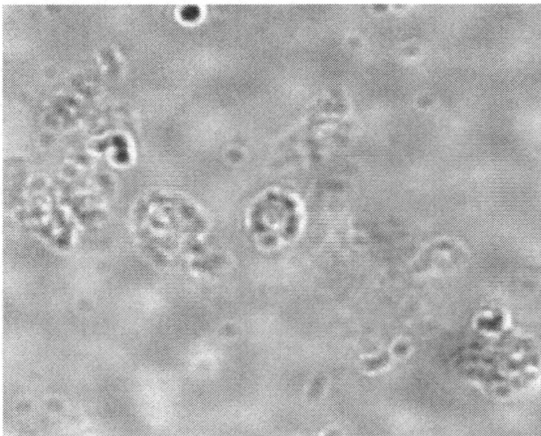
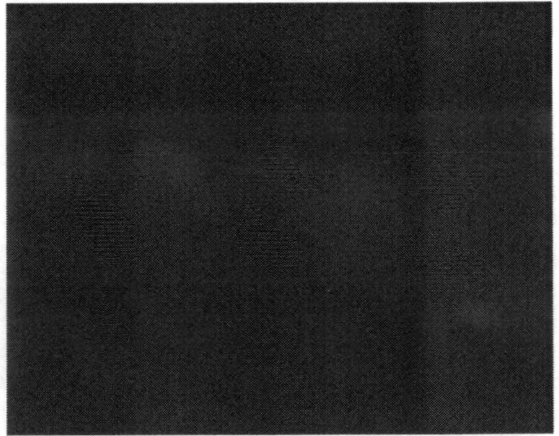


Fig.4 Actin disassembly in lung type II cells upon stimulation. Alveolar type II cells cultured in chamber slides overnight were incubated without (0 min, Panel A, B) or with 10 μM terbutaline for 3 min (Panel C, D) and 5 min (Panel E, F). At the end of incubation, the cells were fixed and stained with 50 nM Oregon Green Phalloidin. Fluorescent staining are shown on the right panels (B, D, F). The left panels (A, C, E) provide a brightfield view of the corresponding cells

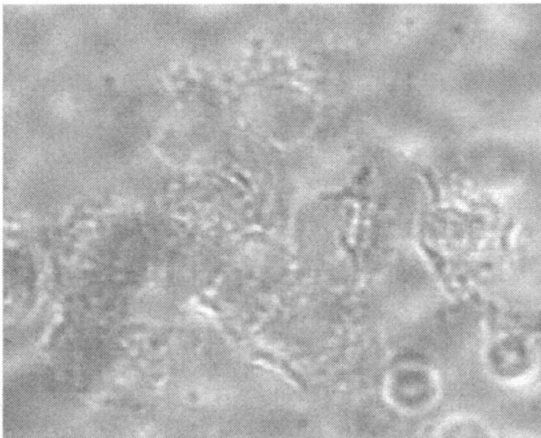
0 min

3 min

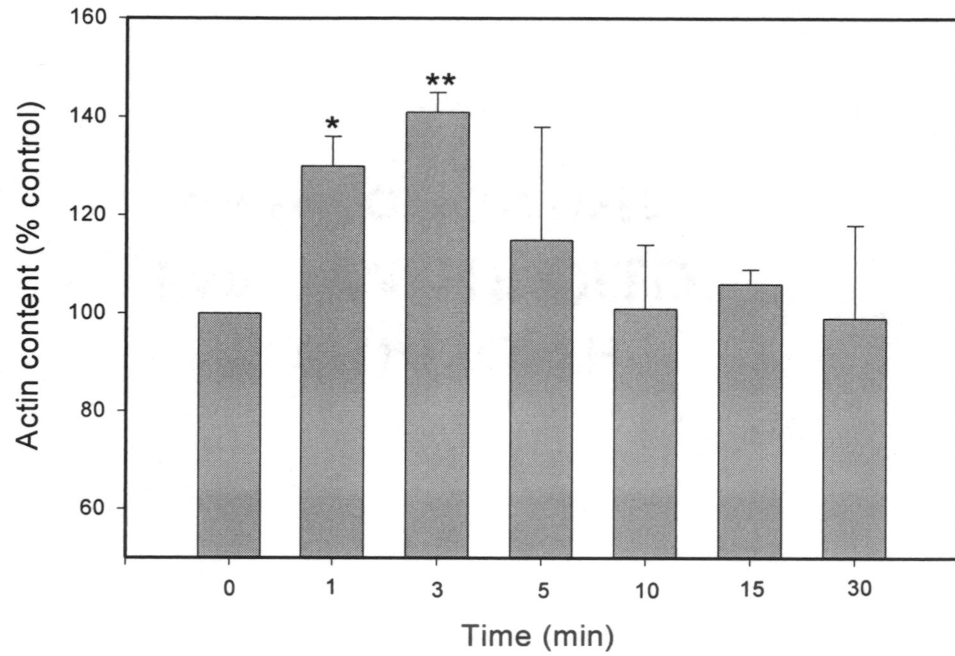
5 min



Redistribution of G-actin and Annexin II in stimulated type II cells

Disassembly of the cytoskeleton is necessary to allow lamellar bodies access to the plasma membranes following stimulation of type II cells. Annexin II has been shown to bundle actin filament in vitro (Glenney, Jr. et al., 1987; Khanna et al., 1990). Possible roles of annexin II in facilitating reorganization of cytoskeleton were investigated by determining the redistribution of annexin II and actin upon stimulation of type II cells. Overnight cultured type II cells were stimulated with 10 μM terbutaline for various times (0, 1, 3, 5, 10 and 30 min) and extracted with the extraction buffer containing 0.5% Triton X-100. The resulting soluble fraction was examined for the content of annexin II and actin by immunoblot using specific antibodies. In order to quantitate data from western blots, samples of the control soluble fractions with various amount of total protein were analyzed using specific antibodies for annexin II and actin. A linear relationship between the amount of protein loaded and the immunolabeling was observed in the range of 0.5-7.5 μg total protein (data not shown). The amount of protein that fell in the linear range was chosen for the following experiments. As shown in Fig. 5, there is an increase of G-actin in the soluble fraction during 1-3 min stimulation. However, G-actin contents returned to the control values after 5-30 min stimulation. Similarly, annexin II in the soluble pool (Fig. 6) was also increased at 1-3 min stimulation. A longer stimulation (5-30 min) resulted in a return of annexin II content to the resting levels. The data suggest a correlation between annexin II and reorganization of cytoskeleton in stimulated type II cells.

Fig.5 Redistribution of G-actin in stimulated type II cells. Rat alveolar type II cells were exposed to 10 μM terbutaline for different time spans. Triton X-100-soluble G-actin fractions were loaded onto SDS-PAGE (10% gel) and transferred to a nitrocellulose membrane. Blots were probed with anti-actin antibody and visualized using ECL. The actin bands were quantitated by ImageQuant. The results were expressed as percentage of the control (0 min). Data shown are mean ± SE (n=3).



* $p < 0.05$ vs control
** $p < 0.005$ vs control

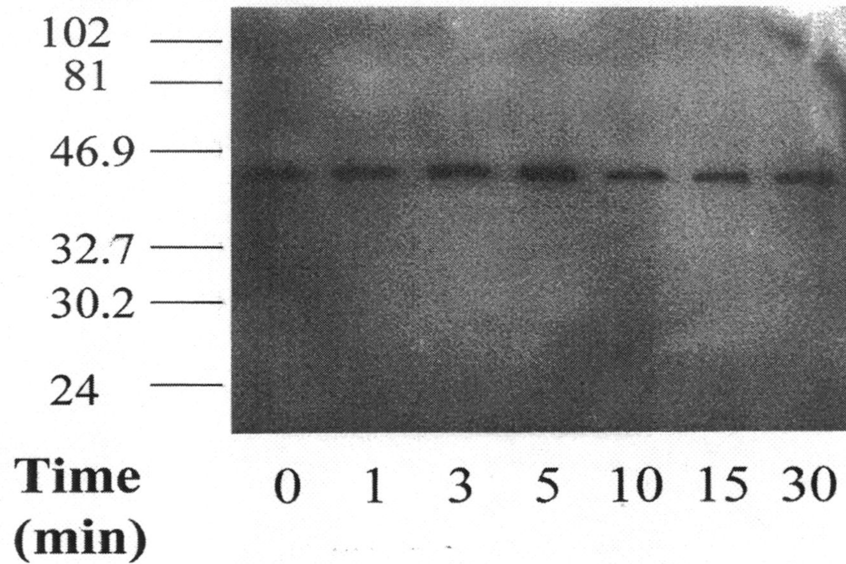
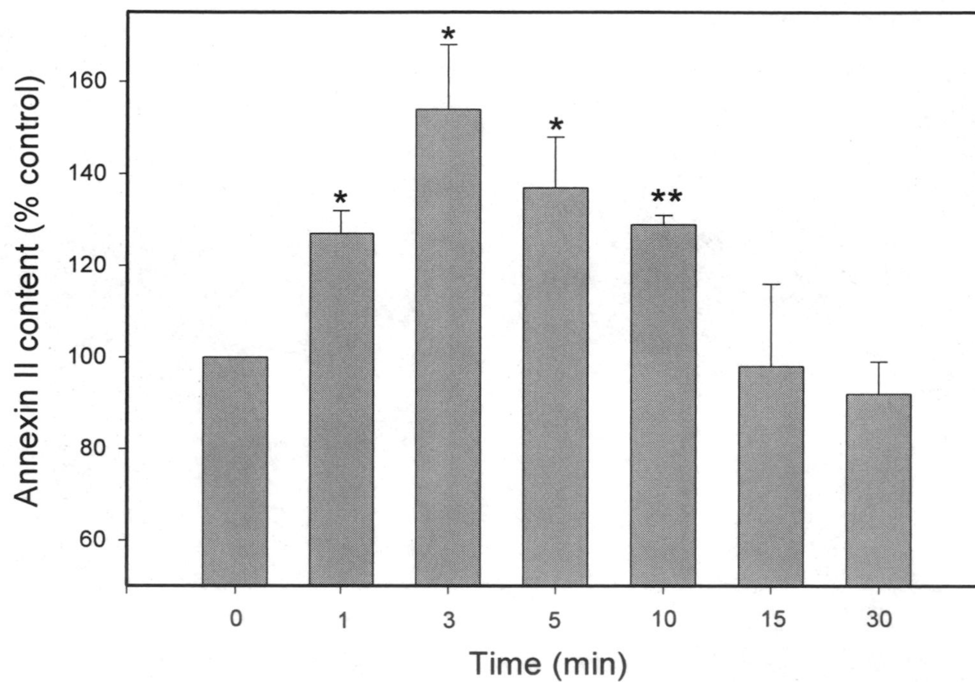
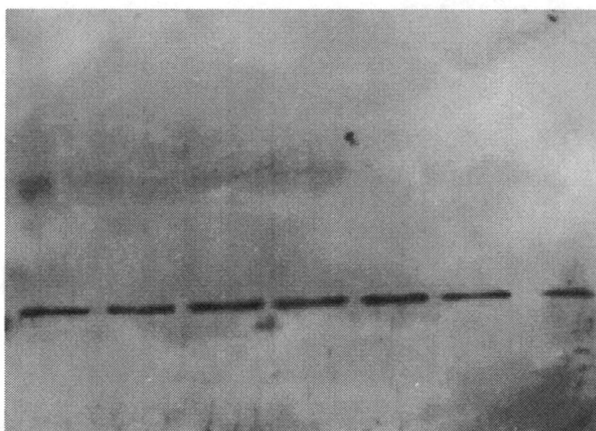


Fig.6 Redistribution of Annexin II in stimulated type II cells. Rat alveolar type II cells were exposed to 10 μM terbutaline for different time spans. Triton X-100-soluble fractions were loaded onto SDS-PAGE (10%) gel and transferred to a nitrocellulose membrane. Blots were probed with anti-annexin II antibody and visualized using ECL. The annexin II bands were quantitated by ImageQuant. The results were expressed as percentage of the control (0 min). Data shown are mean ± SE (n=3).



* $p < 0.05$ vs control
** $p < 0.005$ vs control



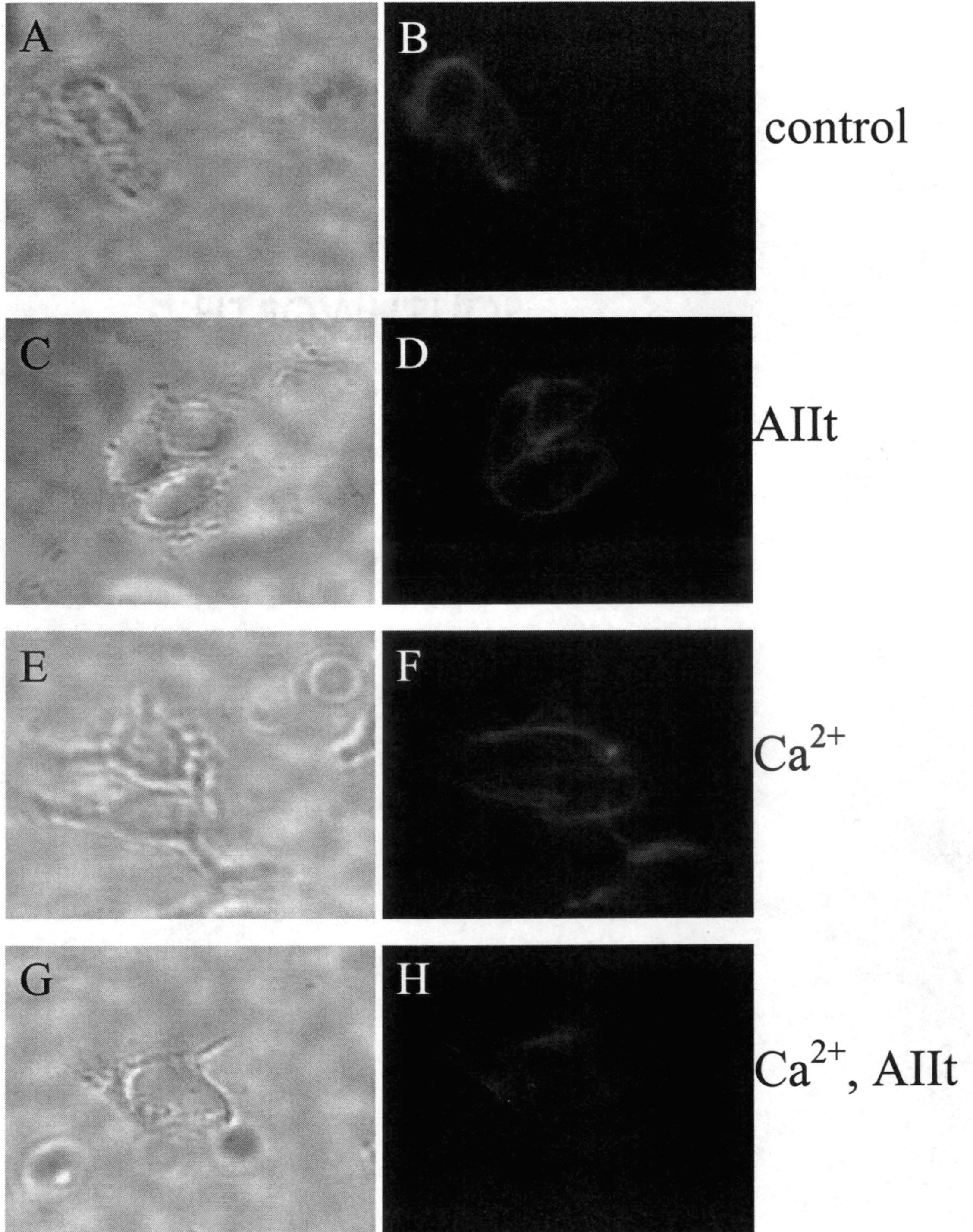
Effect of exogenous AIIt on actin disassembly in permeabilized type II cells

To directly test the role of AIIt in reorganization of cytoskeleton, type II cells were permeabilized with β-escin and thus exogenous AIIt was able to be introduced into the cells. The cortical actin network in permeabilized type II cells was examined by Oregon Green Phalloidin staining. The staining pattern in permeabilized cells was similar to that in intact cells, showing distinct cortical actin staining (Fig. 7 A and B). No changes in phalloidin staining were observed when AIIt was added in the absence of Ca^{2+} and MgATP (Fig. 7 C and D). Addition of 0.5 μM Ca^{2+} and 5 mM MgATP to permeabilized cells resulted in a minor disruption of the cortical actin staining (Fig. 7 E and F). Furthermore, the addition of AIIt along with 0.5 μM Ca^{2+} and 5 mM MgATP caused complete disruption of the cortical fluorescent ring (Fig. 7 G and H). The data presented here suggest that AIIt plays a role in the removal of the cortical actin, which normally acts as a barrier to exocytosis. Furthermore, it is implied that the ability of AIIt to dissociate the F-actin network requires the presence of Ca^{2+} and MgATP.

Distribution of F-actin in PMA treated type II cells

In previous studies, phorbol 12-myristate 13-acetate (PMA) treatment has been shown to potentiate the effect of 14-3-3 proteins on secretion and actin changes in permeabilized chromaffin cells (Roth and Burgoyne, 1995). Phorbol ester treatment also led to a rapid and reversible disassembly of cortical actin in intact chromaffin cells. In newly permeabilized chromaffin cells this disassembly was seen only in the presence of 0.3 μM free Ca^{2+} (Burgoyne et al., 1989). The effect of PMA treatment on F-actin

Fig.7 Effect of exogenous AIIt on actin disassembly in permeabilized type II cells. Type II cells were permeabilized with 40 μM β-escin for 10 min at room temperature, followed by a 10 min incubation without any addition (Panel A, B) or with 50 μg/ml AIIt (Panel C, D), 0.5 μM Ca^{2+} and 5 mM MgATP (Panel E, F), or 50 μg/ml AIIt, 0.5 μM Ca^{2+} and 5 mM MgATP (Panel G, H). The cells were then fixed with formaldehyde and stained for F-actin using Oregon Green Phalloidin. Panels on the right (A, C, E, G) show the fluorescent staining. Corresponding panels (B, D, F, H) on the left provide the brightfield view of the cells.


A
B
control
C
D
AIIt
E
F
Ca^{2+}
G
H
Ca^{2+}, AIIt

distribution, therefore, was examined in permeabilized type II cells using phalloidin staining. Once again, untreated cells exhibited a distinct cortical actin staining (Fig. 8 A and B). In cells treated with PMA, the fluorescence intensity decreased markedly (Fig 8 C and D). Since PMA is a protein kinase C (PKC) activator, PKC may play a role in the disruption of cortical F-actin through the phosphorylation of some endogenous actin-binding proteins.

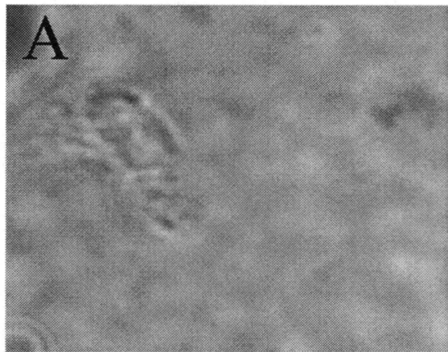
Distribution of F-actin in NEM-treated type II cells

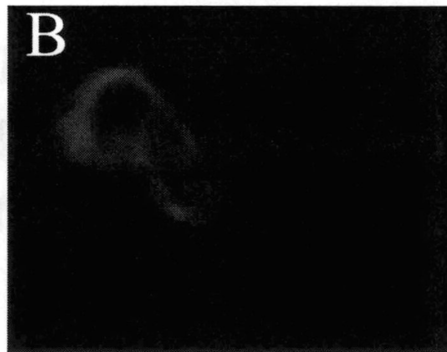
N-ethyl-maleimide (NEM) is a sulfhydryl reagent, which is used to modify cysteine residues in proteins. It has been shown to stimulate secretion in chromaffin cells, possibly via disruption of cortical cytoskeleton network (Wu et al., 1992). The effect of NEM on the distribution of F-actin in lung type II cells was therefore examined by labeling with Oregon Green Phalloidin. In the control cells there was a continuous fluorescent ring near the periphery of the cells (Fig. 9 A and B). However, treatment of the cells with NEM resulted in a complete disruption of this fluorescent ring (Fig. 9 C and D).

Inhibition of AIIt mediated F-actin bundling by NEM

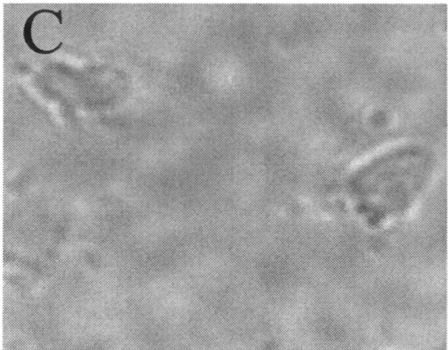
N-ethyl-maleimide caused disassembly of the cortical F-actin could be through inactivating actin-binding proteins. Annexin II tetramer has been shown to bind and bundle F-actin filament in Ca^{2+}-dependent manner (Glenney, Jr. et al., 1987; Khanna et al., 1990). Effect of NEM on the F-actin binding and bundling activity of AIIt was therefore investigated. Annexin II tetramer was treated with 100 μM NEM and excess

Fig. 8 Distribution of F-actin in PMA-treated type II cells. Type II cells were permeabilized with 40 μM β-escin for 10 min and treated with 1 μM PMA in the presence of 0.5 μM Ca^{2+} and 5 mM MgATP for 10 min at room temperature. Untreated (Panel A, B) and treated cells (Panel C, D) were stained with Oregon Green Phalloidin. Panels on the right (B, D) show the fluorescent staining. Panels on the left provide the brightfield view of the corresponding cells.



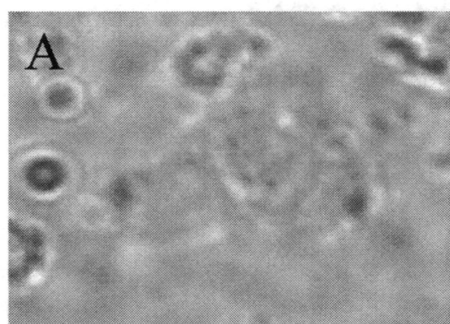


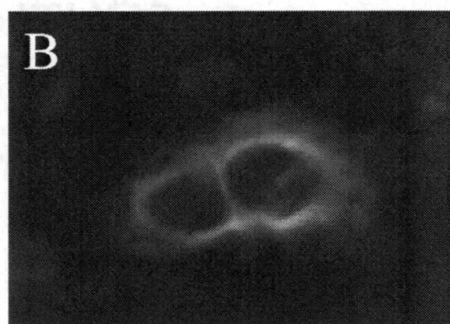
control



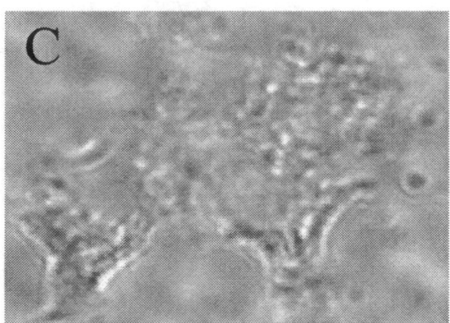
PMA

Fig. 9 Distribution of F-actin in NEM-treated type II cells. Type II cells were incubated without (Panel A, B) or with 30 μM NEM (Panel C, D) for 20 min at room temperature. The cells were then fixed with formaldehyde, stained with 50 nM Oregon Green Phalloidin and observed using a fluorescence microscope. Panels on the right (B, D) show fluorescent staining. Panels on the left (A, C) provide a brightfield view of the corresponding cells.





control



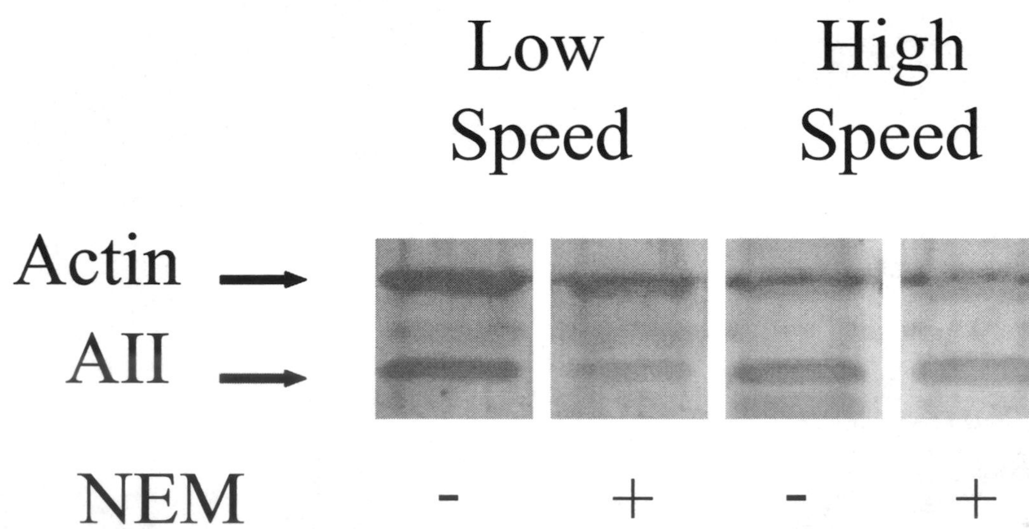
NEM

NEM in the reaction mixture was removed by bio-spin columns. F-actin was incubated with the treated or untreated AIIt and examined by co-sedimentation. F-actin bundling was analyzed by low-speed centrifugation (14,000 x g, 10 min) and F-actin binding by high-speed centrifugation (74,800 x g, 70 min). Under low-speed centrifugation, only F-actin bundles and AIIt associated with these F-actin bundles were sedimented. As shown in Fig. 10, a greater amount of AIIt and F-actin in the untreated sample (Fig. 10, lane 1) was recovered in the pellet from low-speed centrifugation compared to those in the NEM-treated sample (Fig. 10, lane 2). This suggests that NEM inhibits AIIt's ability to bundle F-actin. Since the bundling of F-actin by AIIt requires the binding of the protein to F-actin, it is possible that the NEM-treated AIIt has less ability to bind to F-actin and, therefore, F-actin bundling could not occur. To investigate this possibility, F-actin binding activity of AIIt was examined by high-speed centrifugation. Under those conditions, all F-actin filaments were sedimented. A similar amount of AIIt was found to co-sediment with F-actin in the untreated and NEM-treated samples (Fig. 10, lane 3, 4). These results suggest that treatment of AIIt by NEM has no effect on the ability of AIIt to bind to F-actin.

Inhibition of AIIt-mediated liposome aggregation by NEM

In addition to binding and bundling F-actin, AIIt is also able to bind and aggregate liposomes (Glenney, Jr. et al., 1987; Drust and Creutz, 1988). In order to determine whether the cysteine residues on AIIt play a role in liposome aggregation mediated by the protein, AIIt was pre-incubated with NEM for 10 min at room temperature. The treated

Fig. 10 NEM inhibits AIIt-mediated F-actin bundling without affecting AIIt-mediated F-actin binding. AIIt (6 μg) was incubated in bundling buffer in the absence or presence of 1 mM NEM for 10 min. At the end of incubation, AIIt was passed through a spin column to remove excess NEM. The recovered AIIt was mixed with F-actin (6 μg) in bundling buffer plus 100 μM Ca^{2+}. Samples were analyzed for F-actin bundling by low-speed centrifugation (14,000 x g, 10 min) and for F-actin binding by high-speed centrifugation (74,800 x g, 70 min). AIIt associated with the sedimented F-actin was analyzed on 10% SDS-PAGE. Lanes 1 and 2: low-speed centrifugation of untreated and NEM-treated AIIt; Lanes 3 and 4: high-speed centrifugation of untreated and NEM-treated AIIt.


Low
Speed
High
Speed
Actin
AII
NEM
-
+
-
+

AIIt was tested for its ability to aggregate liposomes in the presence of Ca^{2+}. As shown in Fig. 11, NEM inhibited AIIt-mediated liposome aggregation in a dose-dependent manner. The concentration resulting in 50% inhibition (IC_{50}) was 0.18 mM. At 1 mM NEM concentration, liposome aggregation was inhibited by 91%. The results suggest that modification of the cysteine residues by NEM interfered with AIIt's function.

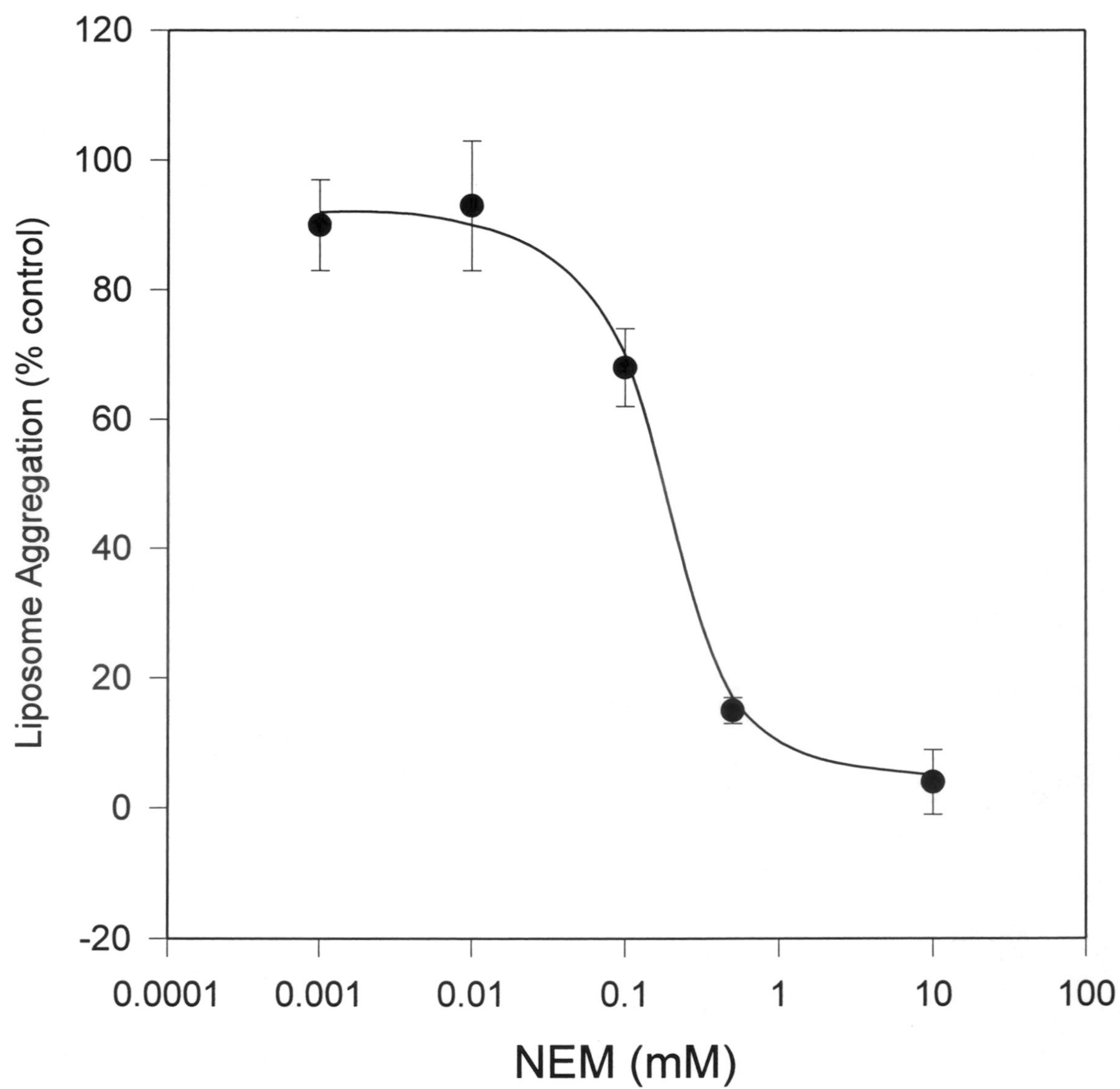
Ca^{2+} sensitivity of AIIt mediated liposome aggregation

Since AIIt is a Ca^{2+}-dependent protein, the effect of Ca^{2+} on AIIt-mediated liposome aggregation in the absence or presence of NEM was investigated. Fig.12 demonstrates that Ca^{2+} increased liposome aggregation in a dose-dependent manner with the EC_{50} of ~2 μM, in agreement with a previous report (Liu et al., 1995a). The greatest increase in liposome aggregation activity occurred between 1 to 10 μM Ca^{2+} concentration. However, when AIIt was pre-treated with 1mM NEM, no liposome aggregation was observed at any of the Ca^{2+} concentrations. This suggests that Ca^{2+} cannot reverse the inhibitory effects of NEM on AIIt.

Effect of Ca^{2+}-induced conformational changes in AIIt on the NEM inhibition

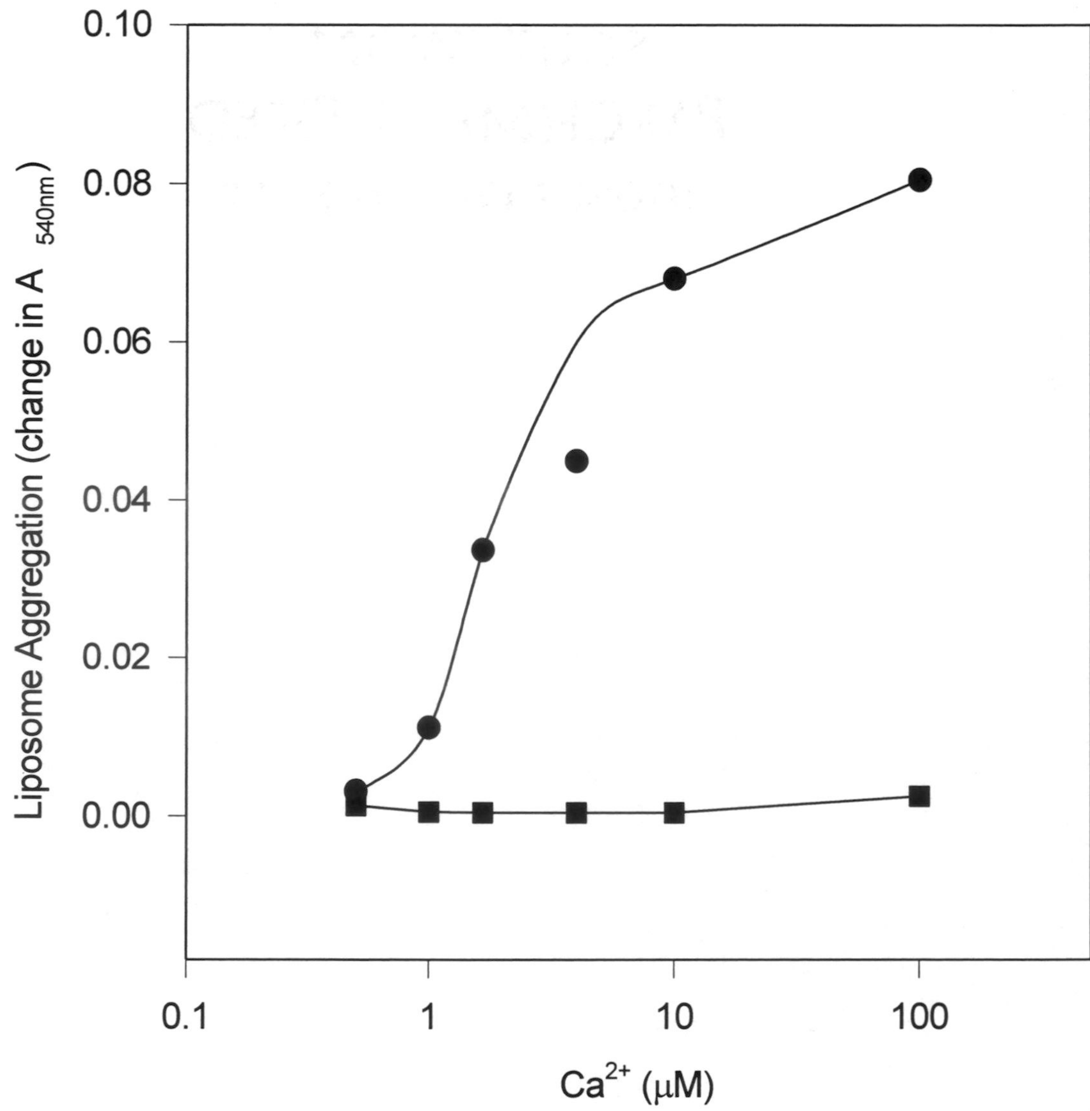
Ca^{2+} causes protein conformational changes in AIIt (Gerke and Weber 1985) and may alter the environment of reactive sites of AIIt by NEM. To test whether such changes influence the NEM inhibition of AIIt's activity, AIIt was preincubated with NEM in the presence of 1 mM EGTA or 1 mM Ca^{2+}. As shown in Fig. 13, a similar inhibition was observed in the presence of EGTA or Ca^{2+}, suggesting that the conformational changes caused by Ca^{2+} had no effect on the NEM inhibition.

Fig.11 NEM inhibits AIIt-mediated liposome aggregation in a dose-dependent fashion. Purified AIIt (5 μg) was incubated in 50 μl of 0.1 M Tris (pH 7.0) containing varying concentrations of NEM at room temperature for 10 min. Liposome aggregation activity was measured by monitoring the turbidity change (A_{540nm}). The aggregation assay was carried out in 1 ml of Ca^{2+}-EGTA buffer containing 1 mM free Ca^{2+} and 100 μg phosphatidylserine liposomes. Liposome aggregation activity was defined as the increase in absorbance after a 30 min incubation over the initial value. The results were expressed as a percentage of control. The data shown are mean ± SE from four experiments.



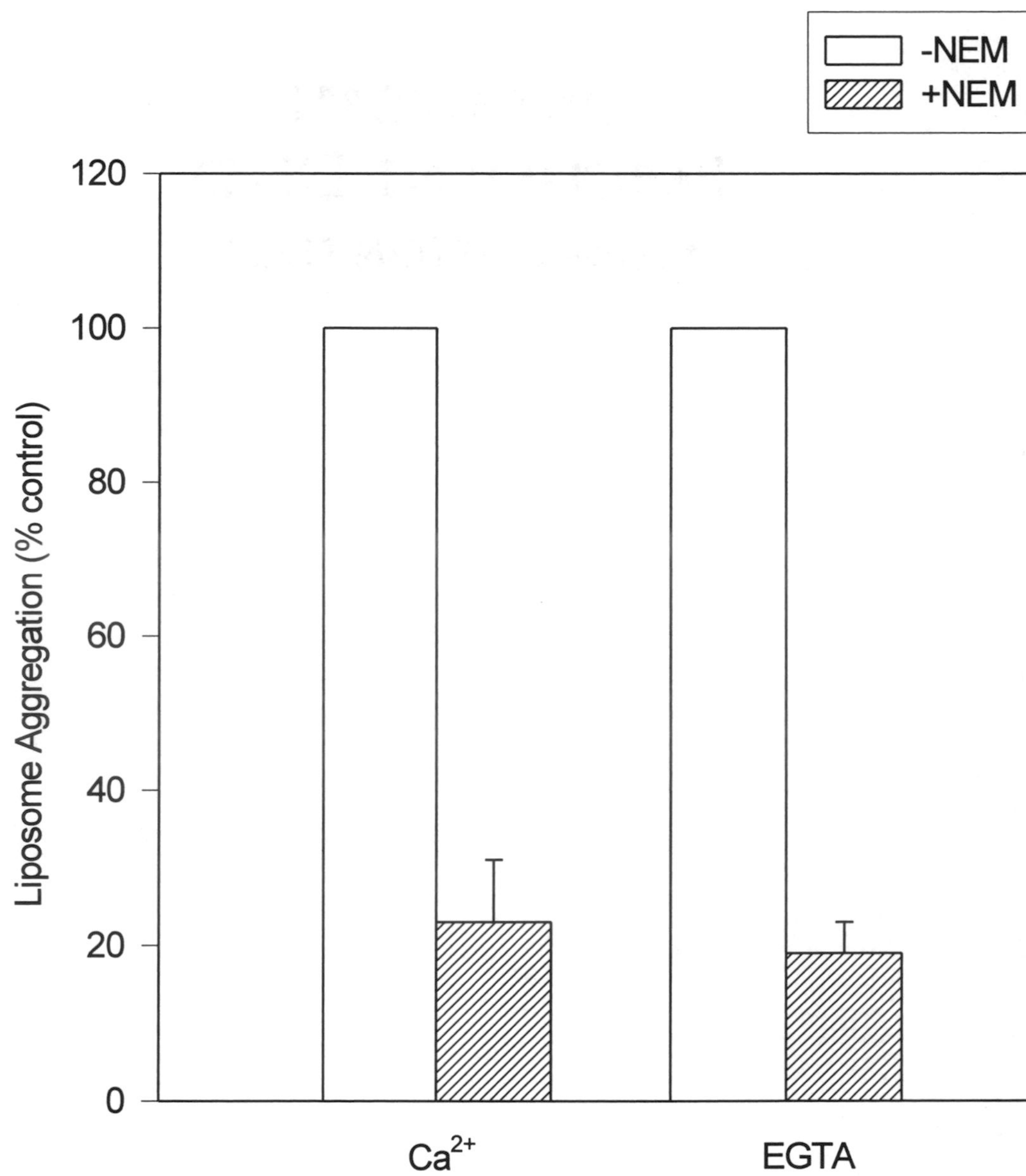
120
100
80
60
40
20
0
-20
Liposome Aggregation (% control)
0.0001
0.001
0.01
0.1
1
10
100
NEM (mM)

Fig. 12 Ca^{2+}-dependence of liposome aggregation by NEM-treated AIIt. AIIt was incubated with 1 mM NEM at room temperature for 10 min. Untreated and NEM-treated AIIt (5 μg each) were assessed for liposome aggregation activity in 1 ml Ca^{2+}-EGTA buffer containing 0.5-100 μM free Ca^{2+} and 100 μg phosphatidylserine liposomes. Liposome aggregation activity was expressed as the change in absorbance at 540 nm between the 30 min and zero time readings.



0.10
0.08
0.06
0.04
0.02
0.00
Liposome Aggregation (change in A 540nm)
0.1
1
10
100
Ca2+ (μM)

Fig. 13 Effect of Ca^{2+}-induced conformational changes in AIIt on the NEM inhibition. AIIt (5 μg) was preincubated in 25 μl of buffer (40 mM HEPES, pH 7.0, 100 mM KCl) containing either 1 mM Ca^{2+} or 1 mM EGTA for 10 min at room temperature. NEM at 1 mM was added and incubated for an additional 10 min at room temperature. Liposome aggregation activity was determined in Ca^{2+}-EGTA buffer containing 1 mM free Ca^{2+} plus 100 μg liposomes. The data were expressed as a percentage of control. Data shown are mean ± SE from 3 experiments.



-NEM
+NEM
120
100
80
60
40
20
0
Liposome Aggregation (% control)
Ca^{2+}
EGTA

NEM insensitivity of AIIt bound to liposomes

After determining the interaction between NEM and AIIt in solution, the effect of NEM on AIIt already bound to liposomes was investigated. After binding to membrane, some residues in AIIt may be hidden by the membrane and no longer accessible to NEM. To test this possibility, AIIt was pre-incubated with liposomes (50 μg) in either 1 mM Ca^{2+} or 1 mM EGTA buffer. After a 10 min incubation NEM was added and the mixture was incubated for an additional 10 min. Then, the mixture was added to Ca^{2+}-EGTA buffer (1 mM free Ca^{2+}) plus 50 μg liposomes and the liposome aggregation activity was determined. As seen in Fig. 14, in the presence of EGTA, NEM still inhibited AIIt's ability to aggregate liposomes as it does in solution, since no AIIt was bound to liposomes during pre-incubation under these conditions. However, in the presence of Ca^{2+}, AIIt was bound to liposomes during pre-incubation. In this case, NEM was unable to alter AIIt's ability to cause liposome aggregation.

Specificity of NEM action

In addition to annexin II, annexins I, II or IV can also aggregate liposome at 1 mM Ca^{2+} (Liu et al. 1996). In order to test whether NEM specifically targets AIIt or acts on all annexins, the effect of NEM on other annexins was tested. Two preparations were used for this purpose. One (AI and IIm) is a mixture composed of approximately 50% annexin I and 50% annexin II monomer. Another (AIII and IV) is a mixture of approximately 50% annexin II and 50% annexin IV (Fig. 15A). These two annexin mixture s along with AIIt were all incubated separately with 1 mM NEM for 10 min

Fig. 14 NEM insensitivity of AIIt bound to liposomes. AIIt (5 μg) was preincubated with liposomes (50 μg) in 25 μl of buffer (40 mM HEPES, pH 7.0, 100 mM KCl) containing 1 mM Ca^{2+} or 1 mM EGTA for 10 min at room temperature. NEM at 1 mM was added and incubated for an additional 10 min. Liposome aggregation activity was determined in Ca^{2+}-EGTA buffer containing 1 mM free Ca^{2+} plus 50 μg liposomes. The data were expressed as a percentage of control (NEM-treated AIIt / untreated AIIt x 100%). Data shown are mean ± SE from 3 experiments.

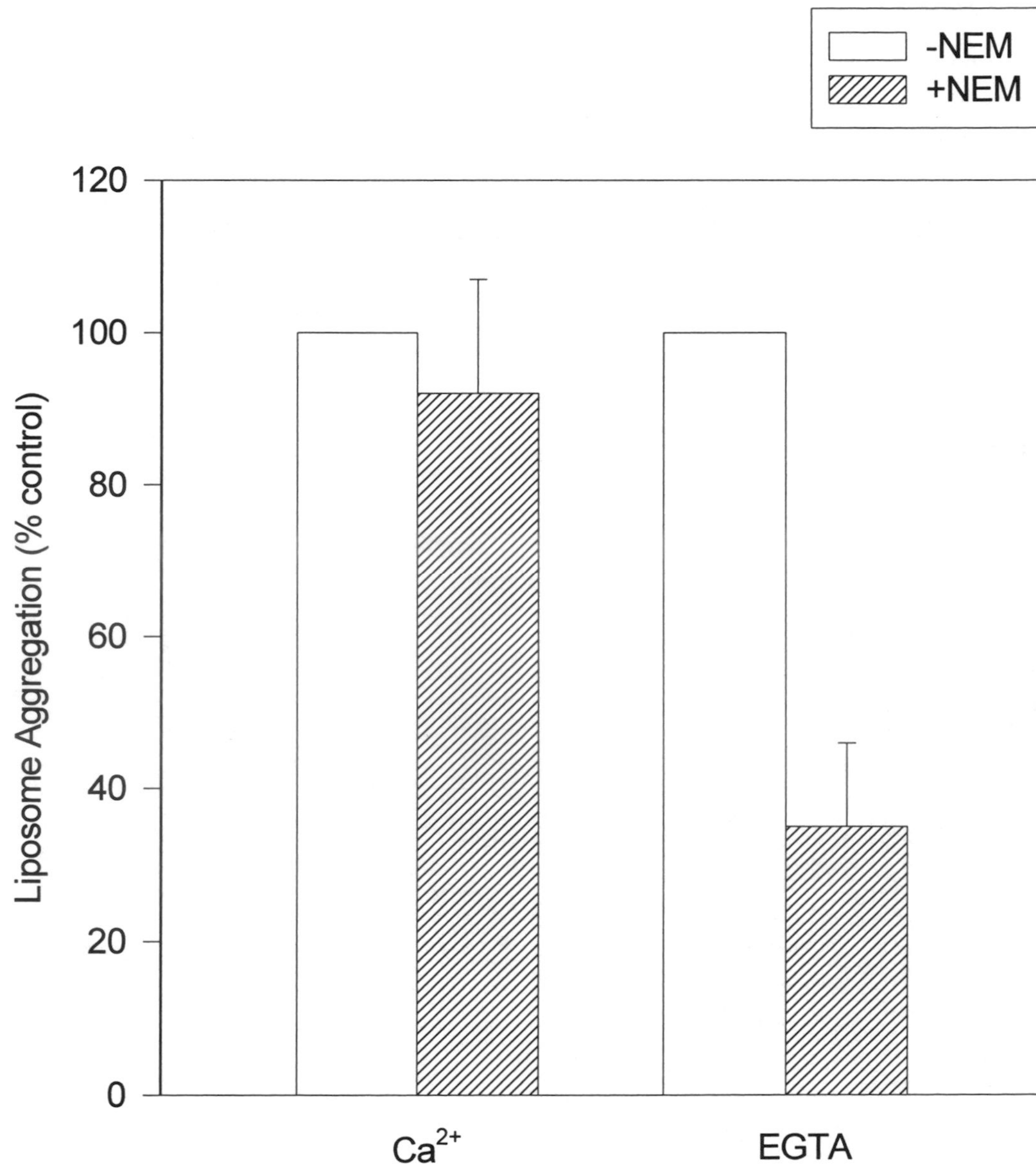
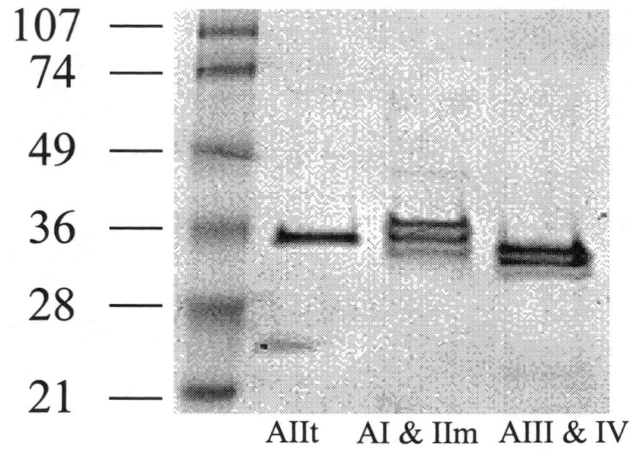
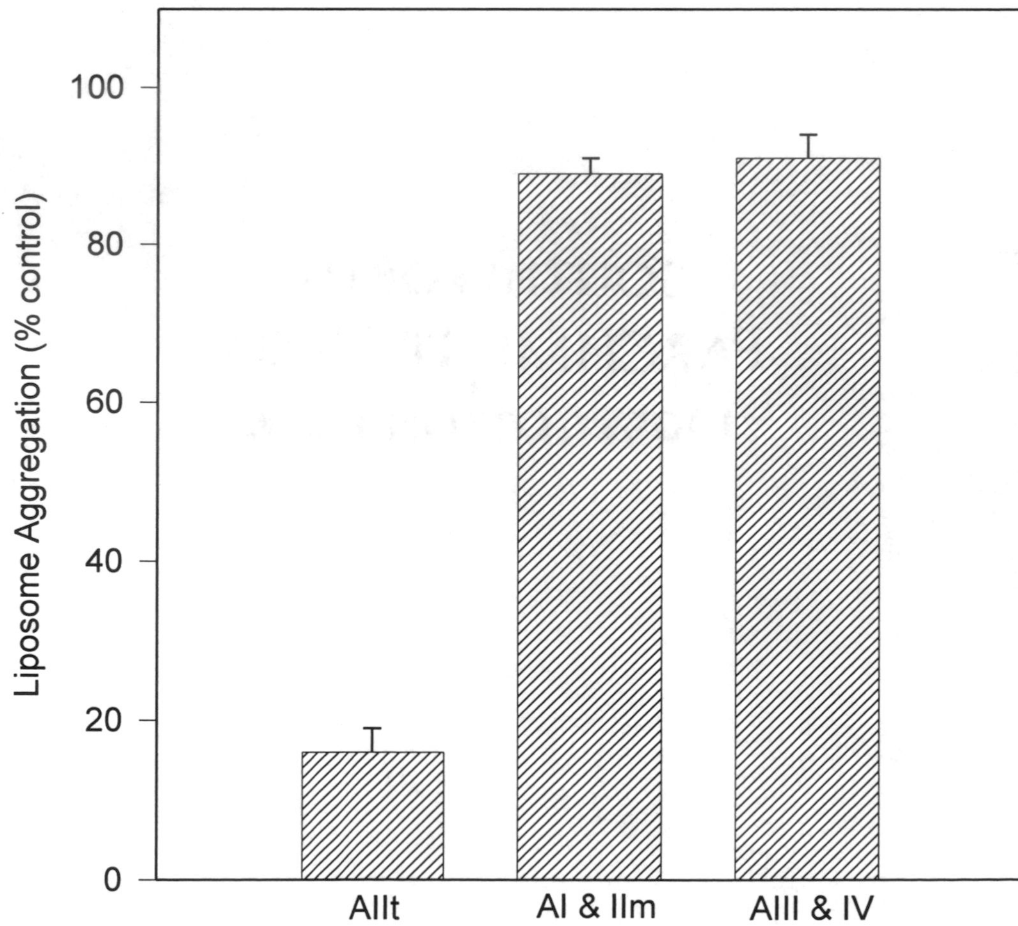

-NEM
+NEM
Liposome Aggregation (% control)
120
100
80
60
40
20
0
Ca2+
EGTA

Fig. 15 NEM inhibits liposome aggregation mediated by AIIt, but not by AI & IIm or AIII & IV. A. All three annexin proteins were analyzed on 10% SDS-PAGE and stained with Coomassie Blue. The bands were quantitated using ImageQuant. Approximately 50% annexin I and 50% annexin II monomer, and, 50% annexin III and 50% annexin IV were present in AI & AIIm and AIII & AIV, respectively. B. Annexins IIt, I & IIm and III & IV (5 μg each) were incubated in 50 μl of 0.1 M Tris (pH 7.0) in the absence or presence of 1 mM NEM for 10 min at room temperature. Liposome aggregation mediated by various annexins was determined. The results were expressed as % control (i.e. activity of NEM-treated annexin / activity of untreated annexin x 100%). The data shown are mean ± SE from four experiments.


kDa
107
74
49
36
28
21
AIIt
AI & IIm
AIII & IV

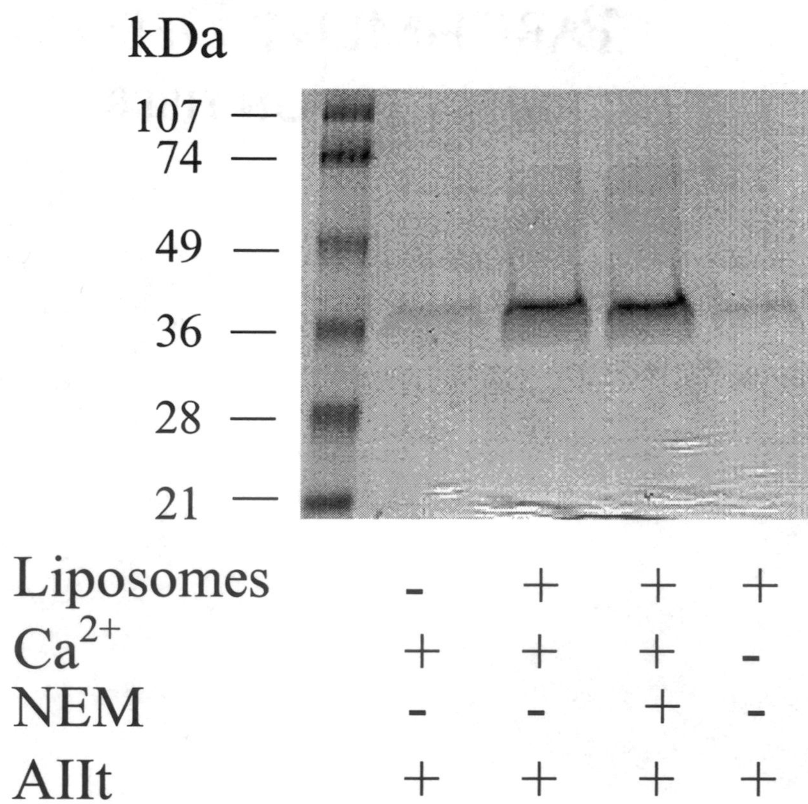

Liposome Aggregation (% control)
100
80
60
40
20
0
AIIt
AI & IIm
AIII & IV

at room temperature and were tested for their aggregation activity. Fig. 15B shows that NEM did not have a significant effect on AI and IIm or on AIII and IV, in contrast to its effect on AIIt. This suggests that NEM specifically targets essential cysteine residues on AIIt and not on other annexins.

Effect of NEM on AIIt's ability to bind liposomes

Since NEM affects AIIt's ability to aggregate liposomes, the loss in liposome aggregation activity of NEM-treated AIIt may correspond with a loss in liposome binding activity. In order to examine the liposome binding activity of AIIt, liposomes were incubated with the NEM-treated or untreated AIIt and were spun down by centrifugation. AIIt in pellet was analyzed on SDS-PAGE. Fig. 16 lanes 2 and 3 show that NEM did not influence AIIt's ability to bind liposomes. In the control experiments, little AIIt was observed, when liposomes or Ca^{2+} were omitted in the incubation medium (Fig. 16 lanes 1 and 4), suggesting that AIIt in the pellet was not due to AIIt aggregate or non-specific binding to the membranes.

Fig. 16 NEM does not affect the binding of AIIt to liposomes. AIIt (10 μg) was incubated in the absence or presence of 1 mM NEM for 10 min. At the end of incubation, AIIt was mixed with PS:PC liposomes (200 μg, 1:1) plus or minus 1 mM Ca^{2+}. After a 20 min incubation at room temperature, liposomes were sedimented by centrifugation and AIIt associated with liposomes was analyzed on 10% SDS-PAGE. Lane 1: untreated AIIt plus 1 mM Ca^{2+}; Lane 2: NEM-treated AIIt plus 1 mM Ca^{2+} and liposomes; Lane 3: untreated AIIt plus 1 mM Ca^{2+} and liposomes; Lane 4: untreated AIIt plus 1 mM EGTA and liposomes.


kDa
107
74
49
36
28
21
Liposomes - + + +
Ca2+ + + + -
NEM - - + -
AIIt + + + +

DISCUSSION

Cytoskeleton and surfactant secretion

Alveolar epithelial type II cells synthesize pulmonary surfactant and store it in secretory granules called lamellar bodies. The binding of agonists to the receptors on plasma membranes initiates the signal transduction leading to the changes in second messengers and eventually an increase of lung surfactant secretion. For example, exposing these cells to β-agonists such as isopoterenol or terbutaline increases the secretion of surfactant in cell culture and perfused lung models (Chander and Fisher, 1990). These agents increase the intracellular cyclic AMP levels via stimulation of adenylate cyclase. Two essential steps involved in the later stage of secretory process include the translocation of lamellar bodies to the apical plasma membrane and fusion of the lamellar body with the plasma membrane. Previous studies have showed close association of actin with lamellar bodies and actin-binding proteins such as spectrins and annexins, beneath the cell surface in type II cells (Liu et al., 1996). Actin exists in two forms: a soluble or G form and a filamentous or F form. Rapid shifts from one form to another correspond with changes in cellular function (Tsilibary and Williams, 1983). It is therefore hypothesized that secretion of surfactant in type II cells may be associated with changes in the cytoskeleton network.

The relationship between cytoskeleton and lung surfactant secretion has been studied using cytochalasins, which act as cytoskeletal disrupting agents. Cytochalasins are fungal products, which prevent actin polymerization by binding to the plus end of

actin filaments (Alberts et al., 1999). Cytochalasins have been known to alter the microfilament organization in several cell types such as mouse adrenal tumor cells (Hall et al., 1981), red blood cells (Jung and Rampal, 1977; Lin and Lin, 1978; Lin and Snyder, 1977) and fibroblasts (Domnina et al., 1982). A previous study demonstrated that cytochalasins A, B, C, and D enhance release of surfactant from cultured type II cells (Rice et al., 1984). Cytochalasin D was the most effective of all the others. Furthermore, cytochalasin B, C, and D had a biphasic effect where at high concentrations surfactant release approached control values. The same study also examined the effect of cytochalasin D on surfactant release induced by terbutaline. Results showed that the surfactant release induced by cytochalasin D was additive to release produced by terbutaline. One explanation for this observation is that terbutaline and cytochalasin D cause the release by distinct mechanisms. Another explanation is that the two agents are affecting different steps in the same pathway. Treatment of type II epithelial cells with cytochalasin D causes alterations in microfilament organization suggesting that cytochalasin-induced increase in surfactant release is somehow associated with reorganization of the cytoskeleton (Rice et al., 1984).

Experiments using cytochalasins to investigate the role of cytoskeleton in the surfactant secretory process give controversial results. The reduction of secretory events as well as the increase in surfactant release are observed (Rice et al., 1984; Wirtz and Dobbs, 1990). The cause of these inconsistencies could be the limited specificity of cytochalasins. In addition to interacting with microfilamentous network, cytochalasins

bind to various cellular target sites (Urbanik and Ware, 1989; Walling et al., 1988). Another reason for the discrepancies may be related to the different dose used by different investigators (Rice et al., 1984).

Cytoskeleton disassembly in type II cells

The cytoskeleton acts as a barrier to exocytosis. In unstimulated cells, the secretory granules are trapped within the actin filament network and their interaction with the plasma membrane is restricted by the F-actin enriched cell cortex. The cortical actin network underneath the cell surface has to reorganize so that the secretory granules can reach the exocytotic sites in stimulated cells. The reorganization might consist of actin filament crosslinking, assembly and interactions with secretory granules and plasma membranes (Burgoyne and Cheek, 1987). Reorganization of the cytoskeleton has been associated with increased levels of cytosolic cAMP and secretion of cellular products in various cells including rabbit neutrophils (Di Virgilio and Gomperts, 1983), human lymphocytes (Greene et al., 1976), and adrenocortical tumor cells (Mattson and Kowal, 1982). Stimulation of adrenal chromaffin cells by nicotinic agonists also results in an increase in exocytosis concomitant with depolymerization of cortical actin filaments. The depolymerization of cortical actin is, however, a transient response to nicotinic stimulation (Cheek and Burgoyne, 1986). Thus, it is postulated that stimulation of secretory cells results in a disruption of the physical barrier, which allows secretory granules access to the plasma membrane.

The present finding, which used A549 cells as a model for type II cells, showed a

similar trend. Stimulation of A549 cells with terbutaline resulted in a transient disassembly of the F-actin when stained with phalloidin, which binds specifically to the filamentous form of actin. The maximal effect was seen at 1 min and a longer stimulation resulted in reassembly of the cortical F-actin. The DNase inhibition analysis confirmed the results from the phalloidin staining.

In the present study, disassembly of cytoskeleton was also studied in primary culture of alveolar type II cells. Western blot analysis revealed an increase in the G-actin fraction in type II cells upon stimulation with terbutaline. The maximum increase was seen at 3 min. Then, G-actin level returned to basal level. A similar study has shown an increase in the G-actin fraction concomitant with a decrease in the cytoskeletal F-actin fraction in adult rat lung type II cells stimulated with terbutaline for 1 min (Bhandari et al., 1997). However, a detailed time course has not been done. Using phalloidin, it was observed that in resting cells the cortical region of the cell was particularly enriched in F-actin. This observation correlates well with previous studies showing the presence of numerous actin filaments in this region connecting the plasma membrane and secretory granules (Nakata and Hirokawa, 1992). Upon stimulation with terbutaline, the phalloidin staining around the cortical region disappeared at 1-3 min and reappeared thereafter, suggesting a transient disassembly of F-actin.

Botulinum C_2 toxin is a binary toxin which ADP ribosylates nonmuscle G-actin. Rose and coworkers (1999) found that the C_2 toxin caused a dose-dependent progressive depletion of the cellular F-actin content and a concomitant increase in the G-actin

content. The F-actin decay induced by C_2 toxin was paralleled by a dose-dependent increase of basal surfactant secretion. At high toxin doses there was more than a twofold increase in baseline PC release. Furthermore, the study showed that pre-exposure of type II cells to C_2 toxin enhanced the surfactant secretory response to the secretagogues and mechanical stretch. In contrast stabilization of F-actin by phalloidin causes a decrease in surfactant secretion. All these observations suggest that the altered state of actin assembly has a significant impact on the surfactant secretory process under basal and stimulatory conditions.

In summary, stimulation of type II cells with terbutaline results in the reorganization of the cortical actin cytoskeleton that normally acts as a barrier to exocytosis of lamellar bodies. This cytoskeletal reorganization causes an increase in lung surfactant secretion. This conclusion is based on the following findings: (i) there is an abundance of F-actin in the cortical region of the type II cells (Fig. 4); (ii) there is a transient increase in G-actin in type II cells (Fig. 5) (Bhandari et al., 1997); (iii) F-actin decay induced by C_2 toxin is paralleled by an increase in surfactant secretion (Rose et al., 1999); and (iv) cytochalasins, which disrupt the microfilament organization, enhance surfactant secretion (Rice et al., 1984) and phalloidin, which stabilize F-actin, decreases the secretion (Rose et al., 1999).

Annexin II and actin disassembly

As discussed above, stimulation of type II cells by terbutaline causes actin reorganization and thus leads to an increase in surfactant secretion. However,

mechanisms of these changes provoked by terbutaline are unknown. One mechanism could be due to changes in actin-binding proteins. One of the candidates is annexin II, which has been shown to bind and bundle F-actin (Waisman, 1995). In the present study, it is found that annexin II undergoes a transient redistribution from the F-actin fraction to the G-actin fraction in type II cells stimulated with terbutaline. Those changes were similar to actin, suggesting a role of annexin II in the reorganization of cytoskeleton. A possible mechanism for annexin II-mediated cytoskeletal reorganization is speculated as follows: terbutaline, a β-adrenergic agonist, raises intracellular cAMP levels by activating the adenylate cyclase pathway. The increased cAMP activates cAMP-dependent protein kinase (PKA) which phosphorylates various proteins in the type II cells. Several targets by PKA in type II cells have been identified (Whitsett et al., 1985). The activated PKA might phosphorylate annexin II, cause its dissociation from F-actin, dismiss its F-actin bundling activity and therefore lead to the disassembly of cytoskeleton. Previous *in vitro* studies have shown that phosphorylated AIIt neither binds to nor bundles F-actin at physiological levels of Ca^{2+} (Hubaishy et al., 1995). Even high levels of Ca^{2+} up to 1 mM could not reverse the inhibitory effects of phosphorylation. Therefore, phosphorylation of AIIt is an inhibitory signal preventing the formation of F-actin filament plus the formation of F-actin bundles or network. Since the phosphorylation state of proteins in terbutaline-stimulated type II cells is transient (Zimmerman et al., 1996), the speculation above correlates well with the present data that redistribution of both annexin II and actin occurs at the early stages of stimulation. This

might be due to the presence of various phosphatases and other dephosphorylating mechanisms. Phorbol 12-myristate 13-acetate, a protein kinase C activator, has been shown to cause a decrease in the content of filamentous actin in the cortical region of the permeabilized rat mast cells (Koffer et al., 1990). This decrease in F-actin content is paralleled by an increase in histamine secretion. In the present study, treatment of permeabilized type II cells with PMA in the presence of Ca^{2+} and MgATP resulted in complete disruption of the cortical F-actin. Protein kinase C is thought to regulate cortical actin disassembly through phosphorylation of an actin-binding protein, which in this case may be AIIt. As mentioned previously, once phosphorylated, AIIt is unable to bind or bundle F-actin thus resulting in the disassembly of cortical F-actin.

Exogenous AIIt and cytoskeleton disassembly

A study done by Koffer and co-workers (1990) examined the changes in actin during exocytotic reaction of permeabilized rat mast cells. After permeabilization, mast cells are capable of exocytosis when stimulated with GTP-γ-S and Ca^{2+}. Treatment of permeabilized cells with cytochalasin E decreases the Ca^{2+} requirement, implicating that disassembly of the cytoskeleton may be necessary for exocytosis. It is thought that this disassembly is controlled by Ca^{2+}-dependent actin regulatory proteins. So far the results from the present study have supported the hypothesis that AIIt is involved in cytoskeletal reorganization in intact type II cells. Furthermore, it has been previously established that addition of AIIt to permeabilized alveolar type II cells enhances Ca^{2+}-dependent PC secretion (Liu et al., 1996). In the present study, the addition of AIIt to permeabilized

type II cells in the presence of MgATP and Ca^{2+} resulted in a significant disruption of the cortical actin. It is possible that this is caused by AIIt's ability to sever F-actin filament in the presence of high concentrations of Ca^{2+} (Martin et al., 1988). This might seem controversial to the previous experiments in this study where AII is thought to bind and bundle F-actin. However, two forms of AIIt may exist in type II cells (Liu, 1999a). One is AIIt bound to cytoskeleton in the resting conditions. Phosphorylation of this form of AIIt leads to the dissociation of AIIt with cytoskeleton and thus disruption of cytoskeleton. Another is soluble AIIt that binds to cytoskeleton in response to a rise of Ca^{2+}. The binding sites of those two forms of AIIt may be different. While the former may directly bind to actin, the latter may bind to actin-binding proteins. Complex formation between AIIt, F-actin and fodrin has been shown to occur at high Ca^{2+} concentrations. The binding of AIIt to fodrin and F-actin is abolished by the addition of EGTA (Gerke and Weber, 1984). Other studies have also demonstrated a calcium-dependent interaction between AIIt and fodrin (Cheney and Willard, 1989). Therefore, it is possible that in the presence of high Ca^{2+} concentration, exogenous AIIt binds to the spectrin/fodrin and inhibits its F-actin cross-linking activity. This explains why the addition of exogenous AIIt in the presence of Ca^{2+} causes the disruption of cortical F-actin.

Previous studies have suggested the involvement of cytosolic Ca^{2+} in inhibition of actin cross-linking. They showed that addition of 10 μM free Ca^{2+} to digitonin-permeabilized cells results in a slower reduction in cytoskeletal actin than the actin

changes in intact cells. However, the reduction of actin in permeabilized cells had a time course similar to that for catecholamine secretion. In chromaffin cells, it is believed that the effect of micromolar Ca^{2+} on actin might involve the Ca^{2+} -dependent actin severing protein gelsolin (Bader et al., 1986). Severing is a Ca^{2+} activated process in which the severing protein attaches to the actin subunit such as F-actin and induces its break with neighboring subunits (Stossel et al., 1985; Pollard and Cooper, 1986). The severing protein prevents the end of the filament form annealing with other actin filaments or binding actin monomers (G-actin). Villin, another actin-severing protein, severs actin filaments at high Ca^{2+} concentrations, but at low Ca^{2+} concentrations it cross-links actin filaments into bundles (Matsudaira and Janmey, 1988). Therefore, an alternative explanation for effects of AIIt on cytoskeleton is that AIIt behaves in a similar manner. Annexin II tetramer binds and bundles F-actin at low, physiological concentration of Ca^{2+}, but in the presence of high Ca^{2+} concentration it disrupts the F-actin bundles.

NEM, cytoskeleton, and surfactant secretion

The results presented above demonstrate that AIIt does play a role in the reorganization of the cytoskeleton in stimulated type II cells. The role of AIIt was further examined by modifying the cysteine residue on AIIt using NEM, a sulfhydryl reagent. Recent studies have shown that nitric oxide can modulate AIIt's activities including membrane aggregation and fusion by S-nitrosylation / formation of disulfide bonds. This modification is reversible by dithiothreitol. This mechanism might represent a novel posttranslational modification and possibly a new regulatory mechanism for annexin II in

cells (Liu et al. 1998). Results from this previous study gave rise to an interest in investigating whether chemical modification of cysteine residues by NEM has an effect on AIIt's function. The present study demonstrates that AIIt-mediated liposome aggregation and F-actin bundling is inhibited by NEM modification.

A study done by Wagner and co-workers (1992) showed that treatment of chromaffin cells with 30 μM NEM resulted in a decrease in the amount of cytoskeletal F-actin. They also observed an increase in the secretory activity in NEM-treated cells (Wu et al., 1992). Depolymerization of the cortical F-actin by NEM would allow the secretory granules to move closer to the plasma membrane in the resting cells. Then when these NEM-treated cells are stimulated, a larger number of secretory granules would be available for fusion thus resulting in an increase in the rate of exocytosis. However, how NEM depolymerizes the cortical F-actin is still unclear. In the present study, when alveolar type II cells were treated with NEM disappearance of cortical F-actin was observed by staining the cells with phalloidin. The results demonstrate that treatment of type II cells with NEM also causes a disassembly of the cortical F-actin network. Taking into account NEM's effect on AIIt *in vitro* it is implied that NEM disrupts the F-actin network by inhibiting AIIt's ability to bundle F-actin.

NEM and AIIt function

NEM inhibits AIIt functions by modifying the sulfhydryl group on cysteine residues. There are several possible mechanisms: (i) binding of NEM to the cysteine residues may lead to conformational changes in the protein and interference of its

function; (ii) NEM can affect the active sites in enzymes or proteins containing these cysteine residues. N-Deacetylase-N-sulfotransferases (NDANST) is involved in the biosynthesis of heparin and heparan sulfate. The derivatization of Cys486 in NDANST with NEM results in total inactivation of the deacetylase activity due to steric hindrance of the active site (Wei and Swiedler, 1999); and (iii) binding sites for substrates or other cofactors may also be affected by NEM. One of the examples is Ssa1p, an ATPase that binds to proteins to guide their folding, assembly, and translocation across membranes. NEM binds to one of the cysteine residues on Ssa1p and sterically interferes with binding of the potassium ion, causing the disruption of interactions between the nucleotide, metal ions, and neighboring amino acid residues (Liu et al., 1996). There are five cysteine residues in each annexin II molecule. Cysteine residues are found on the amino- as well as the carboxyl-terminal region. This raises the possibility that NEM could modify cysteine residues on either terminus. Although it has been shown that NEM interferes with AIIt's ability to aggregate liposomes and bundle F-actin, the exact location of NEM modification on AIIt remains to be determined.

It has been proposed that the ability of AIIt to bundle F-actin could be due to self-association of F-actin-bound AIIt (Jones et al., 1992). After AIIt binds to F-actin, an interaction occurs between F-actin and the F-actin bundling site on AIIt, which results in a conformational change in AIIt. As a result of the conformation change, AIIt is able to interact with other AIIt molecules already bound to F-actin and therefore in their active conformation (Waisman, 1995). The present results demonstrate that AIIt's ability to

bundle F-actin is altered by NEM, however its ability to bind F-actin is not affected. If the proposed model above holds true, then NEM must be acting on the cysteine residues involved in AIIt-AIIt bridge formation and thus preventing the self-association of F-actin-bound AIIt. However, if F-actin bundling is due to the interaction of one annexin molecule to two distinct F-actin filaments, then NEM could be acting on one of the binding sites on AIIt and not the other one, therefore preventing F-actin bundling without interfering with F-actin binding.

Two mechanisms of annexin-mediated membrane aggregation and fusion have been proposed. In the first model it is suggested that a single annexin molecule interacts with two distinct vesicles, implicating that two phospholipid-binding sites exist on one annexin molecule. This is supported by a previous study showing that the second phospholipid-binding site is exposed after the initial binding of annexin to a PS monolayer (Andree et al., 1993). The second model involves the self-association of membrane-bound annexin to form a protein-protein bridge. Some annexins have been known to undergo polymerization in solution or on the membrane in the presence of Ca^{2+} (Creutz et al., 1979; Sudhof et al., 1982). Results from the present study demonstrate that NEM-treated AIIt is unable to aggregate liposomes, but still has the ability to bind liposomes. There are two possible explanations for this finding. One is that, NEM may impair the membrane-bridging activity of AIIt by preventing the formation of the AIIt-AIIt bridge. Another explanation is that, NEM might be affecting one binding site, but not the second binding site on the same annexin molecule, therefore, allowing the binding

of liposomes, but not the aggregation of liposomes. These results also indicate that the liposome aggregation activity and liposome binding activity of AIIt may reside in distinct domains of the protein. There is evidence suggesting that the binding of annexin to phospholipid and annexin-mediated vesicle aggregation are not coupled to each other. Phosphorylation of annexins I or II by PKC causes inhibition of vesicle aggregation without having a significant affect on phospholipid binding by the proteins (Johnstone et al., 1992; Wang and Creutz, 1992; Johnstone et al., 1993). Furthermore, a monoclonal antibody to the annexin I N-terminus inhibits protein-mediated vesicle aggregation without blocking the binding of the protein to PS vesicles (Ernst et al., 1991).

The present results indicate that NEM no longer inhibits AIIt-mediated liposome aggregation once the protein binds to the membrane. Similar results were seen in the nitric oxide study (Liu et al., 1998). One possible explanation for this observation is that after the binding of AIIt to the membrane, the reactive cysteine residues are protected by the membrane. Also, the binding of AIIt to the membranes may result in a protein conformational change (Pigault et al., 1990). Such a change may bury the reactive sites of NEM deep in the protein matrix, thus preventing NEM from binding to the AIIt. It has been previously shown that AIIt can self-associate in the presence of Ca^{2+} (Liu, 1999a). Annexins V and XII have been shown to form trimers and hexamers on the membranes (Concha et al., 1992; Weng et al., 1993; Wirtz and Schmidt, 1996). Therefore, it is also possible that after binding to the membranes, AIIt forms polymers that can hide the reactive sites.

Through UV and fluorescence studies of annexin II it has been shown that Ca^{2+} induces a conformational change in the protein which causes the aromatic amino acids, tyrosine and tryptophan residues to be exposed more to the aqueous phase (Marriott et al., 1990; Pigault et al., 1990). These conformational changes do not appear to affect NEM reaction with AIIt, since a similar inhibition of AIIt-mediated liposome aggregation by NEM was seen in the presence of Ca^{2+} as well as in the presence of EGTA (Fig. 14). It is probably because Ca^{2+} only caused a modest conformational change in the protein.

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