ABSTRACT

PARK, JIN-AH. Role of protein kinase C delta (PKCδ) in airway mucin secretion. (Under the direction of Dr. Kenneth B. Adler).

Mucin hypersecretion is a prominent pathophysiologic feature of chronic airway diseases such as cystic fibrosis, asthma and chronic bronchitis. Exacerbated mucin secretion can lead to impaired mucociliary function, increased susceptibility to bacterial pathogens, potentiation of inflammatory responses, and, in extreme cases, death. Despite these deleterious effects, effective therapy to alleviate mucin hypersecretion remains to be developed.

The intention of this work was to elucidate the mechanism of mucin secretion in response to secretagogues utilizing normal human bronchial epithelial (NHBE) cells *in vitro*. NHBE cells were maintained in air/liquid interface and allowed to differentiate into a normal airway epithelium containing mucin secreting (goblet), ciliated and basal cells. Mucin secretion was measured by an enzyme linked immunoabsorbent assay (ELISA) using antibodies recognizing specific mucin proteins (MUC5AC, MUC5B and MUC2) or pan-secreted mucins.

In manuscript one, we elucidate some aspects of the mechanism whereby human neutrophil elastase (HNE) enhances mucin secretion from NHBE cells. We found that HNE provokes mucin secretion in a concentration-dependent manner, with maximal stimulation (more than two-fold) occurring within a short (15 minute) time period. The mucins, MUC5AC and MUC5B, but not MUC2, were released in response to 500nM HNE. Myristoylated alanine-rich C-kinase substrate (MARCKS) is rapidly phosphorylated in

response to HNE exposure (within 2 minutes), suggesting activation of protein kinase C (PKC) is involved in HNE-induced mucin hypersecretion. PKCδ was the only isoform to translocate from cytoplasm to membrane in response to HNE, and inhibition of PKCδ by a specific pharmacologic inhibitor (rottlerin) attenuated HNE-mediated mucin secretion. Therefore, we concluded that HNE induces mucin secretion via the activation of PKCδ in normal human bronchial epithelial cells *in vitro*.

In manuscript two, we further investigated the role of PKCδ in mucin secretion. Selective activation of PKCδ by bryostatin 1 provoked mucin secretion and induced phosphorylation of MARCKS in NHBE cells. Suppression of PKCδ by rottlerin significantly attenuated HNE-or PMA-mediated mucin secretion as well as phosphorylation of MARCKS. A virally immortalized human bronchial epithelial cell line (HBE-1) was utilized for transient transfections. Transfection of HBE-1 cells with a dominant-negative PKCδ construct (pEGFP-N1/PKCδ^{K376R}) significantly attenuated PMA-induced mucin secretion and phosphorylation of MARCKS compared to cells transfected with empty vector (pEGFP-N1) alone. These results suggest that PKCδ regulates mucin hypersecretion in human airway epithelial cells.

ROLE OF PROTEIN KINASE C DELTA (PKCδ) IN AIRWAY MUCIN SECRETION

by

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This dissertation is dedicated to my parents and my husband for their support and love.

BIOGRAPHY

Jin-Ah Park was born on March 22nd, 1973, in Jeonju, the Republic of Korea. She is a daughter of Jong-Young Park and Kwang-Ja Cheon. She grew up and spent her earlier school years in Jeonju. She went to Chonbuk National University in Jeonju and received a Bachelor of Science Degree in Chemistry in 1994. She continued her graduate education at alma mater, where she focused on investigating the characterization of proteins. She received a Master's Degree in Chemistry with her thesis entitled "Crystallization and Biochemical Studies on Proteinase K Inhibitor-2 from Wheat Germ".

After earning a Master of Science Degree, Jin-Ah worked in the Korea Research Institute of Bioscience and Biotechnology, KIST, in Dajeon. She then joined the company, Fine Life Science. Co. LTD. as a technical consultant for the department of technical support, in Seoul. In 1998, she came back to the research field to work with Dr. Young Deuk Choi, at the Department of Urology, Yonsei University College of Medicine, in Seoul, until she came to the United States to pursue her Ph.D. Degree in the department of Toxicology. In January of 2002, she began to work with Dr. Kenneth Adler. During her Ph.D. study in Raleigh, North Carolina, she met her soul mate, future husband, and married Won in the summer of 2004.

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GENERAL INTRODUCTION

The epithelium protects the interstitial airway by secreting and mobilizing a mucus layer which captures, neutralizes, and expels environmental pathogens and particulates. Mucins, the major structural components of mucus, are expressed at low levels in healthy individuals and contribute to mucus' protective function, both biologically and physically. However, hypersecretion of mucin commonly seen in chronic airway diseases such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), and asthma, is deleterious to airway function. In combination with mucin hypersecretion, neutrophil-predominant inflammation is a prominent feature of chronic airway diseases.

Using background knowledge of the pathophysiological relationship between mucin hypersecretion and neutrophil recruitment, research was performed to elucidate the mechanism of mucin secretion in airway epithelial cells *in vitro*. Results of these studies are presented in two separate manuscripts following a chapter of introductory background information. In the first manuscript, we demonstrated the role of human neutrophil elastase, a major product released by activated neutrophils, on mucin secretion in normal human bronchial epithelial cells. In the second manuscript, we demonstrated the role of protein kinase Cδ in the mucin secretory pathway using both normal human bronchial epithelial cells and a virally transformed human bronchial epithelial cell line *in vitro*.

A summary of the entire body of research, as well as an outline for future studies, concludes the dissertation.

1. Airway epithelium

The mammalian airway is continuously exposed to the external environment. As the first point of contact, the airway epithelium acts both as a physical barrier and a mediator of airway homeostasis. Some of the specific functions of airway epithelium include lung fluid balance, metabolism of exogenous materials, mediation of inflammatory responses, regulation of smooth muscle tone, and secretion of mucins and surfactants (1-3).

The airway epithelium is an integrated structure of pseudostratified columnar cells, attached to a basement membrane, consisting of basal, ciliated, Clara, and goblet cells (reviewed in(3)). Basal cells have a high capacity for proliferation and regeneration (4) and are thought to be progenitors of goblet and ciliated cells (5). In the distal conducting airway, Clara cells may act as progenitors in lieu of basal cells (6). In addition to a role as progenitor cells, basal cells mediate inflammatory response and trans-epithelial water movement, as well as neutralize reactive oxygen species (7). Ciliated epithelial cells, which makeup over 50% of all epithelial cells, have a primary role in mucociliary clearance (8). Ciliated cells are believed to be terminally differentiated cell types arising from either basal cells or Clara cells; however, evidence has demonstrated that trans-differentiation of squamous or goblet cells can also result in ciliary cells (9, 10). Clara cells are located in the bronchi and bronchioles and express Clara cell secretory protein (CCSP) and bronchiolar surfactant. In addition, Clara cells play a role in detoxification by the action of cytochrome p450 and anti-proteases (3, 9). Goblet cells are filled with membrane bound mucin granules and are mainly found in the bronchi and regular bronchioles. They are sparse in the terminal bronchioles and absent in the respiratory bronchioles under normal conditions (6). The major function of goblet cells is production and secretion of mucin.

2. Airway mucus and mucin

Airway mucus

Mucus produced by the epithelium of respiratory, gastrointestinal and reproductive tracts provides a physical barrier between the external environment and the cellular components of the epithelial layer (11). Airway mucus is a heterogeneous mixture of different secretions including water, electrolytes, and organic compounds, such as mucin glycoproteins, proteoglycans, carbohydrates, peptides, serum proteins, soluble proteins, and lipids (reviewed in(12)). Hydrated gel-forming mucus humidifies the airways, and serves to "trap" inhaled deleterious substances as part of the mucociliary clearance apparatus (8).

Mucins

The gel forming properties of mucus are due to highly glycosylated oligomeric glycoproteins termed "mucins". Mucins are predominantly secreted from goblet cells of the airway epithelium and mucous cells of submucosal gland (11, 13). Twenty-one mucins have been currently identified in the human genome. They are categorized as secreted gel-forming mucins (MUC2, MUC5AC, MUC5B, MUC6, and MUC19), secreted non gel-forming mucins (MUC7, MUC8 and MUC9), and membrane-associated mucins (MUC1, MUC3A, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC14, MUC15, MUC16, MUC17, MUC18, and MUC20) (reviewed in(14, 15)).

Mucin glycoproteins are composed of an extensive number of tandem repeats (TR), cysteine-rich domains in its protein backbone, and highly glycosylated side chains bound to the TR domains (reviewed in(11, 15)). TR domains are found in most mucin glycoproteins, with the exception of MUC14, 15 and 18. TR's consist of unique repeating amino acid

sequences including a high number of serine and threonine residues, and at least one proline residue. These serine and threonine regions are responsible for the O-glycosidic linkage of carbohydrate side chains of the mucin backbone. Cysteine-rich domains, unique structures of the amino and carboxyl terminal regions of the mucin protein backbone, are required for oligomerization of secreted mucins (16, 17). Distinct from secreted mucin, transmembrane domains are included in the carboxyl termini of membrane-associated mucins (reviewed in(15, 18)). Glycosylated side chains occupy about 50-90% of the mass of mucin glycoproteins. O-glycosylation is initiated by N-acetylgalactosaminyl peptidyltransferase in the Golgi apparatus and O-glycans are elongated in a stepwise manner by specific glycosyltransferases. Highly evolved glycosylated side chains can serve as signaling receptors for incoming bacterial pathogens and environmental particles in the airway (19).

Physical characteristics of mucins in normal and disease states have been thoroughly described by Thornton and colleagues (20, 21). Mucin monomers are 2-3 mega-Daltons in mass and are approximately 600nm in length. Oligomeric mucin glycoproteins isolated from sputum are 2-40 mega-Daltons in mass and are approximately 0.5-10um in length as measured by light scattering and electron microscopy. These multimeric mucins are stabilized by disulfide bonds located in the cysteine-rich domains. Mucin density in normal airway secretion is about 1.4g/ml, which slightly increases in diseased states (20).

Twelve mucins are known to be expressed in the airway and extensive publication on gel-forming mucins reflects their significance (reviewed in(15)). The four gel-forming mucins (MUC2, MUC5AC, MUC5B, and MUC19) are expressed in the airway and contribute to the viscoelastic properties of airway mucus (19, 22-24). Disulfide bonds present in the cysteine-rich motifs are unique to gel-forming mucins (16, 17). MUC5AC and

MUC5B are the predominant mucins in the airways and their expression has been correlated with chronic airway diseases (15, 25). Two different populations of MUC5B (high-charged or low-charged forms) are present in the normal human airway due to glycosylation differences (26). MUC2 mucin is a minor component of mucus as demonstrated by analysis of sputum collected from COPD patients and measurement of mucin secretion in cultured differentiated epithelial cells (22, 27). MUC19 was recently identified as a gel-forming mucin, however its expression in airway disease has not yet been demonstrated (23). With the exception of MUC19, all gel-forming mucin genes in the human are clustered on chromosome 11p15.5 (28, 29).

3. Exocytosis of mucin granules

Gel-forming mucins are secreted from goblet cells or glandular mucous cells into the airway lumen by a regulated event termed exocytosis (14, 30). Mucins are synthesized and stored within membrane-bound granules (31). Mucin secretion is the consequence of exposure to various secretory agonists which are primarily pathophysiological mediators, such as adrenergic, cholinergic and purinergic agonists, bacterial products, cytokines, proteases, inflammatory mediators and eicosanoids (reviewed in(31, 32)). The intracellular signaling pathway of mucin secretion following secretagogue exposure has been researched for several years, but still little is known.

In contrast to constitutive exocytosis of integral proteins, regulated exocytosis is triggered by an intracellular signal when needed. Regulated exocytosis involves the movement of secretory granules to the inner cell surface, docking, and then fusion of the lipid bilayer of secretory granule to the plasma membrane (reviewed in(14, 30)). These

processes require well-regulated signaling pathways and coordination of the surrounding proteins. One mediator of mucin granule trafficking, ATP, involves activation of protein kinase A (PKA) and protein kinase C (PKC), but downstream components have not been fully elucidated (30).

4. Mucin hypersecretion and airway diseases

In 2002, chronic lower respiratory disease was the fourth leading cause of death in the U.S. according to the National Center for Health Statistics (Source: U.S. National Center for Health Statistics, Health, United States, 2004) and is projected to be the third leading cause of death by the year 2020. Although secreted mucins play a critical defensive function in the airway, mucin hypersecretion is a prominent pathophysiological feature of chronic airway disease. Persistent mucin hypersecretion ultimately leads to increased susceptibility to infection and eventual failure of airway function (reviewed in(33, 34)). Mucin hypersecretion is often followed by neutrophilic inflammation, which can exacerbate the chaotic state of the airway in a diseased individual. Understandably, elucidation of the specific signaling events which regulate mucin hypersecretion is imperative for developing potent therapeutic options to treat chronic pulmonary disease.

The mechanisms involved in mucin gene expression have been thoroughly studied using both *in vitro* and *in vivo* models (reviewed in(35)). Specifically, augmented expression of MUC5AC and MUC5B are well characterized in diseased states including COPD and asthma (22, 36). However, increased amounts of mucin in the airway involve both upregulated mucin gene expression and stimulated mucin granule exocytosis, and less is known about the regulatory pathways of such mucin granule exocytosis.

Asthma

Asthma is a complex disease characterized by bronchial hyperresponsiveness, infiltration of inflammatory cells such as CD4+ T cells, eosinophils and mast cells, and airway obstruction by mucus (37). In addition, specific structural characteristics, termed "airway remodeling", develop in the lung. Airway remodeling is highlighted by continuous damage to the epithelium, increased basement membrane thickness, smooth muscle hyperproliferation, and goblet cell hyperplasia (38). As opposed to COPD, airway flow limitation is reversible during early stages of asthma. However, mucus cell hyperplasia and submucosal gland hypertrophy resulting from chronic inflammation can lead to complete occlusion of the airway in asthma (21, 37). Indeed, widespread occlusion of the distal airway is well-documented in patients who have died from asthma (39, 40).

MUC5AC in the sputum from asthmatic patients is prominently increased compared to non-asthmatic individuals; however, MUC5AC expression in goblet cells is similar in either condition (22). MUC5B is also produced from goblet cells in the asthmatic airway; however, it is only produced from mucous cells of the submucosal gland in normal individuals. Strikingly, the low-charged variant of MUC5B is predominately found in individuals with asthma compared to normal individuals (41). The clinical significance of this observation is not clear.

Cystic fibrosis (CF)

Cystic fibrosis is a common recessive disorder affecting airway surface fluid balance by hyperabsorption of sodium chloride and a reduction in periciliary salt and water content (42). The primary genetic mutation associated with CF is the deletion of a phenylalanine

residue at the 508 position of the cystic fibrosis transmembrane conductance regulator (CFTR) (43). In CF airways, abnormal accumulation of mucus leads to impaired mucociliary clearance, which results in increased mucus retention and a high viscosity of mucus in the airways (42, 44). Prolonged secretion of excessive mucus contributes to CF morbidity by obstruction of airways and impaired clearance of bacterial pathogens such as *Pseudomonas aeruginosa, Staphylococcus aureus* and *Haemophilus influenzae* (34, 45). However, the mechanism regarding bacterial priming in CF patients is still unresolved.

Kirkham et al. demonstrated both MUC5AC and MUC5B are elevated in the sputum of patients with CF and the ratio of MUC5B to MUC5AC is significantly higher compared to ratios in asthmatic patients. Similarly the low-charged variant of MUC5B is also increased in CF patients (22). However, contradictory results regarding the amount of MUC5AC and MUC5B secreted from the airways of CF patients was recently reported by Henke et al. (46). Their results demonstrated that MUC5AC and MUC5B glycoproteins are decreased in CF sputum as observed in Western blot and dot-blot analysis. These contradictory results were thought to be the result of protease degradation of the mucin glycoprotein. However, the ratio of MUC5B to MUC5AC was found to be five-fold greater than in normal individuals, which is consistent with the results of Kirkham et al. (22).

Chronic Obstructive Pulmonary Disease (COPD)

COPD is characterized by slowly progressive and irreversible airway obstruction associated with long-term exposure to toxic gases or particulates including cigarette smoke and coal dust (47, 48). Although the American Thoracic Society (ATS) defined COPD as "airflow limitation due to chronic bronchitis or emphysema", it can often be caused by CF

and chronic asthma (reviewed in(49)). The heterogeneity of the disease makes treatment difficult and patients often suffer high morbidity or even mortality. In COPD, CD8+ T cells and macrophage recruitment are predominantly augmented with neutrophilic infiltration via increased neutrophilic chemokine (epithelial-derived neutrophil attractant-78, interleukin-8) expression during acute exacerbation (50). Proteases released from neutrophils and macrophages are beneficial in host defense; however, protease overproduction due to chronic inflammation can cause lung alveolar breakdown, resulting in emphysema (51, 52). Genetic deficiency of α-1 anti-trypsin often results in the same pathology. An imbalance of oxidants and antioxidants is another factor contributing to COPD development. Reactive oxygen species (ROS) are generated by cigarette smoking, activation of macrophages or neutrophils, and inhalation of environmental pollutants such as ozone, nitrogen dioxide, or sulfur oxide (48). Bacterial pathogens such as *Staphylococcus aureus* and *Haemophilus influenzae* can also commonly exacerbate bronchitis. In addition, bacterial products, including lipopolysaccharide and endotoxin, can cause goblet cell hyperplasia and potentiate ozone-induced goblet cell metaplasia in rodent models (53, 54).

In COPD, mucin hypersecretion results from mucous gland hyperplasia in the large airways and goblet cell metaplasia in the small airways (14, 55). Similar to asthma and CF, increased MUC5AC and MUC5B expression has been demonstrated in COPD (22). MUC5AC expression is increased in the bronchiolar epithelium, while increased MUC5B is found in the bronchiolar lumen compared to normal subjects (56). The ratio of MUC5B to MUC5AC is increased and there is an increase in the low-charged form of MUC5B in chronic bronchitis sputum compared to normals (22).

5. Neutrophils and human neutrophil elastase (HNE)

Neutrophil-predominant inflammation is well-characterized in various lung diseases including chronic bronchitis, bronchiectasis, cystic fibrosis, and asthma (57-59). In the airways, the presence of neutrophils is associated with not only declining airway function, but also increased mucin production (60, 61). Neutrophils contain three distinct enzyme-containing granules within their cytoplasm which are involved in innate immunity. Within azurophilic granules, three serine proteases (elastase, cathepsin, and protease-3) are known to mediate mucin overproduction (62-67). Specific granules and storage granules contain collagenase (matrix metallo proteinase-8) and gelatinase (matrix metallo proteinase-9), respectively. These enzymes mediate cell migration and host defense against bacterial infection. However, persistent or excessive enzymatic activity, as seen in airway disease such as COPD, can result in detrimental effects (even in the absence of α-1 anti-trypsin deficiency) central to the pathogenic processes. For example, it can lead to connective tissue degradation, secretory cell metaplasia, reduced ciliary beating, stimulate excess mucus secretion, bacterial proliferation, and recurrent infections (51, 68, 69). In addition, all three serine proteases present in azurophilic granules can induce secretory cell metaplasia and emphysema pathogenesis in a hamster model (70, 71).

Human neutrophil elastase (HNE)

Among neutrophil proteases, HNE has been of particular focus due to its strong association with airway disease (reviewed in(72)). HNE is a serine protease (EC: 3.4.21.37) composed of 218 amino acid residues with two asparaginyl N-linked side chains and four intramolecular disulfide bridges. HNE functions as a potent hydrolyzer of most protein

components of the extracellular matrix (73). This 24 KDa protease contains a conserved triad of catalytic residues including Ser-195, His-57, and Asp-102. The active-site serine is very nucleophilic, and is inactivated by specific proteases such as diisopropyl phosphofluoridate, phenylmethanesulfonyl fluoride, and 3,4-dichromisocoumarin (reviewed in(74)). In addition, when elastase is released from a neutrophil, it is quickly complexed to natural inhibitors such as α1-antitrypsin or α2-macroglobulin. These complexes are cleared by the liver or by macrophage phagocytosis. However, an imbalance in levels of HNE and protease inhibitors results in chronic disease states which can lead to the destruction of alveolar walls (52, 75). HNE has been associated with many inflammatory disorders such as pulmonary emphysema, acute respiratory distress syndrome, shocked lung, rheumatoid arthritis, and glomerulonephritis (72). Conversely, a HNE-deficient mouse study showed that HNE is critical to host defense against gram negative bacteria including *Klebsiella pneumoniae* and *Escherichia coli* (76). Extensive research to develop potent HNE inhibitors which target its destructive and pro-inflammatory action has been ongoing for years.

HNE is found in high concentration (about 3.3μM) in the sputum of patients with cystic fibrosis and chronic bronchitis (77-80). HNE can degrade a variety of extracellular matrix proteins including elastin, collagen, fibronectin, laminin and proteoglycan with a broad spectrum of substrates (74). Under normal conditions, the proteolytic action of elastase is controlled by endogenous inhibitors such as α1-antitrypsin, α2-macroglobulin and the secretory leukocyte protease inhibitor, elafin (74, 81). In addition, persistent exposure to HNE leads to impaired ciliary motility, goblet cell metaplasia or hyperplasia, increased mucin production/secretion, and enhanced mucin gene expression (62, 78, 80, 82, 83).

Several mechanisms have been proposed regarding HNE effects on mucin gene expression. Voynow and colleagues demonstrated that HNE induced MUC5AC expression by enhancing mRNA stability via oxidant-dependent mechanism in a lung adenocarcinoma cell line and normal human bronchial epithelial (NHBE) cells (84, 85). In addition, they demonstrated that upregulated MUC4 expression and activation of the ErbB2 receptor are involved in epithelial recovery following HNE exposure (86). Nadel et al. showed that upregulated MUC5AC expression is the result of the sequential activation of protein kinase C, reactive oxygen species, and TNF-alpha-converting enzyme in human pulmonary mucoepidermoid carcinoma cell lines and NHBE cells (87, 88). Goblet cell metaplasia was demonstrated in animal models after HNE exposure resulting in mucin hypersecretion similar to what is seen in the small airways of patients suffering chronic airway diseases (62, 83). Voynow et al. suggested that the proteolytic activity of HNE initiates an inflammatory process leading to goblet cell metaplasia by assessing keratinocyte-derived chemokine and IL-5 expression following HNE exposure with and without an HNE inhibitor (methoxysuccinyl Ala-Ala-Pro-Val chloromethylketone (AAPV-CMK)) in mouse lung (74, 83). *In vitro* investigation of the role of HNE in mucin secretion is difficult due to limited appropriate culture systems; however, some studies have been published recently. Kim et al. showed that HNE mediated mucin release from primary hamster tracheal epithelial cell cultures *in vitro,* and that an active catalytic site of HNE is required for both release and degradation of mucin (63). Studies from our laboratory demonstrated that HNE provokes mucin secretion via activation of the PKCδ isoform in well-differentiated NHBE cells (89).

6. Protein kinase C (PKC)

Classification

Protein kinase C (PKC) is a serine/threonine kinase involved in various cellular events such as proliferation, differentiation, gene expression, apoptosis, tumoriogenesis, muscle contraction and exocytosis (reviewed in(90)). PKC isoforms have been classified into three subfamilies depending on their mode of activation: conventional (α, β, γ) PKCs, which are activated by phosphatidylserine (PS), diacylglycerol (DAG) or phorbol esters (12-O-tetradecanoyl-phorbol-13-acetate (TPA) or phorbol 12-myristate 13-acetate (PMA)), novel (δ, ε, η, θ, (μ)) PKCs, which are PS, DAG or TPA dependent but Ca^{2+} independent; and atypical (ζ, ι/λ) PKCs, which are either Ca^{2+} or PS, DAG or TPA independent for their activation (90, 91). In addition, newly discovered member of the PKC family, called the protein kinase C-related kinase (PRK; PRK1,2,3), has sequence homology to PKC (92). The PRK family does not require DAG or PMA for their activation (93). There is still some controversy as to whether or not they really should be classified as part of the PKC family. Newly found novel type PKCs are termed protein kinase D (PKD) (94, 95). Initially, the PKD family was designated as novel type isoforms due to their requirement for DAG or PS for activation; these include PKCμ and PKCν (96).

Structure

All PKCs contain both a regulatory and catalytic domain, each of which consists of four conserved regions and five variable regions. Two distinctive domains are separated by the V3 “hinge” region. Four conserved regions distributed in the regulatory domain (C1 and C2) and catalytic domain (C3 and C4) are responsible for activation of these proteins. In

addition, a pseudosubstrate or autoinhibitory domain is located in the amino terminus near the C1 domain (90). The C1 domain consists of two cysteine-rich zinc finger regions that bind to DAG or PS during activation. The C2 domain binds to anionic phospholipids on the cellular membrane in the presence of Ca^{2+}, which is required for the activation of conventional PKCs (91). Enzymatic activity of PKC is derived from its catalytic domain upon the binding of the C3 and C4 regions to ATP and substrate, respectively.

Mode of activation

Traditionally, PKC activation is accomplished in a stepwise manner that involves the translocation of inactive PKC localized within the cytosol to the cellular membrane (90). PKC in the cytosol remains inactive by binding of a pseudosubstrate domain to C4 regions in its catalytic domain. Conversion of PKC into the active form occurs when the affinity of the pseudosubstrate domain for the C4 domain decreases following the binding of DAG to the C1 domain. Additional interaction with the anionic phospholipids on the lipid bilayer membrane is followed by a critical conformational change which allows PKC to bind to the substrate as well as ATP. Then active PKC mediates the phosphorylation of the substrate at the serine and threonine residues. Sustained PKC activation is followed by degradation of PKC by a protease (calpain) or by the ubiquitin-proteasome pathway (97-99).

The activation mechanism of PKC is focused on subcellular localization. More recently, phosphorylation at its own serine/threonine residues appears to generate enhanced thermal stability and catalytic activity, and resistance to protease or phosphatase (100, 101). Activation of PKC includes phosphorylation at the activation loop, autophosphorylation site and hydrophobic region. Phosphorylation of the activation loop (Thr-500) is required for the

complete activation of conventional and atypical PKCs, while it is not required for novel PKCs (Thr-505).

Distribution in tissue

The distribution of PKCs shows tissue specific patterns. PKCα expression is ubiquitous, while PKCγ is expressed exclusively in the central nervous system, while PKCβ, δ, and ε are widely expressed in most tissues (102). All isoforms (except PKCγ) are expressed in mammalian lung tissue (103). We have found that primary normal human bronchial epithelial cells in culture contain all except θ and γ. PKC isoforms expressed in the lung have been implicated in a number of cellular responses including permeability, smooth muscle contraction, inflammatory cell migration, proliferation, differentiation, hypertrophy, apoptosis, and secretion (103-106).

7. Protein kinase Cδ (PKCδ)

Structure

PKCδ is the most widely studied member of the novel PKC family due to its extensive roles in a variety of cellular signaling pathways including cell growth, differentiation, proliferation, apoptosis, tumor promotion, and carcinogenesis (reviewed in (107, 108). PKCδ protein is expressed in brain, heart, spleen, lung, liver, ovary, pancreas, and adrenal tissues and in a variety of inflammatory cells (102, 104).

PKCδ was cloned from a rat brain cDNA library by Ono et al. in 1987 (109). The amino acid sequence of PKCδ shows 58% homology to PKCα. The PKCδ gene is clustered at human chromosome 3p21, rat chromosome 19p14, and mouse chromosome 14 (110, 111).

The PKCδ comprises 18 exons in human and mouse, and 19 exons in rats. It is composed of 676 amino acids in human and 673 amino acids in rat, and its genomic structure is highly conserved in mammals. As a novel PKC, it includes the common primary structure of novel PKCs. Its C1 domain is composed of two cysteine-rich zinc-finger domains which bind to DAG and PS resulting in its activation. Of the two domains, the second cysteine-rich domain (Cys2) has higher binding affinity to PMA and mutational studies also showed that Cys2 plays a key role in PKCδ translocation in response to PMA (112). The crystal structure of the Cys2 and PMA complex revealed that five of six cysteine residues and two histidine residues are critical for their binding (113). More recently, the Cys2 domain has been recognized as a phosphotyrosine binding domain by crystal structure determination (114). Following their translocation to the membrane, some PKCs require phosphorylation of the active loop for their complete catalytic activity (115). However, PKCδ is synthesized as a catalytically competent enzyme that is able to autophosphorylate (107).

Biological function

PKCδ has been shown to play multiple roles in cell growth, proliferation, differentiation, apoptosis, and muscle contraction.

Growth and proliferation

The loss of PKCδ causes cell transformation in fibroblasts (116) and increased B-cell proliferation via transcriptional regulation of interleukin-6 (117). Expression of a dominant negative PKCδ mutant in cells expressing c-Src results in transformation of fibroblasts, and inhibition of PKCδ by rottlerin, a specific inhibitor, leads to transformation of c-Src-

overexpressing cells (116, 118). PKCδ shows a unique feature among the PKC family, in that its overexpression results in inhibition of cell growth in many cell types including CHO cells, smooth muscle cells, fibroblast, and human glioma cells. Overexpression of PKCδ leads to G2/M arrest of the cell cycle in CHO cells (119) and inhibition of vascular smooth muscle cell proliferation by suppressing G1 cyclin expression (120). Mouse lungs exposed to asbestos showed increased PKCδ protein expression as well as membrane localization, which reflect PKC activation. Similar findings were observed in C10 alveolar epithelial cells (121). Increased PKCδ is mainly detected in PCNA-positive cells in the membrane, which suggest that asbestos fibers mediate proliferation of epithelial cells in a PKCδ-dependent manner. Activation of PKCδ is also involved in the mechanism whereby several drugs work. Microtubule-active anticancer drugs, including taxol, vinblastine, and vincristine, induce MnSOD gene expression in a lung adenocarcinoma cell line (A549) via PKCδ activation (122).

Apoptosis

Various apoptotic stimulants such as UV radiation (123-125), hydrogen peroxide (H_2O_2) (126), TNF-ligand (127, 128), PMA (129) result in PKCδ activation and translocation into distinct cellular compartments including mitochondria (123, 129), Golgi apparatus (130) and the nucleus (128) where apoptotic responses generate. Following PKCδ activation in response to any DNA damaging agents, PKCδ translocates to the mitochondria where it activates caspase-9 pathways. Active caspase-9 sequentially cleaves caspase 3, which in turn cleaves PKCδ resulting in the release of the catalytic fragment. The catalytic fragment of PKCδ binds directly to the C-terminus of DNA-dependent protein kinase (DNA-PK) and

phosphorylates, it resulting in dissociation of DNA-PK from DNA. DNA-PK is an essential kinase that repairs double-strand breaks, thereby the phosphorylation of DNA-PK enhances DNA fragmentation induced by apoptosis (131, 132). Matassa and colleagues demonstrated that PKCδ is required for mitochondria-dependent apoptosis that is mediated by etoposide, ultraviolet radiation, and taxol (133). In their report, overexpression of wild type PKCδ leads to a robust induction of apoptosis indicated by DNA fragmentation, while the dominant negative PKCδ blocks apoptosis in response to diverse stimuli in human salivary epithelial cell line, C5. A specific inhibitor of PKCδ, rottlerin has shown its anti-apoptotic effect on DNA-damage induced apoptosis by blocking both the activation of caspase -3 and proteolytic cleavage of PKCδ in human cervical carcinoma cell line (134).

Inflammation

PKCδ plays a negative role in tumor necrosis factor (TNF) α -mediated degranulation and oxygen radical release in adherent neutrophils (135). TNF has two receptors, p60TNFR and p80TNFR, which differ in molecular weight. TNF-α triggers free radical production by binding to p60TNFR on the surface of neutrophils (136). The resulting receptor phosphorylation and desensitization regulate its function in pro-inflammatory cellular responses. PKCδ phosphorylates the serine residue of the p60TNFR that is expressed on the surface of neutrophils, and rottlerin inhibits phosphorylation of p60TNFR. PKCδ enhances expression of inducible nitric oxide synthase, which indicates an anti-inflammatory role, by suppressing granulocyte colony stimulating factor (GM-CSF) (137). However, PKCδ also can play a pro-inflammatory role by inducing transcription of the pro-inflammatory mediators, GM-CSF, RANTES, and ICAM-1, which are upregulated in patients with asthma

(138). Further evidence that PKCδ is involved in the pro-inflammatory signaling pathway was demonstrated in substance-P (SP) induced IL-8 production and nuclear factor kappa B (NFκ-B) activation in colon epithelial cells (139). In addition, Koon et al. demonstrated that SP-induced IL-8 production via activation of NFκ-B is attenuated by the inhibition of PKCδ activity using rottlerin, suggesting the positive role of PKCδ in pro-inflammatory cytokine production. Additionally, PKCδ activity is involved in the regulation of ion transport of Cl^-, K^+, Na^+ in airway epithelial cells (140, 141). Liedtke et al. demonstrated that Na-Cl-K cotransport is rapidly increased by PKCδ in response to the α1-adrenergic agonist, methoxamine, in human airway epithelial cells (142).

Exocytosis

A large body of evidence suggests that PKCδ controls various exocytotic events including the secretion of insulin (143, 144), gastric peptides (145), enzymes (146), platelet dense granules (147) and inflammatory cell degranulation (148-150). In human platelets, PKCδ is activated in response to protease-activated receptor agonist peptides (SFLLRN and AYPGKF) leading to dense granule release (147). In addition, PAR agonist peptide-induced dense granule release is blocked by rottlerin (a specific PKCδ inhibitor), but not by Go6976 (a conventional PKC inhibitor). Ishikawa et al. demonstrated that carbachol-stimulated insulin secretion is associated with translocation of PKCδ in rat pancreatic islets (144). Carbachol-stimulated insulin secretion is not reduced by Go6976, but significantly suppressed by an ambiguous PKC inhibitor, chelerythrine. Ishikawa's report suggested that one of the novel PKC isoforms, δ or ε, might play a regulatory role in carbachol-stimulated

insulin secretion. Cho et al. demonstrated that PKCδ activation is involved in antigen induced-mast cell degranulation, which is subsequently inhibited by rottlerin or transfection of a dominant negative mutant of PKCδ (149). These results were in contrast to those of Leitges et al. who suggested that PKCδ is a negative regulator of antigen induced-mast cell degranulation (150). Despite the controversy, PKCδ appears to be a key molecule in antigen induced-mast cell degranulation *in vivo* and *in vitro*.

Until now, few studies have been conducted regarding the role of PKCδ in mucin secretion. Abdullah et al. showed that the PKCδ isoform plays a role in the mucin secretion pathway in response to purinergic agonists (ATPγS) and PMA in mouse goblet cells (151). Park et al. also demonstrated that the PKCδ isoform modulates mucin secretion in response to HNE in well differentiated NHBE cells (89).

8. Myristoylated alanine-rich kinase C substrate (MARCKS)

MARCKS, widely distributed in many cell types (including epithelial cells) is a well known PKC substrate. MARCKS has been implicated in many cellular functions such as cell motility, phagocyte activation, exocytosis, membrane trafficking and mitogenesis through regulation of cytoskeletal structures (152-154). In humans, MARCKS consists of 323 amino acids. Its expected molecular weight based on the number of amino acids is 40kDa, but it is detected near 85kDa by Western blot analysis due to its rod-like shape. MARCKS has three conserved domains: the amino-terminus myristoylated domain, the multiple homology 2 (MH2) domain and the phosphorylation site domain (PSD) (reviewed in(155)). The N-terminal glycine is the site of myristoylation, which allows for effective binding of

MARCKS protein to the plasma membrane (156, 157). Synthetic peptides corresponding to the first 24 sequences of the N-terminus inhibit constitutive and cholinergically-stimulated mucin secretion in ovalbumin sensitized mice (158). The function of the MH-2 domain has not been elucidated. Recently, it was shown that deletion mutation of the MH-2 domain of MARCKS reduces mucin secretion when the construct is transfected into HBE-1 cells (unpublished result by Adler et al.). The phosphorylation target of PKC, the PSD site, has the ability to bind to membranes with a highly basic region containing 12 to 13 positively charged Lys/Arg residues as well as to actin where it crosslinks actin filaments together. These functions can be disrupted by Ca^{2+}/calmodulin or phosphorylation of MARCKS by PKC (159, 160).

MARCKS is phosphorylated by PKC at serine residues 152, 156, and 163 that are located in its phosphorylaton site domain (PSD) (161). Phosphorylation of MARCKS in the PSD region has been shown to be central to the function of MARCKS protein (152, 159, 162, 163). MARCKS is a prominent PKC substrate and its phosphorylation is differentially regulated depending on the specificity of each PKC isoform. Hagret and colleagues performed PKC phosphorylation assays *in vitro* to demonstrate the differential efficiency of each PKC isoform in phosphorylation of MARCKS (161). MARCKS is phosphorylated by PKCα, β1, β2, γ, δ, ε, but not by ζ. Additionally, it has been suggested that the intact MARCKS protein is an excellent substrate for PKCβ1, δ, and ε. Interestingly, PKCδ appears to be the most potent isoform responsible for the phosphorylation of MARCKS, followed by the ε and β1 isoforms, as determined by Vmax/Km catalytic efficiency ratios. This finding was supported by a study conducted by Fujise et al. which also demonstrated that PKCδ has a high affinity for MARCKS (164).

Involvement of MARCKS in exocytosis in conjunction with PKC activation has been reported. Transfection of deletion mutation constructs lacking the PSD site results in attenuation of mucin secretion in response to PMA in HBE-1 cells (154). Glutamate exocytosis from synaptosome is increased by PKC in cooperation with phosphorylation of MARCKS and increased intracellular calcium concentrations (165). MARCKS protein is involved in chromaffin cell secretion (166). Tifaro et al. suggested chromaffin cell secretion is the result of two cooperating pathways: calcium influx into cells with scinderin activation and phosphorylation of MARCKS by PKC. They also suggested those two separate steps lead to control of actin filaments which serve as a barrier under the membrane. Association of actin with MARCKS during exocytosis has been demonstrated in oxytocin exocytosis from bovine luteal cells in response to prostaglandin F2-α (153).

9. Phosphorylation and granule associated proteins involved in exocytosis

As mentioned above in section 3, exocytosis involves fusion of secretory granule membranes with the plasma membrane following trafficking and docking of the granule (diagramed in figure 2 in ref(30)). Recent studies have focused on the role of granule-associated proteins facilitating fusion of granules to the plasma membrane. Those granule associated proteins include cysteine string protein (CSP), soluble N-ethylmaleimide-sensitive factor attachment protein (SNAP), Munc18/nSec1, and SNAP receptor (SNARE) including a complex of vesicle-associated membrane protein (VAMP), synaptosome-associated proteins (SNAP-23/25), and syntaxin (reviewed in(30)). The isoform expression of VAMP, SNAP, and syntaxin is cell specific.

The role of SNARE assembly in exocytosis has been studied extensively. The SNARE complex is composed of two helices of SNAP-25, a helix of syntaxin, and a helix of VAMPS. After the granule moves close to the plasma membrane, it binds to the lipid bilayer via a fully assembled SNARE complex (diagramed in Figure 6 in ref(30)). Before the SNARE complex is completely assembled, one of the SNARE members, syntaxin, is not free to bind to other SNARE proteins due to its association with Munc18. Following dissociation with Munc18, syntaxin is able to associate with the other members of SNARE. Dissociation of Munc18 from syntaxin may be mediated by its phosphorylation. Interactions between the SNARE proteins are demonstrated by *in vitro* binding assays and co-immunoprecipitation (167). Foster et al. demonstrated three different interactions; SNAP-23 and syntaxin-4, VAMP-2 and syntaxin-4, and VAMP-2 and SNAP-23. Granule fusion is the important step in exocytosis, and regulating the interaction of each of the proteins with each other and the membrane may represent a potential target to modulate mucin secretion.

There also is good evidence that the binding affinity between proteins involved in exocytosis is modulated by their phosphorylation. Using *in vitro* phosphorylation of recombinant proteins, Evans et al. demonstrated that proteins associated with granules are phosphorylated by PKA (cAMP-dependent kinase) or PKC (168). Munc18, SNAP-25, and Rab3A are phosphorylated by PKC and CSP is phosphorylated by PKA. They have also demonstrated that phosphorylation of CSP at the Serine10 residue inhibits its interaction with syntaxin *in vitro*, while it does not affect its interaction with heat shock protein 70. In addition, phosphorylation of cysteine string protein (CSP) results in marked reduction in chromaffin cell secretion, suggesting that phosphorylation of CSP and CSP interactions with syntaxin have a role in exocytosis. Phosphorylation of Munc 18 at the Serine 313 residue

inhibits interaction with syntaxin *in vitro* (169, 170). Interaction of SNAP-23 with syntaxin-4 is inhibited by either phosphorylation of synataxin-4 (171) or SNAP-23 (172). Chung et al. demonstrated (171) that syntaxin is phosphorylated in human platelets treated with thrombin, and inhibition of syntaxin phosphorylation by PKC inhibitors reduces thrombin-mediated dense granule release from platelets. They also showed that recombinant syntaxin is phosphorylated by various PKC isoforms *in vitro*, including PKCα, β, γ, δ, ε, and ζ. They suggested that among the PKC isoforms, a novel type PKC is a probable regulator of thrombin-mediated platelet secretion. Thus, either the δ or ε isoform of PKC can be the target kinase regulating SNARE assembly.

HYPOTHESES AND RATIONALES

Manuscript I : Human Neutrophil Elastase Induces Hypersecretion of Mucin From Well-Differntiated Human Bronchial Epithelial Cells in vitro via a Protein Kinase δ – mediated mechanism.

Chronic airway diseases such as cystic fibrosis, chronic bronchitis, and asthma have two common pathologic features: mucus hypersecretion of the airways, and neutrophil-predominant airway inflammation. Although neutrophil elastase released from activated neutrophils has been shown to be a potent secretagogue for mucin secretion, intracellular mechanisms regulating HNE-induced mucin secretion have not been elucidated. Therefore, we generated three hypotheses related to the mechanisms of HNE-induced mucin hypersecretion utilizing well-differentiated human primary bronchial epithelial (NHBE) cells.

1) HNE provokes mucin secretion by airway epithelial cells *in vitro*.
2) HNE provokes release of the mucin glycoproteins, MUC5AC and MUC5B.
3) HNE-induced mucin hypersecretion is regulated by a Protein Kinase C - mediated mechanism.

Manuscript II : Protein Kinase C delta Regulates Airway Mucin Secretion via Phosphorylation of MARCKS Protein.

In the second manuscript, we further investigated the potential role of protein kinase Cδ (PKCδ) in airway mucin secretion, based on conclusions published in the first manuscript regarding HNE stimulation of mucin secretion via activation of PKCδ in normal human bronchial epithelial cells *in vitro*. We generated three hypotheses and utilized either well-differentiated primary NHBE cells or the virally-transformed human bronchial epithelial cell line (HBE-1) as *in vitro* model systems. HBE-1 cells were used for effective transfection of the PKCδ construct.

1) The PKCδ isoform regulates, at least in part, mucin secretion by airway epithelial cells *in vitro*.
2) Phosphorylation of MARCKS is required for PKCδ-mediated mucin hypersecretion.
3) Overexpression of the dominant negative PKCδ decreases mucin hypersecretion as well as MARCKS phosphorylation in airway epithelial cells *in vitro*.

MANUSCRIPT I

Human Neutrophil Elastase Induces Hypersecretion of Mucin From Well-Differntiated Human Bronchial Epithelial Cells *in vitro* via a Protein Kinase δ – mediated mechanism

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ABSTRACT

The presence of mucus obstruction and neutrophil-predominant inflammation in several lung disorders, such as cystic fibrosis, suggests a relationship between neutrophils and excess mucus production. Mechanisms of human neutrophil elastase (HNE)-induced mucin secretion by well-differentiated normal human bronchial epithelial (NHBE) cells maintained in air/liquid interface culture were investigated. HNE increased mucin secretion in a concentration-dependent manner, with maximal stimulation (more than twofold) occurring within a short (15 minutes) time period. Mucins MUC5AC and MUC5B, but not MUC2, were released in response to HNE. Stimulation of mucin secretion required partial elastase enzymatic activity and did not appear to involve a soluble product released by the cells. HNE-stimulated secretion involved activation of protein kinase C (PKC), as HNE exposure rapidly provoked PKC enzymatic activity that was attenuated by the general PKC inhibitors calphostin C and bisindoylmaleimide I. Of the different isoforms, PKCα, δ, ζ, λ, ι, and ε were constitutively expressed in NHBE cells while PKCβ, η, and μ were PMA-inducible. PKCδ was the only isoform to translocate from cytoplasm to membrane in response to HNE. Inhibition of PKCδ attenuated HNE-mediated mucin secretion. The results suggest HNE stimulation of mucin release by human airway epithelial cells involves intracellular activation of PKC, specifically the δ isoform.

INTRODUCTION

Neutrophils are involved in a variety of inflammatory lung disorders including chronic bronchitis, bronchiectasis, cystic fibrosis, and probably asthma. In these diseases, the pathological findings of mucus obstruction and neutrophil-predominant inflammation in airways[1-6] suggest a relationship between neutrophil recruitment/infiltration and excess mucus production and secretion. Neutrophils store three proteases that have been implicated in airway mucin secretion: elastase,[7-9] cathepsin G,[10] and proteinase-3.[11, 12] Of these, human neutrophil elastase (HNE), a major component of primary or azurophilic granules,[13] is the most widely studied with regard to enhanced mucus secretion. Levels of HNE are elevated in airways of patients with chronic bronchitis and cystic fibrosis,[14] and levels in patients' sputum may exceed 100 μg/ml (3.3×10^{-6} mol/L).[15-17] Purified HNE has been shown to provoke secretion of mucin by isolated airway epithelial cells and glands from several species.[7, 8, 10, 18] Although there have been suggestions that interactions between HNE and epithelial cell surfaces may be involved in the response,[9, 19] intracellular mechanisms and signaling pathways associated with HNE-induced mucin hypersecretion have not been elucidated.

In this study, well-differentiated primary normal human tracheobronchial epithelial (NHBE) cells maintained in vitro in air/liquid interface were exposed to HNE, and the secretory response assessed. Elastase proved to be a potent mucin secretagogue for NHBE cells, eliciting a robust (greater than twofold) increase in mucin secretion within 15 minutes. The mucin gene products released included those of MUC5AC and MUC5B, but not of MUC2. The mechanism appeared to involve activation of protein kinase C (PKC), as HNE

exposure rapidly provoked phosphorylation of MARCKS (myristoylated alanine-rich C kinase substrate) protein, a cellular substrate of PKC, and the mucin secretory response to HNE was attenuated by two different PKC inhibitors. Additional studies provided compelling evidence that PKCδ is the specific PKC isoform involved in the secretory pathway.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade or higher. NHBE cells, bronchial epithelial basal medium, and supplements for air/liquid interface cell cultures were purchased from Cambrex (San Diego, CA). Endotoxin-free HNE purified from human sputum was purchased from Elastin Products Company (EPC, Owensville, MO). Cytotoxicity was evaluated with CytoTox 96 nonradioactive cytotoxicity assay kits obtained from Promega Corp. (Madison, WI). A specific HNE substrate, MeO-SUC-AL-AL-PRO-VAL-PNA, and an HNE inhibitor, chloromethyl ketone-modified tetrapeptide (CMK), also were purchased from EPC and the HNE inhibitor elastatinal was obtained from Calbiochem (La Jolla, CA). 17Q2 pan mucin antibody was purchased from Babco (Richmond, CA) and anti-MUC5AC (45M1) was purchased from Neomarkers (Fremont, CA). A monoclonal antibody (11C1) against human MUC5B was generously provided by Dr. Reen Wu, University of California at Davis, Davis, CA. The epitope for this antibody, which was generated from the secreted mucin of well-differentiated airway epithelial cells, is not known, but by immunohistochemical staining and Western blot analysis, it appears to recognize the MUC5B peptide. A monoclonal antibody that cross reacts with human MUC2, raised against the guinea pig 522-bp gene sequence analogous to the human D4 domain located in the carboxy-terminal region of the *Muc2* gene sequence established previously in our laboratory, was used to detect MUC2 mucins.[20] An ImmunoPure (G) IgG purification kit used for purification of antibodies for enzyme-linked immunosorbent assay (ELISA) was from Pierce (Rockford, IL). For Western blot analysis of PKC isoforms expressed in NHBE cells, a PKC sampler kit and E-cadherin antibody were

obtained from BD Biosciences (San Jose, CA). Goat anti-PKCζ and mouse anti-α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against phosphorylated (ser) PKC substrate and phosphorylated MARCKS were from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase-conjugated goat anti-mouse IgG and donkey anti-goat IgG also were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated goat anti-rabbit IgG was purchased from Upstate Biotechnology (Lake Placid, NY). Enhanced chemiluminescence development kits and Hyperfilm were from Amersham Pharmacia Biotech (Piscataway, NJ). All PKC-related inhibitors (ie, calphostin C, bisindoylmaleimide, PKCepsilon and zeta inhibitor peptides, rottlerin) were purchased from Calbiochem. A PepTag assay for nonradioactive detection of PKC activity was purchased from Promega. Other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO). Transwell-Clear culture inserts and high-binding 96-well assay plates were purchased from Corning Inc. (Corning, NY).

Epithelial Cell Culture

Primary cultures of NHBE cells were established using an air/liquid interface cell culture system described previously.[21] Briefly, NHBE cells were expanded once and cells collected and frozen in liquid nitrogen (referred to as passage-2 cells). Air/liquid interface cultures of NHBE cells were established on Transwell-Clear culture inserts thin-coated with rat-tail type I collagen. The basic medium used for NHBE cells was a 1:1 mixture of bronchial epithelial basal medium and high glucose (4.5 g/L) Dulbecco's modified Eagle's medium. The complete medium was composed of basic medium containing a final concentration of 0.5 ng/ml human recombinant epidermal growth factor, 0.5 μg/ml

hydrocortisone, 5 μg/ml insulin, 10 μg/ml transferrin, 0.5 μg/ml epinephrine, 6.5 ng/ml triiodothyronine, 50 μg/ml gentamicin, and 50 ng/ml amphotericin-B. In addition, the media contained 0.13 mg/ml bovine pituitary extract made according to the protocol of Bertolero and colleagues,[22] 5 x 10^{-8} mol/L all-*trans* retinoic acid, 1.5 μg/ml bovine serum albumin, and 20 U/ml nystatin.

Frozen NHBE cells were recovered and seeded at a density of ~2 x 10^4 cells/cm^2 onto the apical surface of the inserts. Media were changed the next day, then every other day until the cells reached ~90% confluence. At this point, the air/liquid interface was established by removing the apical media, whereas basolateral media were changed daily for up to 21 days. A mucin phenotype was observed at ~14 days in culture (~7 days in air-liquid interface culture) and cilia were apparent by 18 days in culture. Mucin secretion reached maximal levels at ~18 days in culture, so cells cultured for ~18 to 21 days were used for the experiments described below.

Exposure of cells to HNE

HNE stock was made as 10 mg/ml (339 μmol/L) in a 1:1 mixture of glycerol and 0.02 mol/L NaOAc, pH 5.0. The stock was diluted into the culture medium to the final concentration indicated. In all studies, the above solvent appropriately diluted was used as a negative control.

Quantification of mucin Secretion

NHBE cells were exposed to HNE from both apical and basolateral sides for 15 minutes (unless otherwise indicated). At the end of each treatment, apical medium containing

the secreted mucin was collected and quantified. Briefly, 0.25 ml of media containing secreted mucin was collected, 0.5 ml of 1 mmol/L dithiothreitol in phosphate-buffered saline (PBS) was added into each well, and the plates were gently agitated and allowed to stand for 3 minutes before the dithiothreitol/PBS plus mucin was collected in the same tube. Finally, 0.5 ml of 10 μmol/L CMK in PBS was added and collected the same way. Approximately 1.25 ml of the collected mucin mixture with dithiothreitol and CMK was centrifuged at 8000 rpm for 5 minutes to remove cell debris, and then collected in a fresh tube. Phenylmethyl sulfonyl fluoride was added to a final concentration of 1 mmol/L.

Baseline and treatment mucin secretions were collected from each culture plate. Baseline mucin secretion was collected to normalize variations from well to well, and to control for possible release of mucin in response to the stress of media change or washing. After the baseline mucin secretion sample was collected, the cells were rested overnight and exposed to test agents the next day for indicated periods of time. Mucin samples were quantified using specific ELISA methods. Firstly, total mucin was quantified by a double-sandwich ELISA using a pan-mucin antibody, 17Q2, that cross reacts with a carbohydrate epitope on human mucins, as described previously.[21] Additional studies were performed using ELISAs for secreted protein products of the mucin genes *MUC5AC*, *MUC5B*, and *MUC2* to determine which mucin gene products were being released on exposure to HNE. MUC5AC was measured via ELISA as described by Takeyama and colleagues[23] using the 45M1 antibody. MUC5B protein was assayed via a standard double-sandwich ELISA method using the 11C1 monoclonal antibody against MUC5B provided by Dr. Reen Wu, University of California, Davis, Davis, CA, as described previously.[24, 25] The *MUC2* gene product was quantified by modification of an ELISA as described previously.[20]

Assay of HNE activity

HNE activity assays were performed following the manufacturer's protocol (EPC). HNE substrate was prepared in substrate buffer (Tris-NaCl buffer: 0.1 mol/L Tris, pH 7.5, containing 0.5 mol/L NaCl and 0.01% Na_3N). Briefly, 3 ml of substrate solution at 25°C was added to test tubes, 1.0 μg of HNE then was added, and the developed color was read immediately and continuously thereafter at 1 minute intervals. Elastase activity was reflected by the rate increase in absorbance in time units (minutes). Color development was read at 410 nm on a spectrophotometer UV160U (Shimadzu, Kyoto, Japan). The specific activity of HNE was expressed as U/mg, and results expressed as percentage of activity of native HNE for each treatment.

Effects of HNE enzymatic inhibition

Effects of enzymatic inhibition of HNE were investigated using three different elastase inhibitors: 1) elastatinal, a natural HNE inhibitor produced by *Actinomycetes*;[26] 2) CMK, a synthetic tetrapeptide;[27] and 3) α1-antitrypsin (α1-AT), a physiological HNE inhibitor.[28] The inhibitors were added directly to HNE, incubated for 15 minutes at 37°C, and then added directly to the cells for another 15 minutes. At the end of this exposure, secreted mucin was collected and quantified as described above.

To determine whether HNE enzymatic activity was directly required for stimulated mucin secretion, or if a secondary product(s) released by NHBE cells after exposure to HNE could be involved in the secretory response, NHBE cells were exposed to HNE (or vehicle) for 5 minutes. After exposure, the conditioned medium was collected and treated with 5 μmol/L of the HNE enzymatic inhibitor, α1-AT, for 15 minutes, at which time this α1-AT-

treated medium was added to a new set of NHBE cells and effects on mucin secretion quantified as described above.

Effects of Protein Kinase Inhibition

The PKC inhibitors, bisindolylmaleimide I (10, 100, 1000 nmol/L)[29] or calphostin C (5, 50, 500 nmol/L)[30] were used to determine PKC involvement in HNE-induced mucin secretion. NHBE cells were preincubated with these agents (or vehicle control) for 15 minutes, then HNE was added for another 15 minutes before mucin secretion was quantified as described above.

PKC Activity Assay

PKC activity in NHBE cells after exposure to HNE was assessed using a PepTag assay for nonradioactive detection of PKC (following the manufacturer's protocol). Briefly, 10 µg of protein extracted from each treatment of NHBE cells was added into the PKC reaction buffer (20 mmol/L HEPES, pH 7.4, 1.3 mmol/L $CaCl_2$, 1 mmol/L dithiothreitol, 10 mmol/L $MgCl_2$, 1 mmol/L ATP) containing 1 mg/ml phosphatidylserine and PepTag C1 PKC substrate peptide (P-L-S-R-T-L-S-V-A-A-K) conjugated with fluorescent dye, and incubated for 30 minutes at 30°C. The reaction was stopped by boiling at 100°C for 10 minutes. Reaction mixtures were separated on 0.8% agarose gels and proteins quantified by Labworks image acquisition and analysis software (UVP Bioimaging System, Upland, CA). Phosphorylation of MARCKS was detected by Western blot using an antibody against phophospecific-MARCKS.

PKC isoform analysis

After treatments, NHBE cells were washed with ice-cold PBS twice and then scraped into lysis buffer (50 mmol/L Tris, pH 7.5, 1 mmol/L ethylenediamine tetraacetic acid, 100 mmol/L NaCl, 1 mmol/L phenylmethyl sulfonyl fluoride) using a rubber policemen. The collected cells were lysed by sonication. For separation of cytosolic and membrane fractions, the lysates were spun at 400,000 x *g* in a Sorvall Discovery 100S ultracentrifuge (Sorvall, Inc. Newtown, CT) for 1 hour. The supernatant was reserved as the cytosolic sample. The pellet was resuspended in the same lysis buffer containing 0.05% Triton-100, dissolved by sonication, and incubated on ice for 30 minutes. After incubation, the same ultracentrifugation as described above was performed on the pellet mixture, and the supernatant separated from the pellet mixture was reserved as the membrane fraction. For preparation of whole cell crude lysates, the disrupted cellular mixture was centrifuged at 15,000 rpm in an Eppendorf 5417R centrifuge (Eppendorf Corp., Hamburg, Germany) for 1 hour at 4°C. The supernatant was collected as the whole crude NHBE cell lysate.

The protein concentration of cell lysate samples was quantified by a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Each sample was boiled in 2x sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer for 10 minutes, loaded on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and transferred to a polyvinylidene difluoride membrane (Micron Separation Inc., Westborough, MA). After blocking with 5% skim milk, the antigen was captured by the specific PKC antibody and further amplified by binding to horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies. Anti-α-tubulin and E-cadherin antibodies were used for cytosolic and membrane controls, respectively, for each sample. Final development was accomplished by the

enhanced chemiluminescence method. The amount of each PKC isoform was analyzed by Labworks image acquisition and analysis software.

Effects of PKC isoform-specific inhibitors on HNE-induced PKC activation and mucin secretion

Because the studies above indicated that PKCδ was the only isoform to translocate to membranes in response to HNE, additional studies were performed with rottlerin, an inhibitor of PKCδ and θ.[31] (Because PKCθ was not expressed in NHBE cells under basal or stimulated conditions, rottlerin is referred to below as a specific inhibitor of PKCδ). Rottlerin has the following potency against PKC isoforms: PKCδ (IC_{50} = 3 to 6 μmol/L); PKCθ (IC_{50} = 50 μmol/L); PKCα, PKCβ, and PKC γ(IC_{50} = 30 to 42 μmol/L); PKCε, PKCη, and PKCζ (IC_{50} = 80 to 100 μmol/L). It also can inhibit CaM kinase III (IC_{50} = 5.3 μmol/L).[31, 32]

Cells were preincubated with rottlerin (1, 5, and 10 μmol/L; IC_{50} = 3 to 6 μmol/L) for 20 minutes before exposure to HNE, and effects on PKC activity [using detection of phosphorylated (ser) PKC substrate] and on HNE-induced mucin secretion were assessed. As additional controls, the potential role of other PKC isoforms present in these cells was assessed: cells were exposed to the following specific inhibitors for 15 minutes before exposure to HNE and assay for mucin secretion: The PKC α/β inhibitor, Gö 6976 (10 nmol/L; IC_{50} = 2~ 6 nmol/L);[29] a PKCζ peptide inhibitor (50 μmol/L; Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu; IC_{50} = 10 μmol/L);[33] or a PKCε peptide inhibitor (3 ~300 μmol/L; Glu-Ala-Val-Ser-Leu-Lys-Pro-Thr; IC_{50} = 80.3 μmol/L).[34, 35]

Statistical analysis

Data were expressed as the ratio of treatment to the corresponding vehicle control. Results were evaluated using one-way analysis of variance with Bonferroni posttest correction for multiple comparisons.[36] A P value of <0.05 was considered significant.

RESULTS

Cytotoxicity

All reagents used were tested for cytotoxicity using a Promega Cytotox 96 nonradioactive cytotoxicity assay kit according to the manufacturer's instructions. The data were expressed as the ratio of released lactate dehydrogenase to total lactate dehydrogenase. Released lactate dehydrogenase never exceeded 10% of total lactate dehydrogenase (data not shown) in any of the experiments below.

Effects of HNE on mucin secretion

As illustrated in Figure 1, HNE stimulated mucin secretion by NHBE cells. Maximal mucin secretion was elicited after 15 minutes exposure to HNE (Figure 1A) so this time point was chosen for additional experiments. HNE increased mucin secretion in a concentration-dependent manner, with 0.01 to 1.0 μmol/L HNE increasing secretion significantly over vehicle control (Figure 1B).

Mucin gene products released by NHBE cells in response to HNE

Secretion of major gel-forming mucins, including MUC2, MUC5AC, and MUC5B, was investigated after exposure to HNE. As illustrated in Figure 2, HNE enhanced release of both MUC5AC and MUC5B mucins from NHBE cells in a concentration-dependent manner. Secretion of MUC2 mucin was significantly decreased by HNE.

HNE enzymatic activity and HNE-stimulated mucin secretion

Elastatinal appeared to be the weakest of the three HNE inhibitors used in this study because the highest concentration used, 100 μmol/L, blocked only 50% of HNE enzymatic activity and did not affect HNE-stimulated mucin secretion (Figure 3A). CMK proved to be a more potent HNE enzymatic inhibitor because 50 μmol/L CMK completely blocked the enzymatic activity of 1 μmol/L HNE, whereas lower concentrations partially inhibited HNE activity in a concentration-dependent manner. CMK also showed an inhibitory effect on HNE-stimulated mucin secretion in a concentration-dependent manner with 50 μmol/L CMK almost completely blocking the secretory effect of HNE (Figure 3B). AT was the most potent HNE inhibitor among the three tested because 5 μmol/L AT blocked 90% of HNE enzymatic activity and completely inhibited its mucin-secretory effect (Figure 3C). Elastatinal (100 μmol/L), CMK (50 μmol/L), or AT (5 μmol/L) by themselves did not affect constitutive mucin secretion (data not shown). As illustrated in Figure 3D, boiling HNE inactivated the enzyme rapidly: boiling for 5 minutes inhibited 70 to 80% HNE activity, while boiling for 15 minutes inhibited HNE activity. Interestingly, HNE boiled for 5 minutes still was able to induce mucin secretion, but boiling for 15 minutes abolished the ability of HNE to provoke mucin secretion.

Finally, as illustrated in Figure 4, media collected from NHBE cell cultures exposed to HNE, called conditioned medium, was capable of stimulating mucin secretion when applied to a different set of NHBE cells. However, this effect was inhibited by AT, indicating that the secretory response was only due to the presence of HNE in the conditioned medium. Thus, effects of HNE on mucin secretion do not appear to involve a soluble secondary product released by NHBE cells.

Effects of kinase inhibitors on HNE-induced mucin secretion

As illustrated in Figure 5, A and B, both PKC inhibitors, bisindolylmaleimide I and calphostin C, attenuated HNE-stimulated mucin secretion in a concentration-dependent manner.

Effects of HNE on PKC Activity

As illustrated in Figure 5C, exposure of NHBE cells to HNE caused a rapid (within 2 minutes) phosphorylation of MARCKS protein, reflecting activation of PKC.

PKC isoforms in HNE-induced mucin secretion

Western blot studies on cytosolic and membrane fractions of NHBE cells were performed under constitutive conditions and after stimulation with HNE (0.5 μmol/L) or a positive control, PMA (100 nmol/L). As illustrated in Figure 6, PKCα, δ, ε, λ, ζ, and ι were expressed constitutively in nonstimulated NHBE cells, while PKCβ and η were induced in the cytosol and PKCμ in the membrane only after exposure to PMA. PKC γ or θ were not detected under any conditions. Of these isoforms, only PKCδ translocated from cytosol to membrane after HNE exposure (Figure 6). Preincubation of cells with rottlerin, a specific PKCδ inhibitor, for 20 minutes before HNE exposure resulted in reduction of total PKC activity and attenuation of HNE-induced mucin secretion (Figure 7). None of the other PKC isoform-specific inhibitors affected HNE-induced mucin secretion (data not shown).

DISCUSSION

The results of the studies reported here indicate that elastase causes rapid release of preformed mucins from primary cultures of human bronchial epithelial cells maintained in air-liquid interface, a cell culture system that maintains characteristics of well-differentiated airway epithelium in vitro, including mucin synthesis and secretion.[21, 37] The concentrations and time course for HNE-induced mucin release from NHBE cells were similar to those reported for HNE in studies using the MM39 human submucosal gland cell line[38, 39] and hamster goblet cells in vitro.[9] The concentrations of HNE (0.01 to 1 μmol/L) that elicited a significant secretory response in NHBE cells in the present study appear to be achievable in inflamed airways. For example, it has been reported that HNE concentrations in sputum of cystic fibrosis patients may exceed 3.3 μmol/L (100 μg/ml).[15, 16] Interestingly, in studies related to mucin production in response to HNE, Voynow and colleagues[40] showed that a much lower HNE concentration (25 nmol/L) can increase MUC5AC mRNA and protein expression in human airway epithelial cells.

The majority of the secretion measurements in our study were done via an ELISA using the pan-mucin antibody, 17Q2, which is directed toward a carbohydrate epitope and therefore recognizes mucins derived from different MUC genes.[41] It has been reported previously that HNE has an ability to release mucins from airway goblet cells and then to degrade the released mucins.[9] However, because we measured pan-mucins using the 17Q2 antibody that recognizes carbohydrate epitope(s), it is very likely that the amount of mucins detected would be virtually the same regardless of the intactness of mucins. Therefore,

degradation of mucins by the hydrolytic effects of HNE would not affect the results or the conclusion of this study.

The mucin proteins secreted in response to elastase appear to be a combination of the gene products of MUC5AC and MUC5B, as ELISA-based analysis revealed significant secretion of both mucins in response to HNE. MUC5AC mucin is usually considered to be the major mucin protein produced and secreted by airway epithelial cells, with MUC5B restricted mostly to submucosal glands.[42, 43] However, Chen and colleagues[44] and Holmen and colleagues[45] independently reported recently that cultured human airway epithelial cells express MUC5B, as does epithelium in situ in both human bronchitis and mouse models of asthma.[44] Thus, MUC5B might also be a relevant mucin gene to investigate in relation to the pathogenesis of mucus overproduction and hypersecretion in inflammatory airway disease. MUC2 is not produced in large amounts by human airway epithelium under normal conditions, and the absolute amounts released in control or HNE-exposed cells were miniscule compared to MUC5AC or MUC5B. The statistically significant decrease in response to HNE could be the result of degradation, because the antibody is against the peptide core. Although it is possible that some degradation of MUC5AC and MUC5B also occurred in these experiments, it would be masked by the high levels of release of these proteins, whereas the effects on MUC2, already at low levels, would be observable. The story has become even more complicated with the recent description of a newly discovered respiratory mucin, MUC19, that also could be involved in inflammation-associated responses of the secretory apparatus in airways.[46] The exact contribution(s) of each of these mucin genes to human airway mucin secretions in health and disease remains to be determined.

In the studies reported here, we found that the secretagogue activity of HNE was linked to its enzymatic activity. However, although complete inhibition of HNE activity also blocked HNE-induced mucin secretion (Figure 3), inhibition of HNE enzymatic activity by 50% [as caused by addition of 100 μmol/L elastatinal (Figure 3A), 5 μmol/L CMK (Figure 3B), or 0.5 μmol/L AT (Figure 3C)] did not alter the secretory response, as the increase in mucin secretion by cultures in response to HNE that had been treated with any of these agents, and thus contained only approximately half of its original enzymatic activity, still reached the same levels as that induced by intact HNE. However, as indicated in Figure 1B, 0.1 μmol/L of HNE had essentially the same effect on mucin secretion as 1.0 μmol/L, so the relationship between enzymatic activity and secretory stimulation does not appear direct, at least at the higher concentrations. It is possible that attenuation of enzymatic activity at lower concentrations of HNE could have had more of an effect on secretory activity. In any case, given that concentrations of HNE in airways of patients with airway inflammation can reach micromolar levels,[15-17] potential anti-elastase therapy in such diseases might need to significantly diminish the enzymatic activity of HNE before it could become effective against its secretory action.

There is a possibility that a potential HNE-related product that acts as a secondary stimulus, rather than HNE directly, could be responsible for HNE-stimulated secretion. If this were true and the potential product(s) were soluble, then the product might be found in the conditioned medium from cells exposed to HNE, and this conditioned medium would induce mucin secretion if added to other cells in a manner such that an elastase inhibitor, such as AT, would not block the enhanced secretion if added directly to the conditioned medium. As illustrated in Figure 4, AT did in fact inhibit conditioned medium-induced mucin secretion,

suggesting that a soluble product released on HNE exposure was not involved. Of course, a soluble product could be released and bind rapidly to the NHBE cells, so these results do not preclude the existence of secondary mediators in the secretory response to HNE. These findings also do not preclude either an HNE substrate or HNE cleavage product that could influence secretion being present on epithelial cell membranes (or even intracellularly). In this regard, Takeyama and colleagues[19] have reported that contact between neutrophils and epithelial cells enhances neutrophil chemoattractant-stimulated goblet cell degranulation, and Kim and colleagues[9] suggested that HNE binding to epithelial cell surfaces maximized its stimulatory activity.

Whatever the actual stimulatory product, be it HNE directly or a secondary product, the secretory response appears to be mediated by PKC. The PKC family contains three types of isoforms: classical (cPKCs:α, β1, β2, γ), novel (nPKCs:δ, ε, η, θ, μ), and atypical (aPKCs:ζ, ι/λ). The classical isoforms are calcium- and phorbol ester-activated, the novel are calcium-insensitive but activated by phorbol esters, and the atypical isoforms are both calcium- and phorbol ester-insensitive, with all isoforms activated by phosphatidylserine.46 Our studies demonstrating that human tracheal epithelial cells express α, β1, β2, δ, ε, and ζ isoforms of PKC are in general agreement with previous reports. [47-49]

Involvement of PKC in secretion of airway mucin in response to various stimuli has been indicated previously.[50-55] The specific PKC isoenzyme(s) (singly or in combination) that contribute to PKC-induced mucin secretion have not been determined, although PKCζ and PKCδ have been suggested as potential candidates.[50, 53, 54] In our studies with HNE, both the DAG-binding site inhibitor of PKC (calphostin C) and the ATP-binding site inhibitor (bisindolylmaleimide I) blocked mucin release in concentration-dependent manners,

suggesting that a cPKC or nPKC isoform(s) is involved in the secretory response. When we examined translocation of PKC isoforms in response to HNE, the only isoform that moved from cytoplasm to membrane in NHBE cells exposed to stimulatory concentrations of HNE was PKCδ (Figure 6). When NHBE cells then were exposed to PKC isoform-specific pharmacological inhibitors, the only inhibitor that attenuated secretion was rottlerin, which also appeared to attenuate enhancement of PKC activity in cells exposed to HNE (Figure 7). Further verification of the involvement of the PKCδ isoform in the secretory process using molecular constructs, such as dominant-negative PKCδ , siRNA, and so forth, would be valuable, but at this time problems with transfection of these constructs into primary cells and the presence of a heterogeneous cell population in NHBE cultures make these kinds of studies difficult to perform.

As illustrated in Figure 6, there were effects of PMA and HNE on expression of other isoforms of PKC. For example, in response to PMA, PKCβ appeared in the cytosol and PKCμ appeared in the membrane fraction, whereas neither isoform appeared to be expressed constitutively. PMA also caused an apparent decrease of protein expression of PKCε in the cytosolic fraction. Since these blots were performed within 15 minutes of exposure, there is little if any probability that transcription could have occurred. There are (at least) two possible explanations for these responses. Firstly, they could reflect rapid posttranscriptional and/or translational events. Secondly, PMA is known to bind to PKCs in the C1 regulatory domain of the enzyme, and it is possible that such binding could induce conformational changes that could expose previously masked epitopes, or, in the case of PKCε , block access of an antibody to such epitopes. Relatedly, PKCε expression in the membrane fraction appeared to be decreased after exposure to HNE, and it could be that HNE effects on

membranes, alluded to previously, could affect expression of several membrane proteins, including some PKC isoforms. In any case, these alterations in expression of several isoforms require further study to determine the mechanisms of action of PMA and/or HNE.

The involvement of PKCδ in the secretory response to HNE is interesting in light of recent findings that this particular PKC isoform has a number of potentially important functions in airway epithelial cell pathophysiology. These include regulation of nuclear factor-κB-dependent expression of proinflammatory genes in a human airway epithelial cell line,[47] control of both NKCC1 function and Na-K-2Cl co-transport in airway epithelial cells,[49, 56] and regulation of asbestos-induced apoptosis in alveolar epithelial cells.[57] Interestingly, Abdullah and colleagues[50] reported that mucin secretion in response to purinergic stimulation in SPOC1 cells, a rat airway cell line, also may involve activation of PKCδ. In addition, a recent report implicated PKCδ in HNE-induced mucin gene expression in airway epithelium.[58]

A role for PKCδ in mucin secretion fits in nicely with previous studies from our laboratory showing that MARCKS protein, a widely expressed PKC phosphorylation target, is a key molecule regulating mucin secretion in airway epithelium,[21, 59] as PKCδ is activated by PMA and can phosphorylate MARCKS protein. Interestingly, as illustrated in Figure 5C, MARCKS is rapidly phosphorylated when HNE is added to NHBE cells, but is quickly dephosphorylated. This pattern of phosphorylation-dephosphorylation of MARCKS was shown in a previous report from this laboratory to be a critical step in the mucin secretory pathway after stimulation[21] so these results are consistent with previous studies implicating MARCKS protein in airway mucin secretion. Whether or not the secretagogue action of HNE depends on MARCKS, as shown with other stimuli that enhance mucin secretion, presently

is under study, and if so would provide additional information supporting the existence of a common intracellular secretory pathway involving MARCKS present in goblet cells in response to a variety of pathophysiological stimuli in the airways.

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FIGURE LEGENDS

Figure 1. NHBE cells were exposed to HNE throughout a range of times and concentrations. A: NHBE cells were exposed to 1 μmol/L HNE or vehicle control for 5, 10, 20, 30, or 60 minutes, and mucin secretion was quantified by ELISA as described in the text using the 17Q2 antibody. Mucin secretion was rapidly increased after exposure to HNE and appeared to plateau at 10 to 15 minutes. B: HNE stimulates mucin secretion from NHBE cells in a concentration-dependent manner after 15 minutes of exposure. Cells were exposed to HNE for 15 minutes and mucin secretion was quantified by ELISA as described in text using 17Q2 antibody. *, Significantly different from vehicle control ($P < 0.005$). †, Significantly different from vehicle control ($P < 0.001$). Data are presented as mean ± SEM ($n = 6$ at each point in A, 12 at each point in B).

Figure 2. Differential effect of HNE on secretion of mucin gene products by NHBE cells. Cells were exposed to HNE for 15 minutes and secretion of MUC5AC, MUC5B, and MUC2 protein quantified by ELISA as described in text. *, Significantly greater than vehicle control ($P < 0.005$); †, significantly greater than vehicle control ($P < 0.001$); ‡, significantly less than vehicle control ($P < 0.05$). Data are presented as mean ± SEM ($n = 6$ at each point).

Figure 3. Effect of elastase enzymatic inhibitors on HNE activity and HNE-induced mucin secretion from NHBE cells. **A:** Elastatinal, 1 to 100 μmol/L; **B:** CMK, 0.5 to 50 μmol/L; **C:** α1-AT, 0.5 to 5 μmol/L; and **D:** boiling HNE for indicated times. HNE activity: 1 μmol/L HNE was incubated with or without the indicated inhibitor for 15 minutes at 37°C or boiled

for the indicated time. HNE activity in each mixture then was measured as described in text. †, Significantly different from HNE alone ($P < 0.001$). Data are presented as mean ± SEM ($n = 6$ at each point). Mucin secretion: HNE was incubated with or without the indicated inhibitor for 15 minutes at 37°C (or boiled), and the mixture then added to NHBE cells for another 15 minutes at 37°C. *, Significantly different from vehicle control ($P < 0.001$). ‡, Significantly different from HNE alone ($P < 0.05$). Data are presented as mean ± SEM ($n = 12$ at each point).

Figure 4. α1-Antitrypsin (AT) blocks mucin secretion stimulated by medium from cells exposed to HNE. NHBE cells were exposed to medium alone or medium plus 1.0 μmol/L HNE for 5 minutes. Medium from cells was then collected and incubated under cell-free conditions with or without AT (5 μmol/L) for an additional 15 minutes at 37°C. At the end of this incubation, a new set of NHBE cells was exposed to the different mixtures for 15 minutes at 37°C, and secreted mucin quantified as described in text. AT added to medium from cells exposed previously to HNE attenuated the stimulatory effect of that medium on new NHBE cells, indicating that a secondary soluble product is not involved in the secretory response to HNE. *, Significantly different from vehicle control ($P < 0.05$). †, Significantly different from HNE alone ($P < 0.05$). Data are presented as mean ± SEM ($n = 12$).

Figure 5. HNE appears to stimulate mucin secretion by a PKC-dependent mechanism. **A** and **B:** PKC inhibitors attenuate HNE-induced mucin secretion by NHBE cells. NHBE cells were pre-exposed to PKC inhibitors or solvent alone for 15 minutes, after which HNE or vehicle control was added to cells at indicated concentrations for an additional 15 minutes, and

secreted mucin was then quantified as described in text. Both PKC inhibitors: bisindolylmaleimide I (Bis I) (**A**) and calphostin C (Cal C) (**B**) attenuated HNE-stimulated mucin secretion in a concentration-dependent manner. (Bis V is bisindolylmaleimide V, a functionally inactive control for Bis I). *, Significantly different from vehicle control ($P < 0.05$); †, significantly different from HNE alone ($P < 0.05$). Data are presented as mean ± SEM ($n = 12$). **C:** Addition of 1 μmol/L HNE to NHBE cells results in a rapid (within 1.5 minutes) activation of PKC, as reflected by phosphorylation of the PKC substrate MARCKS protein at that time point. MARCKS appears to be rapidly dephosphorylated within the next 10 to 15 minutes. Blot is representative of three replicate experiments. **D:** Phosphorylated MARCKS and MARCKS protein from blot **C:** were quantified by densitometry. Phosphorylated MARCKS was normalized to total MARCKS protein. A clear increase in phosphorylated MARCKS is apparent.

Figure 6. Several PKC isoforms are expressed by NHBE cells *in vitro*, but only PKCδ translocates from cytosol to membrane in response to HNE. All proteins were detected by Western blot from both cytosolic and membrane fractions. All PKC isoforms were detected using the same original gel. Under nonstimulated conditions, cells contain PKC α, δ, ε, ζ, ι, and λ in the cytosol, and α, δ, and ι in membrane fractions. On stimulation with PMA (100 nmol/L; 15 minutes), PKCβ, η and μ appear induced in the cytosol or membrane fraction, and only PKCδ and ε translocate to the membrane. In response to HNE (0.5 μmol/L; 15 minutes), however, only PKCδ translocates to the membrane. α-Tubulin and E-cadherin are

used as controls to show cytosolic and membrane protein expression, respectively. Blots are representative of experiments repeated at least three times.

Figure 7. Effects of the PKCδ inhibitor, rottlerin, on HNE-induced PKC activity and mucin secretion in NHBE cells. Cells were preincubated with rottlerin or vehicle control for 20 minutes, and then exposed to HNE (0.5 μmol/L) for an additional 15 minutes, at which time PKC activity and mucin secretion were assessed as described. **A:** Rottlerin attenuates HNE-induced PKC activity in a concentration-dependent manner. Data are representative densitometric scans averaged from two replicate experiments. **B:** Rottlerin inhibits, in a concentration-dependent manner, secretion of mucin (MUC5AC) in NHBE cells exposed to HNE. Rottlerin also significantly decreased constitutive mucin secretion. *, Significantly different from vehicle control ($P < 0.05$); †, significantly different from HNE alone ($P < 0.05$). Data are presented as mean ± SEM ($n = 4$).

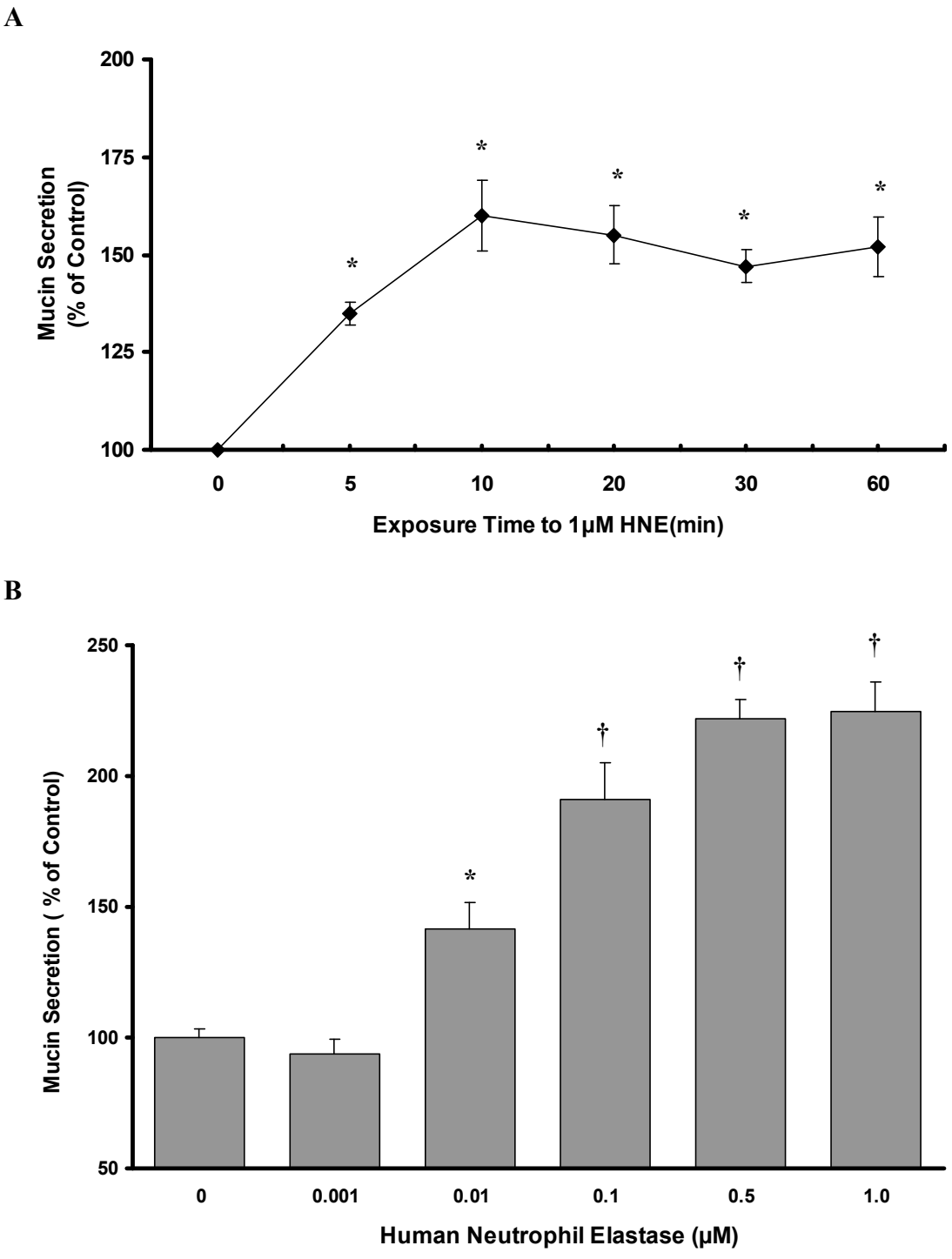


Figure 1. NHBE cells were exposed to HNE throughout a range of times and concentrations

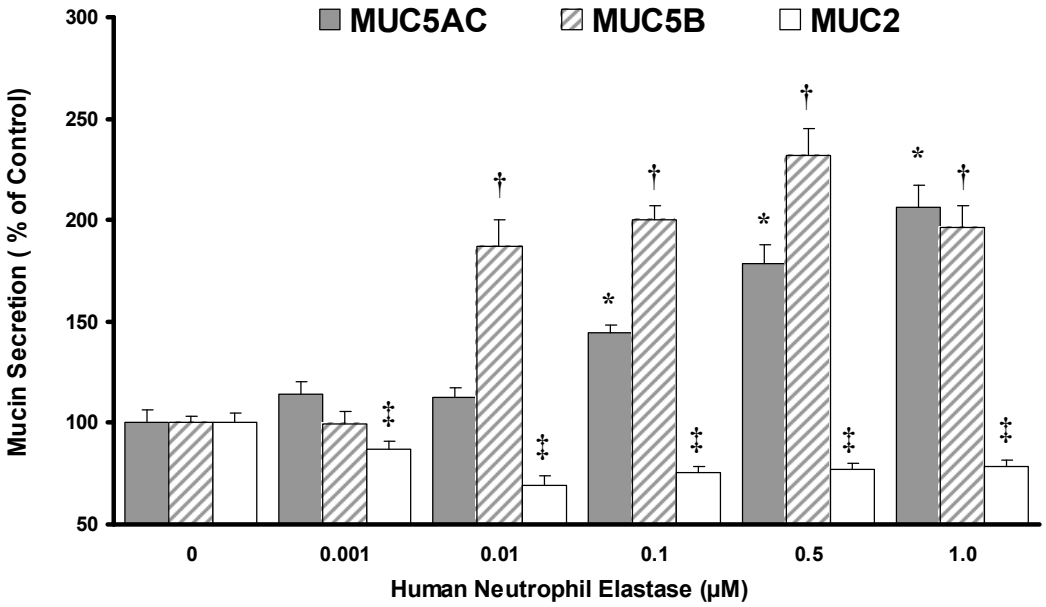
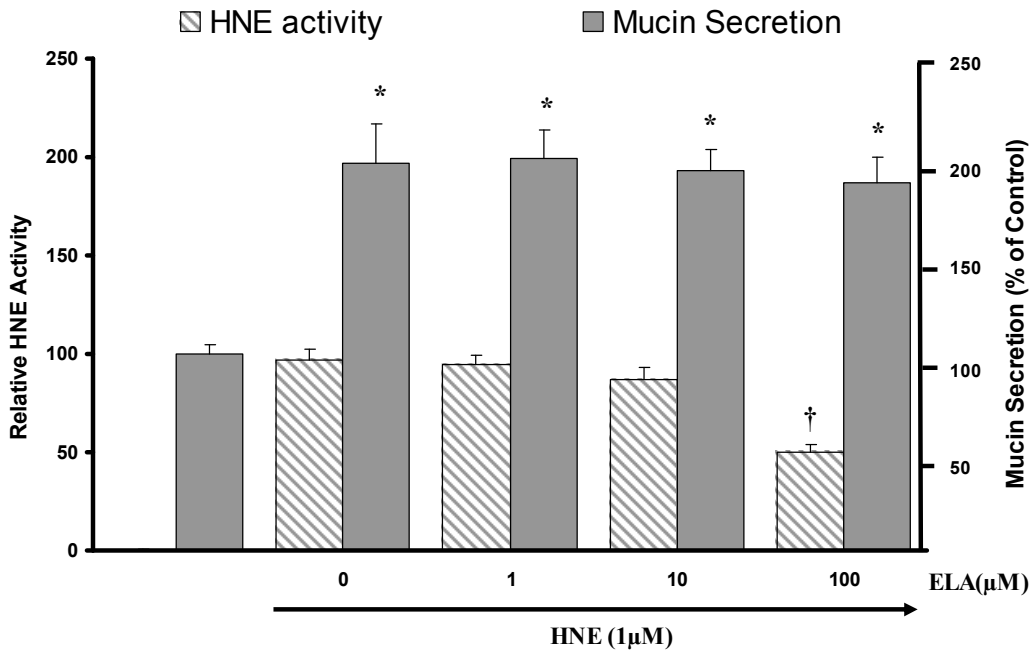


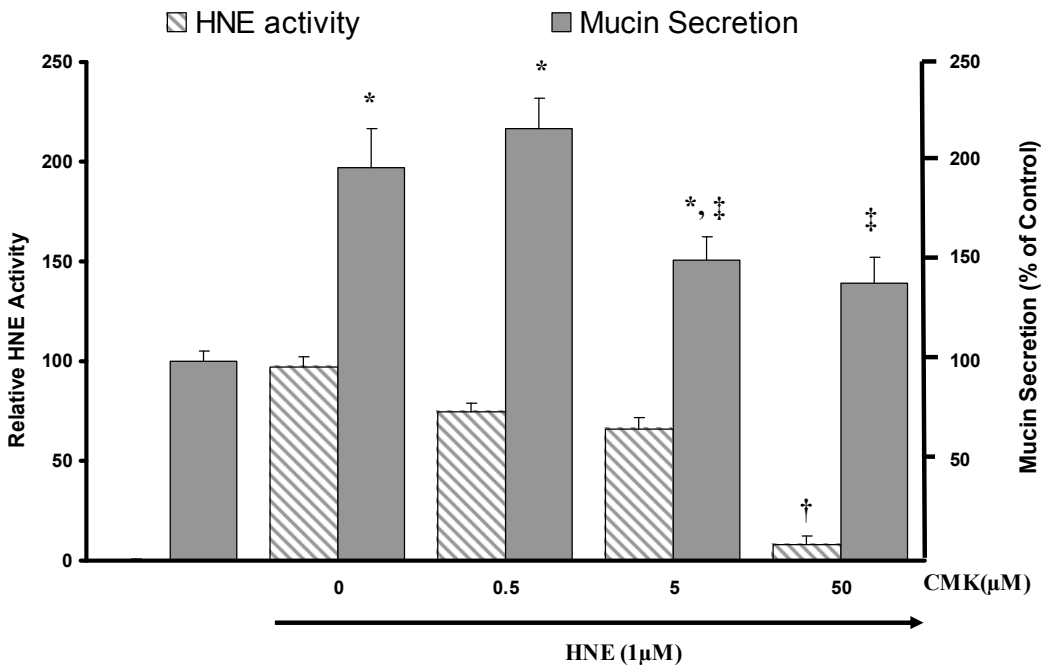
Figure 2. Differential effect of HNE on secretion of mucin gene products by NHBE cells

Figure 3. Effect of elastase enzymatic inhibitors on HNE activity and HNE-induced mucin secretion from NHBE cells

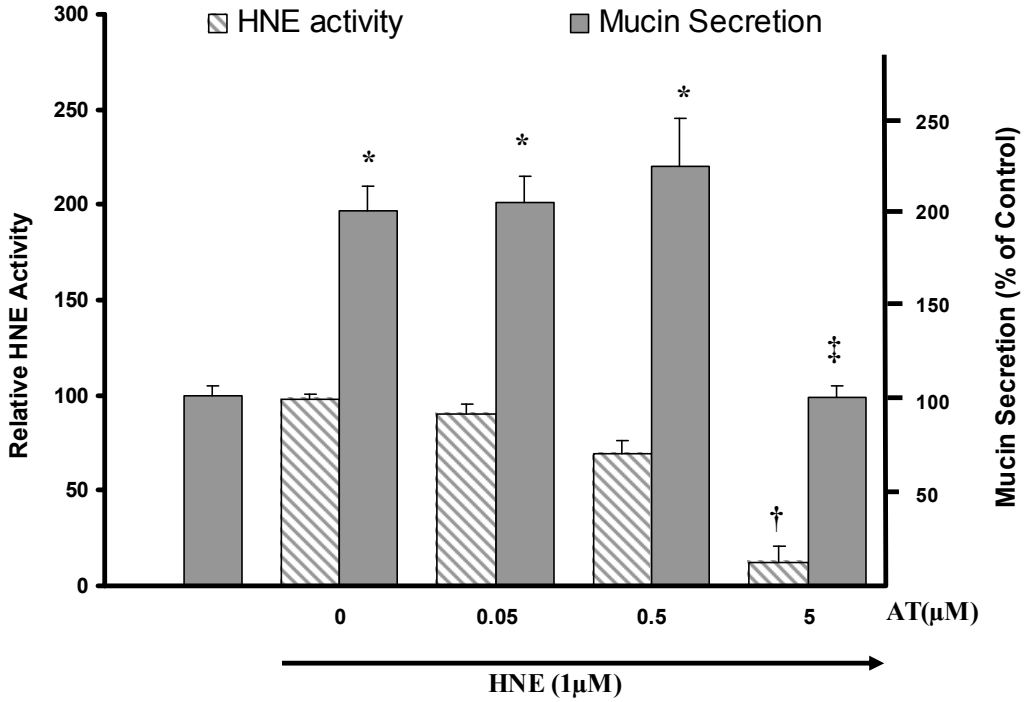
A


HNE activity
Mucin Secretion
Relative HNE Activity
Mucin Secretion (% of Control)
250
200
150
100
50
0
*
*
*
*
†
0
1
10
100
ELA(μM)
HNE (1μM)

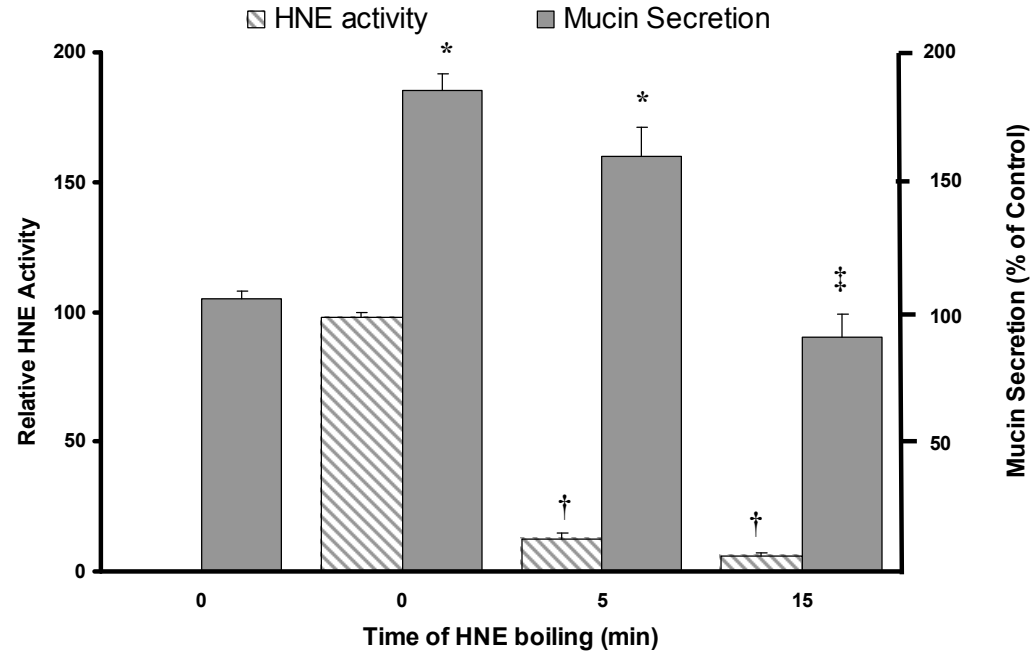
B


HNE activity
Mucin Secretion
Relative HNE Activity
Mucin Secretion (% of Control)
250
200
150
100
50
0
*
*
*, ‡
‡
†
0
0.5
5
50
CMK(μM)
HNE (1μM)

C


HNE activity
Mucin Secretion
Relative HNE Activity
Mucin Secretion (% of Control)
300
250
200
150
100
50
0
0
0.05
0.5
5
AT(μM)
HNE (1μM)
*
†
‡

D


HNE activity
Mucin Secretion
Relative HNE Activity
Mucin Secretion (% of Control)
200
150
100
50
0
0
0
5
15
Time of HNE boiling (min)
*
†
‡

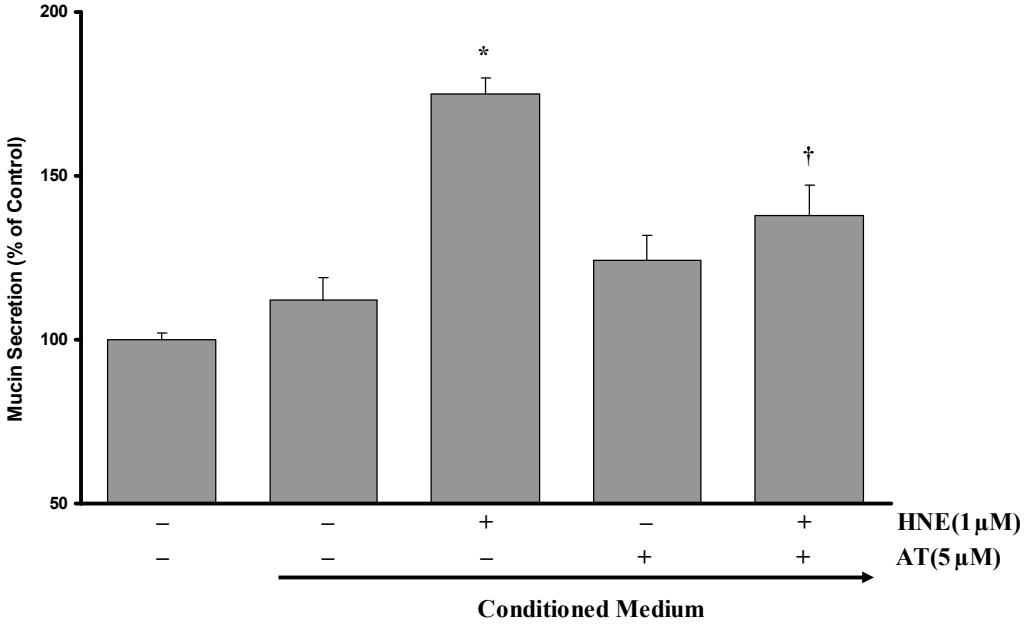
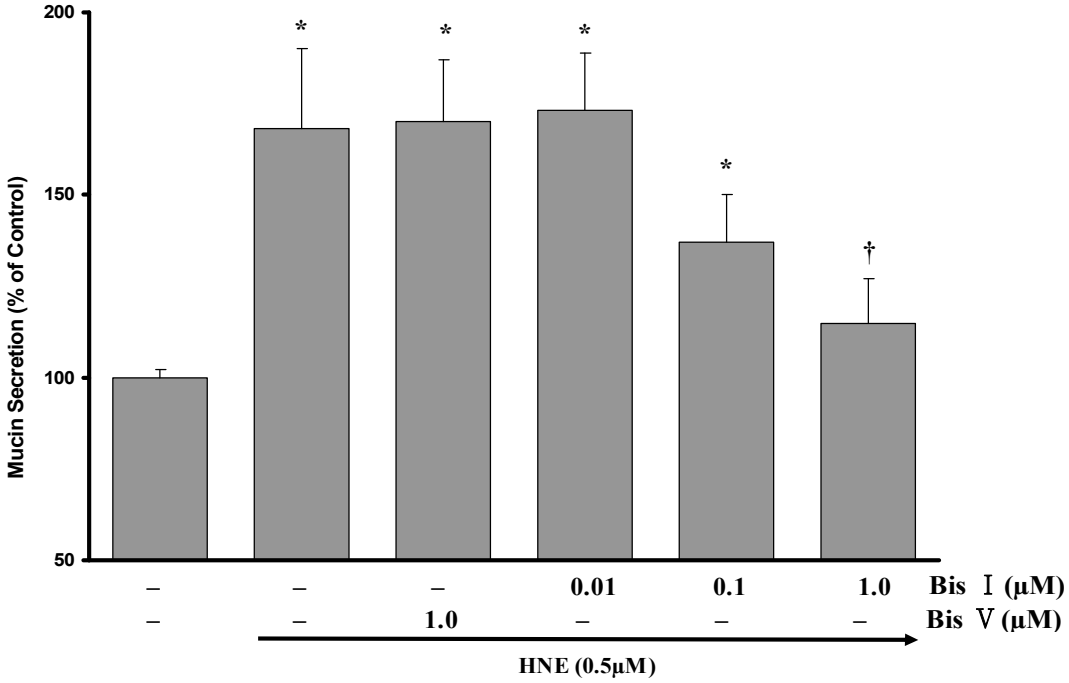


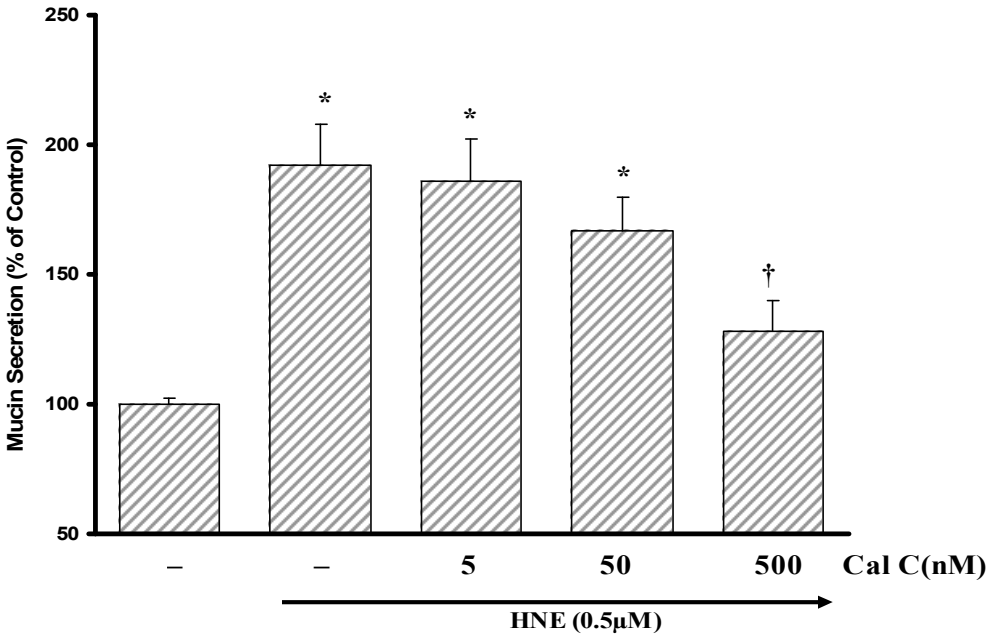
Figure 4. α1-Antitrypsin (AT) blocks mucin secretion stimulated by medium from cells exposed to HNE

Figure 5. HNE appears to stimulate mucin secretion by a PKC-dependent mechanism

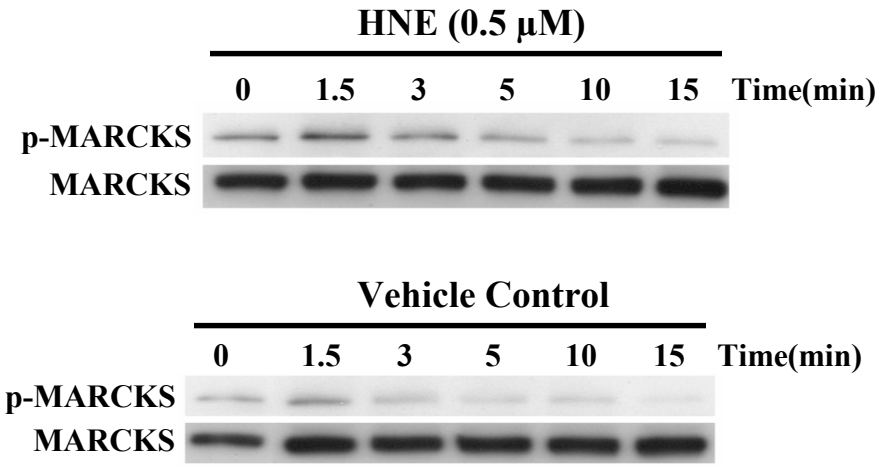
A


Mucin Secretion (% of Control)
200
150
100
50
*
*
*
*
†
– – – 0.01 0.1 1.0 Bis Ⅰ (μM)
– – 1.0 – – – Bis Ⅴ (μM)
HNE (0.5μM)

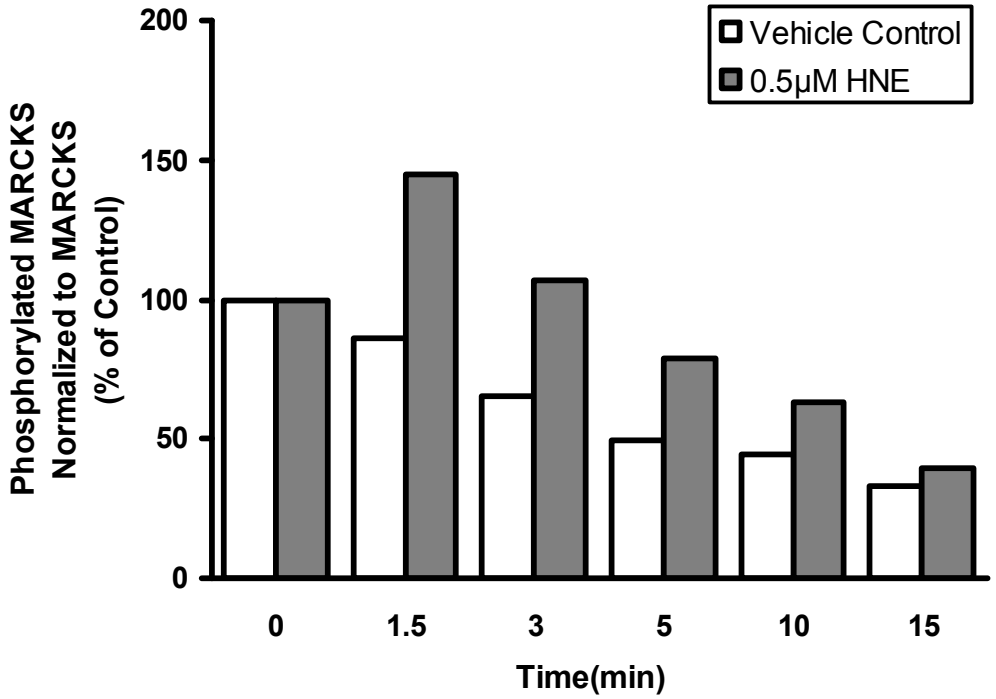
B


Mucin Secretion (% of Control)
250
200
150
100
50
*
*
*
†
– – 5 50 500 Cal C(nM)
HNE (0.5μM)

C


HNE (0.5 μM)
0
1.5
3
5
10
15
Time(min)
p-MARCKS
MARCKS
Vehicle Control
0
1.5
3
5
10
15
Time(min)
p-MARCKS
MARCKS

D


Vehicle Control
0.5μM HNE
Phosphorylated MARCKS
Normalized to MARCKS
(% of Control)
200
150
100
50
0
0
1.5
3
5
10
15
Time(min)

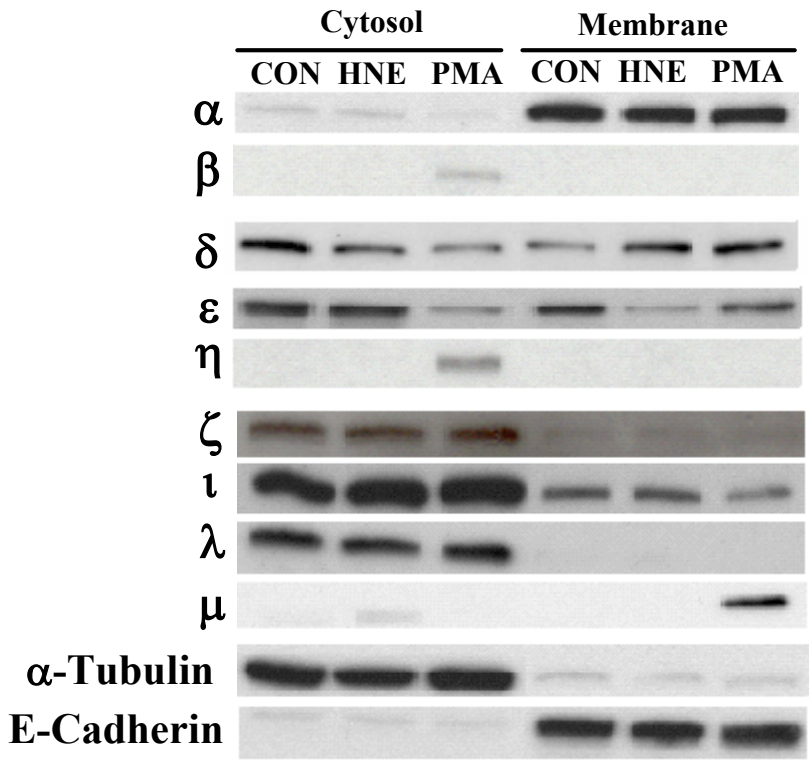
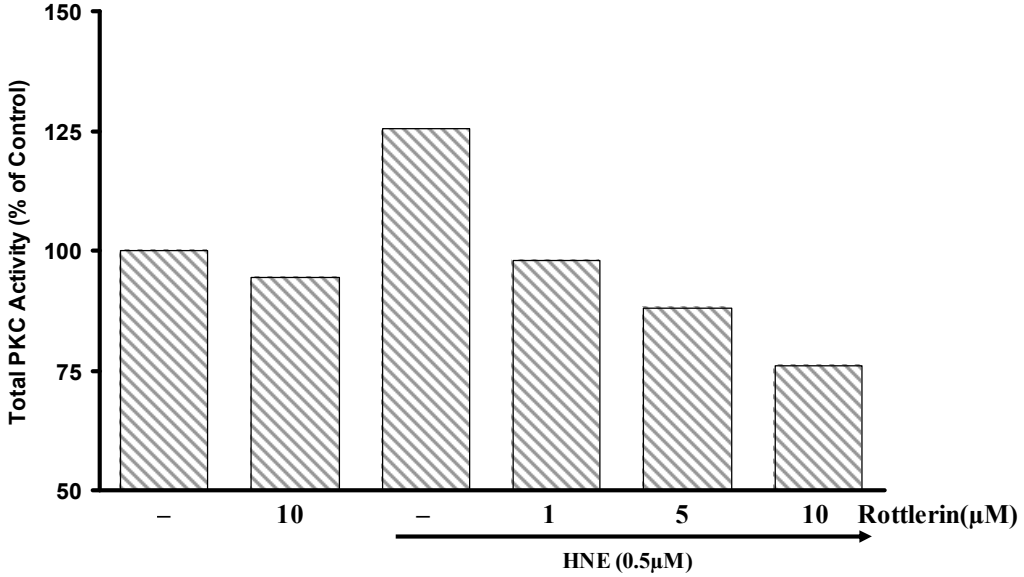


Figure 6. Several PKC isoforms are expressed by NHBE cells *in vitro*, but only PKCδ translocates from cytosol to membrane in response to HNE

A



B

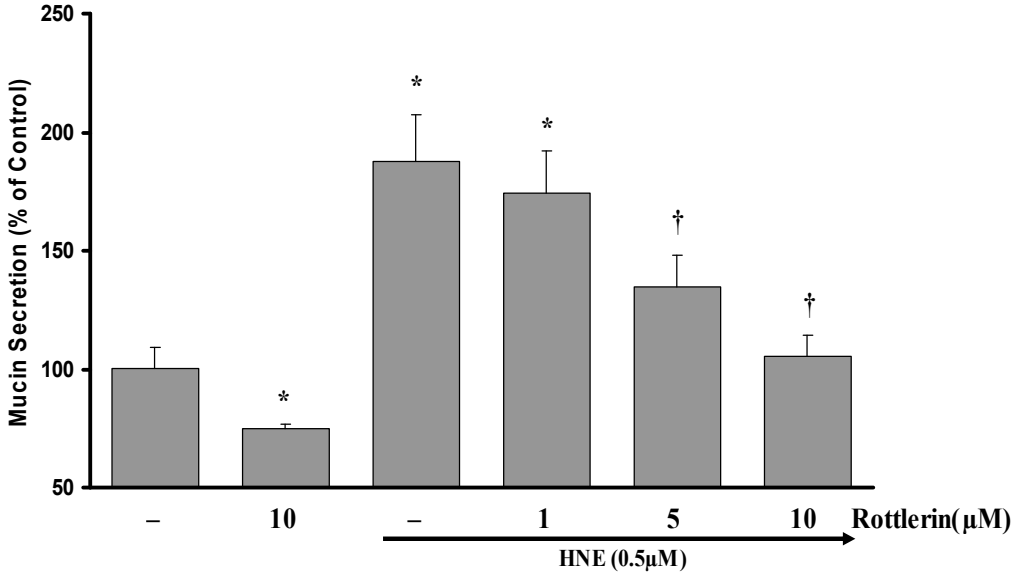


Figure 7. Effects of the PKCδ inhibitor, rottlerin, on HNE-induced PKC activity and mucin secretion in NHBE cells

MANUSCRIPT II

Protein Kinase C delta Regulates Airway Mucin Secretion via Phosphorylation of MARCKS Protein

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Running title: PKC delta and airway mucin secretion

This manuscript will be submitted to *The American Journal of Respiratory Cell and Molecular Biology* and is in the format required of that journal

ABSTRACT

Mucin hypersecretion is a major pathological feature in pulmonary diseases such as asthma, chronic bronchitis and cystic fibrosis. Cellular mechanisms regulating secretion of mucin under normal or diseased conditions have not been fully elucidated. Previously, we reported that mucin hypersecretion induced by human neutrophil elastase (HNE) appears to involve activation Protein Kinase C (PKC) specifically the delta isoform (PKCδ). Here, we investigated further the potential role of PKCδ in mucin hypersecretion using either well-differentiated human primary bronchial epithelial (NHBE) cells or the virally-transformed human bronchial epithelial cell line (HBE-1) as *in vitro* model systems. Both mucin secretion and phosphorylation of MARCKS were significantly enhanced by exposure of cells to the PKCδ – activator, bryostatin 1. Mucin hypersecretion and enhanced phosphorylation of MARCKS in response to either HNE or phorbol myristate- 13-acetate (PMA) was attenuated by pre-incubation with the PKCδ – specific inhibitor, rottlerin. To selectively suppress endogenous PKCδ kinase activity, a dominant negative PKCδ construct (pEGFP-N1/PKCδ^{K376R}) was transfected into HBE-1 cells. Transfection with this dominant-negative PKCδ construct significantly attenuated both PMA-induced mucin secretion and phosphorylation of MARCKS compared to cells transfected with empty vector (pEGFP-N1) alone. Transfection of a wild-type construct increased PKCδ in these cells and resulted in enhanced mucin secretion and MARCKS phosphorylation in response to PMA. These results suggest that PKCδ plays an important role in regulating mucin secretion by airway epithelium.

INTRODUCTION

Mucus produced by epithelium of the respiratory, gastrointestinal and reproductive tracts provides a barrier between the external environment and cellular components of the epithelial layer. Mucins, the glycoprotein component of mucus, constitute a family of large, highly glycosylated macromolecules that impart physical (aggregation, viscosity, viscoelasticity, lubrication) and biological (protection) properties to mucus (reviewed in(1)). Airway mucus is an integral component of the mucociliary clearance system in the trachea and bronchi and thus serves to protect the lower airways and alveoli from impingement of particulate matter and pathogens. However, mucin secretion is abnormally augmented in disease states such as asthma, emphysema, chronic bronchitis and cystic fibrosis, increasing morbidity and mortality in these patients (reviewed in(1, 2)) Mucin hypersecretion is potentiated by many pathophysiologic mediators such as bacterial proteinases and endotoxin, adenine and guanine nucleotides, cytokines, inflammatory mediators and eicosanoids (reviewed in(3)). However intracellular mechanisms and signaling molecules involved in the secretory process have not been fully elucidated.

Protein kinase C (PKC) is a serine/threonine kinase involved in various exocytotic events in different cell types including secretion of mucin granules (4, 5), insulin (6), neurotransmitters (7), and platelet dense granules (8). Previously, we demonstrated that mucin secretion is regulated by PKC via phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) (9, 10). In addition, we demonstrated that mucin hypersecretion in response to human neutrophil elastase (HNE) appears to be mediated by the PKCδ isoform in human airway epithelial cells (11). Not surprisingly, PKCδ, a novel PKC isoform, has a

strong affinity for MARCKS and can phosphorylate MARCKS both *in vitro* and *in vivo* (12-14). Indeed, increasing evidence suggests that PKCδ mediates exocytosis of mucin granules (4), insulin (15), platelet dense granules (8), as well as coordination of inflammatory cell degranulation (16, 17).

Here we further elucidate the role of PKCδ in the mucin secretory pathway in airway epithelial cells *in vitro*. Mucin secretory response and the phosphorylation of MARCKS were assessed after phorbol-12- myristate-13- acetate (PMA; a general PKC activator) or bryostatin 1 (a PKCδ/ε activator) exposure to well differentiated normal human bronchial epithelial (NHBE) cells. In addition, a dominant negative PKCδ construct (K376R) was transfected into the human bronchial epithelial cell line (HBE-1) to demonstrate the importance of PKCδ activity on mucin secretion.

MATERIALS AND METHODS

Culture of Bronchial Epithelial Cells

Primary human normal bronchial epithelial (NHBE) cells purchased from Cambrex Bioscience (Walkersville, MD) were expanded and maintained in a humidified air, 5% CO_2 incubator as described previously (9). Briefly, passage-2 NHBE cells were plated on Transwell® plates (Corning, Inc., Corning, NY) coated with type 1 rat tail collagen (BD Biosciences, San Jose, CA) at a density of 2×10^4 cells/cm^2. Cells were maintained submerged in medium until cultures reached confluence, at which time the medium was removed to expose the apical cell surface to ambient air. Media were replaced every other day while submerged and every day after exposure to air. The medium consisted of a 1:1 mixture of bronchial epithelial basal medium (Cambrex Bioscience, Walkersville, MD) and supplemented high glucose (4.5 g/L) Dulbecco's modified Eagle's medium (Mediatech, Herndon, VA), as described previously (11).

Passage 31 or 32 of human papilloma virus-transformed human bronchial epithelial cells (HBE-1) (18) were seeded and maintained as described for NHBE cells. The basic medium used for HBE-1 cells was a 1:1 mixture of Ham's F12 (Mediatech, Herndon, VA) and high glucose (4.5g/L) Dulbecco's modified Eagle's medium. The complete medium was composed of basic medium containing a final concentration of 10ng/ml human recombinant epidermal growth factor, 0.1μM dexamethasone, 5μg/ml insulin, 5μg/ml transferrin, 20ng/ml cholera toxin, 50μg/ml gentamicin, 50 ng/ml amphotericin B, 30nM all-*trans*-retinoic acid, and 20 U/ml nystatin. In addition, the medium contained 0.13 mg/ml bovine pituitary extract

made according to the protocol of Bertolero and colleagues (19). Transient transfection of HBE1 cells was performed after 10 days of air exposure.

Exposure of Cells to Inhibitors or Secretagogues

Well-differentiated NHBE cells were exposed to test agents both apically and basolaterally for 15 minutes (unless otherwise indicated). Transfected HBE-1 cells were exposed to phorbol-12- myristate-13- acetate (PMA) (EMD Biosciences, La Jolla, CA) apically only. Rottlerin (EMD Biosciences, La Jolla, CA), a PKCδ specific inhibitor, was preincubated for 20 minutes prior to PMA exposure. Initial stock solutions of rottlerin or PMA were prepared in DMSO and keep at -20ºC until used, and then finally diluted in growth medium before use. When cells were exposed to PMA in the presence or absence of rottlerin, PMA was "spiked" into each well at the indicated concentration.

Measurement of Mucin Secretion

Mucin was collected both at baseline and after treatments as described previously (9). Baseline mucin secretion was used to normalize well-to-well variation. Briefly, accumulated mucus on the apical surface of the cells was removed by washing with phosphate-buffered saline (PBS), pH 7.2, containing 1mM dithiothreitol. After baseline mucin samples were collected, cells were rested overnight and exposed to test reagents the next day for indicated periods of time. After each treatment period, the secreted mucin was collected and quantified by sandwich ELISA using the 17Q2 antibody (Covance Research Product, Berkeley, CA), a monoclonal antibody that reacts specifically with a carbohydrate epitope on human airway mucins (20). The 17Q2 antibody was purified using the ImmunoPure (G) IgG purification kit

(Pierce Biotechnology, Rockford, IL) following manufacturer's protocol and then conjugated with alkaline phosphatase (EMD Biosciences, La Jolla, CA). Levels of mucin secretion were reported as percentage of the non-treated control.

Subcellular Localization of PKC isoforms

Activation of PKCδ was assessed by subcellular fractionation following the protocol described by Kajstura et. al. (21) and subsequent Western blot analysis using a PKCδ-specific antibody. Briefly, cells were washed with cold PBS and scraped into lysis buffer [20mM Tris-Cl, pH, 7.5, 1mM ethylenediamine tetraacetic acid, 100mM NaCl, 1mM phenylmethyl sulfonyl fluoride, 1mM dithiothreitol, 1% (V/V) protease inhibitor cocktail and phosphatase inhibitor cocktail (Sigma, Saint Louis, MO)]. The lysate was then sonicated and pelleted at 20,000g (Eppendorf, 5417 centrifuge) for 40 min. The supernatant was collected and kept as the cytosolic fraction at -80ºC until assayed. The remaining pellet was resuspended in lysis buffer containing 1% Triton X-100, sonicated and centrifuged at 20,000g for 40 min. The supernatant membrane fraction was stored at -80ºC until analyzed via Western blot.

Western Blot Analysis

Green fluorescence protein (GFP), phosphorylated MARCKS, and PKCδ protein levels were measured via Western blot. The protein concentrations of cell lysates were quantified by a Bradford assay (Bio-Rad Laboratories, Hercules, CA). Sample lysates were prepared by boiling in 2× sodium dodecyl sulfate sample buffer [125mM Tris-Cl (pH 6.8), 25% glycerol, 4% sodium dodecyl sulfate, 10% β-mercaptoethanol, 0.04% bromophenol

blue] for 10 minutes. Sample lystaes (30-60μg) were loaded on 10% or 12% sodium dodecyl sulfate-polyacrylamide gels and then transferred to a polyvinylidene difluoride membrane (PVDF) (Schleicher & Schuell BioScience, Inc. Keene, NH) following electrophoresis. PVDF membranes were blocked with 5% non-fat milk, probed with an appropriate dilution of primary antibody followed by horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies. Chemiluminescent detection was performed using ECL detection reagents (GE Healthcare Life Sciences, Piscataway, NJ) following the manufacturer's protocol. Protein amounts were analyzed using Labworks image acquisition and analysis software 4.0. (Ultra Violet Products, Ltd., Upland, CA)

Antibodies against α-tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and E-cadherin (BD Biosceinces, San Jose, CA) were used as loading controls for cytosolic and membrane fractions, respectively. Phosphorylated MARCKS (at serine 152/156) was detected with a specific antibody (Cell Signaling Technology, Inc., Danvers, MA). After detection, the membrane was stripped in 62.5 mM Tris-Cl (pH 6.5), 10% sodium dodecyl sulfate, and 100 mM β-mercaptoethanol for 10 minutes at room temperature and reprobed with a monoclonal antibody against total MARCKS protein (Clone # 2F12; Upstate, Charlottesville, VA) to verify equal loading.

Transient Transfection of PKC constructs

Transient transfection of vectors overexpressing wild-type or dominant-negative PKCδ protein in HBE-1 cells was performed using the FuGene 6® transfection reagent (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol. The pEGFP-N1 vectors containing a wild type PKCδ cDNA (22) and a dominant negative PKCδ

mutant cDNA (Lysine (AAG)→Arginine (AGG) mutation, position 376) (23) were generously provided by Dr. Arti Shukla (University of Vermont) and Dr. Peter Blumberg (National Cancer Institute). The K376→R mutation in the ATP binding site of the catalytic domain has been demonstrated previously to inhibit PKCδ kinase activity (24). Briefly, HBE1 cells grown in air/liquid interface were dissociated by Versene solution (Invitrogen, Carlsbad, CA) and re-seeded in 12-well culture plates at 1×10^5 cells/cm^2. After overnight incubation, cells were transfected with the pEGFP-N1 vector alone or the pEGFP-N1 vector containing either a wild-type or dominant negative PKCδ cDNA (K376R). Cells were subsequently cultured for 48 hrs to allow for detectable protein expression. Transfection was confirmed by fluorescence microscopy and assessment of the expression of GFP-tagged PKCδ by Western blot analysis using monoclonal antibodies against PKCδ or GFP (Cell Signaling Technology, Inc., Danvers, MA).

Cytotoxicity Assay

All treatments used were tested for cytotoxicity using a CytoTox 96® Non-Radioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The data were expressed as the ratio of released lactate dehydrogenase to total lactate dehydrogenase. Released lactate dehydrogenase never exceeded 10% of total lactate dehydrogenase (data not shown) with any of the treatments.

Statistical Analysis

Data were expressed as the ratio of treatment to the corresponding vehicle (DMSO or water) control. Results were evaluated using one-way analysis of variance with a Bonferroni

post-test correction for multiple comparisons (25). A p-value of less than 0.05 was considered significant.

RESULTS

Effect of Rottlerin on PMA-induced mucin secretion and MARCKS phosphorylation in NHBE cells

To determine whether or not PKCδ is an important regulatory molecule in mucin secretion, we investigated the effect of rottlerin, a PKCδ specific inhibitor, on PMA-induced mucin secretion in well-differentiated NHBE cells. As illustrated in Figure 1, pretreatment of cells with rottlerin attenuated PMA-induced mucin secretion. PMA at 100nM provoked translocation of PKCδ from cytosol to membrane in these cells (Figure 1A). Inhibition of PKCδ activity by pretreatment of cells with 15μM rottlerin for 20 min significantly attenuated PMA-induced mucin secretion (by ~ 70%) as compared to control (pretreatment with PMA alone; Figure 1B). Phosphorylation of MARCKS mediated by PMA in the presence or absence of 15μM rottlerin was analyzed by Western blot analysis. HNE, previously shown to stimulate mucin secretion in NHBE cells via a rottlerin-inhibitable mechanism (11) was used as an additional control. As shown in Fig. 1C, both PMA- and HNE- induced phosphorylation of MARCKS were decreased by pretreatment with rottlerin.

Bryostatin 1 provokes mucin secretion and MARCKS phosphorylation in NHBE cells

Bryostatin 1, a PKCδ/ε activator, was used to investigate further the relationship between PKCδ and mucin secretion in NHBE cells. As illustrated in Figure 2, exposure of NHBE to bryostatin 1 over a range of concentrations (10~1000nM) for 15 min resulted in translocation of PKCδ from cytosol to membrane in response to all concentrations tested

(Figure 2A). Mucin secretion also was increased significantly by bryostatin 1 in a concentration-dependent manner (Figure 2B). As shown in Figure 2C, phosphorylation of MARCKS also was induced in these cells in response to bryostatin 1 (from 10nM to 1000nM) with maximal phosphorylation at 100nM. None of these treatments induced a cytotoxicity as measured by LDH release assay (data not shown).

PKC activation stimulates mucin secretion in HBE-1 cells

To investigate further a role for PKCδ as a regulator of mucin secretion using molecular manipulation of PKCδ activity, the HBE-1 cell line was utilized. As illustrated in Figure 3, exposure of HBE-1 cells to 500 nM PMA for 15 minutes significantly increased mucin secretion (by ~ 1.7-fold compared to medium control) and also induced phosphorylation of MARCKS.

PKCδ regulates mucin secretion in airway epithelial cells

As illustrated in Figure 4, suppression of PKCδ activity in HBE-1 cells by transfection of the dominant negative PKCδ construct attenuated PMA-induced mucin secretion. Transient transfection of the PKCδ construct into HBE-1 cells was confirmed by fluorescence microscopy and Western blot analysis. After 48 hrs transfection, Green Fluorescence Protein (GFP) expressed in the transfected cells was detected with a fluorescence microscope prior to PMA exposure. After exposure to PMA, cells were lysed to detect expression of PKCδ protein fused with GFP via Western blot analysis using PKCδ and GFP antibodies.

Mucin secretion by the transfected HBE-1 cells was stimulated by exposure to PMA at 500nM. Transfection of HBE-1 cells with the dominant negative construct (pEGFP-N1/PKCδ^{K376R}) resulted in significant reduction of PMA-induced mucin secretion (~ 45%) whereas cells transfected with the wild type PKCδ construct (pEGFP-N1/PKCδ) showed significant enhancement of PMA-induced mucin secretion (~ 40%) compared to control cells transfected with no DNA or an empty vector (pEGFP-N1). Phosphorylation of MARCKS was decreased in response to PMA in the HBE-1 cells transfected with the dominant negative construct (pEGFP-N1/PKCδ^{K376R}), but increased in cells transfected with the wild type PKCδ construct (pEGFP-N1/PKCδ).

DISCUSSION

Airway mucus is a heterogeneous mixture of water, mucins, enzymes and anti-enzymes, salts, endogenous and exogenous bacterial agents, organic compounds, and cell debris (reviwed in(26, 27)). Airway injury or disease often leads to deleterious alterations in mucin production and/or in the content of mucin in the mucus. Increased amounts of mucin have been consistently reported in patients with asthma and chronic inflammatory diseases (2, 28-30). Despite recognition of mucin hypersecretion as a major pathophysiological feature in many diseases, little is known about the actual intracellular mechanisms that regulate secretion under normal or pathological conditions

Previously, we reported that mucin secretion and PKCδ activity are both increased in normal human airway epithelial cells in response to human neutrophil elastase (HNE). In addition, pretreatment of cells with a PKCδ specific inhibitor resulted in the attenuation of HNE-induced mucin secretion (11). PKC is a Serine/Threonine Kinase, containing three types of isoforms: conventional (α, β_1, β_2, γ); novel (δ, ε, η, θ, μ); and atypical (ζ, ι/λ). They are categorized by cofactors which activate them. The involvement of PKC activation during airway mucin secretion in response to various secretagogues has been reported (9, 31-33). However, involvement of any specific isoform has not been fully characterized. Among other PKC isoforms, PKCδ isoform has specifically been proposed to mediate mucin secretion in response to purinergic agonists (ATPγS) and PMA in mouse goblet cells (4). In addition, our previous report also suggested that the PKCδ isoform serves as a regulatory molecule in airway mucin secretion (11).

Involvement of PKCδ has been indicated in the secretory pathways of other cell types. In human platelets, PKCδ is activated in response to protease-activated receptor (PAR) agonist peptides (SFLLRN and AYPGKF), leading to dense granule release (8), a process attenuated by the PKCδ inhibitor, rottlerin, but not by Go6976 (a conventional PKC inhibitor). Ishikawa et. al. demonstrated that carbachol-stimulated insulin secretion in rat pancreatic islets was associated with translocation of PKCδ (15). In that study, carbachol-stimulated insulin secretion was not reduced by Go6976, but was significantly suppressed by an ambiguous PKC inhibitor, chelerythrine, suggesting that a novel PKC isoform, δ or ε, might play a regulatory role in carbachol-stimulated insulin secretion. Cho et al. demonstrated that PKCδ activation was involved in antigen induced-mast cell degranulation, which was subsequently inhibited by rottlerin or transfection of a dominant negative mutant of PKCδ (17). In contrast, Leitges et al. who reported that PKCδ is a negative regulator of antigen induced-mast cell degranulation (16).

Here we demonstrate that increased mucin secretion in response to PMA is significantly attenuated by rottlerin (Fig. 1), suggesting that the mucin secretory response involves activation of PKCδ. Additionally, phosphorylation of MARCKS, a well-known PKC substrate, was significantly reduced by rottlerin prior to exposure of NHBE cells to two secretagogues, PMA and HNE. Previously, we demonstrated that MARCKS protein is a key molecule in the mucin secretory pathway (9, 34). MARCKS is phosphorylated by PKC at serine residues 152, 156, and 163 located in its phosphorylation site domain (PSD) (13). Although MARCKS is a prominent PKC substrate, its phosphorylation is differentially regulated depending on the specificity of each PKC isoform. Hagret and colleagues

demonstrated the differential efficiency of each PKC isoform to phosphorylate MARCKS by performing PKC phosphorylation assays *in vitro* (13). It was shown that PKC's α, β1, β2, γ, δ, ε, but not ζ, can phosphorylate MARCKS, with PKCδ being the most potent isoform followed by ε and β1, as determined by Vmax/Km catalytic efficiency ratios (13). This finding was supported by results of studies by Fujise et al. which showed that PKCδ indeed has the highest affinity for MARCKS among the isoforms (12).

In the present study, we demonstrate that mucin secretion correlates well with PKCδ-mediated phosphorylation of MARCKS in both NHBE and HBE-1 cells. Two mucin secretagogues (HNE and PMA) (Figure 1) and bryostatin 1 (a PKCδ activator) (Figure 2) induce mucin secretion and phosphorylation of MARCKS. In addition, pretreatment with rottlerin decreases phosphorylation of MARCKS in response to HNE or PMA, and also attenuates mucin secretion.

Since the above-described results were derived from experiments using pharmacologic activation/inhibition, the potential influence of non-specific effects on the measured outcomes cannot be determined. Therefore, transfection of HBE-1 cells with a dominant negative construct of PKCδ allowed targeted investigation of PKCδ mediation of the mucin secretion pathway. Competition of endogenous PKCδ kinase activity with overexpression of the dominant negative construct resulted in significant reduction of both PMA induced mucin secretion and phosphorylation of MARCKS (Figure 4). Additionally, overexpression of wild type PKCδ significantly enhanced both mucin secretion and phosphorylation of MARCKS in PMA-stimulated HBE-1 cells. Collectively, the results

suggest that active PKCδ, via phosphorylation of MARCKS, appears to be a key regulatory molecule in the mucin secretion pathway in airway epithelial cells *in vitro*.

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FIGURE LEGENDS

Figure 1. Rottlerin, a PKCδ specific inhibitor, attenuates PMA-induced mucin secretion and phosphorylation of MARCKS in well-differentiated NHBE cells. To investigate the effect of rottlerin on PMA-induced mucin secretion and phosphorylation of MARCKS mediated by PMA or HNE, cells were pre-incubated with 15μM rottlerin (or DMSO control media) for 20 min prior to addition of 100nM PMA (B, C) or 500nM human neutrophil elastase (C). (A) PKCδ translocates from cytosol to membrane in response to PMA (100nM) for 15 min. After detecting PKCδ, the PVDF membrane was stripped and reprobed with antibodies against α-Tubulin and E-Cadherin which were used as controls to detect the cytosolic and the membrane fraction, respectively. (B) Rottlerin significantly attenuates PMA-induced mucin secretion in NHBE cells. * = significantly different from vehicle control ($p<0.05$); † = significantly different from PMA alone ($p<0.05$). Data are presented as mean ± SEM ($n = 4$). (C) Rottlerin attenuates phosphorylation of MARCKS in NHBE cells exposed to two different mucin secretagogues, HNE (500 nM) or PMA (100 nM). Phosphorylated MARCKS was detected by Western blot using a specific antibody against phosphorylated MARCKS at Serine 152/156. After detecting phosphorylated MARCKS, the PVDF membrane was stripped and reprobed with an antibody against total MARCKS protein as a loading control for each lane. Blots are representative of two replicate experiments.

Figure 2. Effect of bryostatin 1, a PKCδ activator, on mucin secretion in well-differentiated NHBE cells. NHBE cells were exposed to bryostatin 1 over a range of concentrations from 1

to 1000nM for 15min. (A) PKCδ translocates from cytosol to membrane in response to bryostatin 1. α-Tubulin and E-Cadherin were used as control for the cytosolic and the membrane fraction, respectively. Blots are representative of three replicate experiments. (B) Bryostatin 1 provokes mucin secretion in a concentration-dependent manner in NHBE cells. Significantly different from vehicle control (*=$p<0.05$), (†=$p<0.001$), (‡=$p<0.005$). Data are presented as mean ± SEM (n =4). (C) Phosphorylation of MARCKS is induced by bryostatin 1 in a concentration-dependent manner. Blots are representative of three replicate experiments.

Figure 3. HBE-1 cells secrete mucin in response to PKC activation. HBE-1 cells maintained in air/liquid interface were exposed to 100 or 500nM PMA for 15min. Mucin secretion and phosphorylation of MARCKS were assessed by ELISA and Western blot analysis, respectively. (A) Mucin secretion is significantly enhanced by PMA at 500nM (but not at 100nM) in HBE-1 cells. * = significantly different from vehicle control ($p<0.05$). Data are presented as mean ± SEM (n = 4). (B) Phosphorylation of MARCKS is increased by exposure of HBE-1 cells to 500nM PMA. Blots are representative of three replicate experiments.

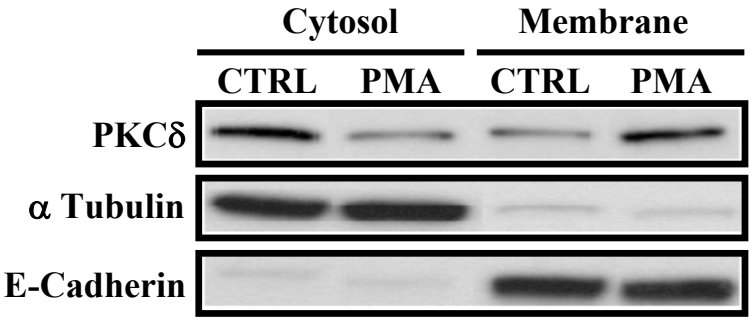
Figure 4. Transient transfection of HBE-1 cells with a dominant negative PKCδ construct results in reduction of mucin hypersecretion. HBE-1 cells were transiently transfected with either the empty vector (pEGFP-N1), the wild type PKCδ construct (pEGFP-N1/PKCδ) or the dominant negative construct (pEGFP-N1/PKCδ^{K376R}) using the FuGene 6® transfection

reagent as described above. (A) After 48 hrs transfection, cells were exposed to 500nM PMA (Lane 2~6) or vehicle control (Lane 1) for 15min, media were collected and mucin secretion assessed by ELISA. Significantly different from vehicle control (*=$p<0.05$), (**=$p<0.001$); †, ‡ = significantly different from cells transfected with no DNA or empty vector and exposed to PMA ($p<0.05$); §=significantly different from cells transfected with the pEGFP-N1/PKCδ^{K376R} and exposed to PMA ($P<0.05$). Data are presented as mean ± SEM (n = 3~4). (B) After collecting media from transfected HBE-1 cells exposed to PMA, cell lysates were analyzed by Western blot for phosphorylation of MARCKS at Serine 152/156 residues. Decreased phosphorylation of MARCKS in cells transfected with the dnPKCδ construct is apparent. Blots are representative of three replicate experiments.

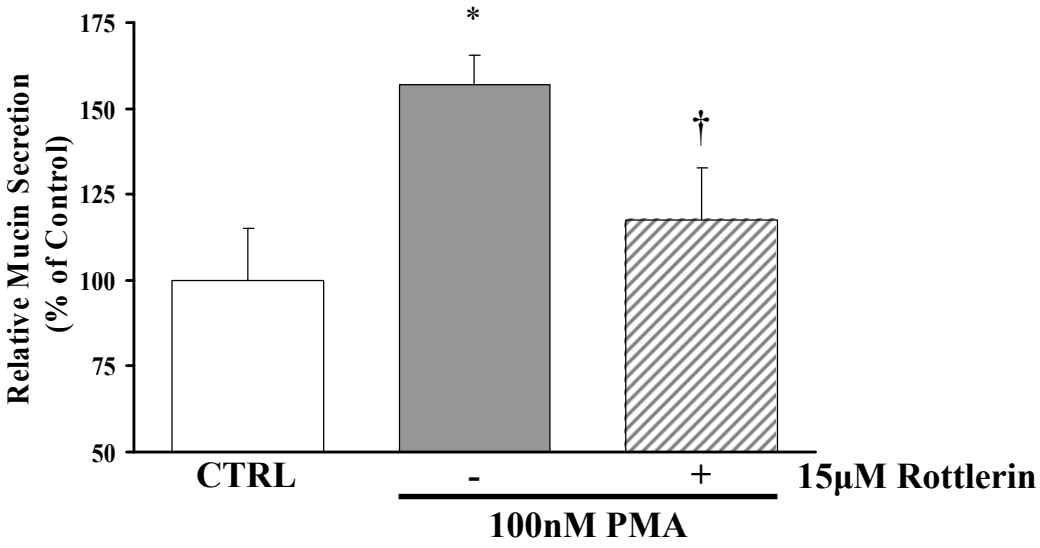
Constitutive, non-stimulated mucin secretion, as well as phosphorylation of MARCKS, was similar in non-transfected control cells, cells treated with transfection reagent alone, cells transfected with the dominant negative, or the wild type PKCδ construct (data not shown).

Lane 1= control medium; Lane 2 = PMA + control medium; Lane 3 = PMA + mock transfection; Lane 4 = PMA + empty vector (pEGFP-N1); Lane 5 = PMA + wtPKCδ (pEGFP-N1/PKCδ); Lane 6 = PMA + dnPKCδ (pEGFP-N1/PKCδ^{K376R}).

A



B



C

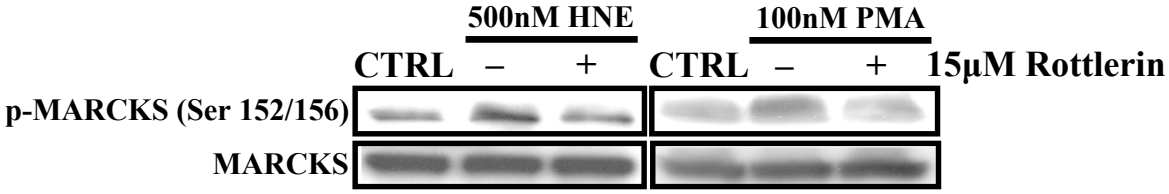
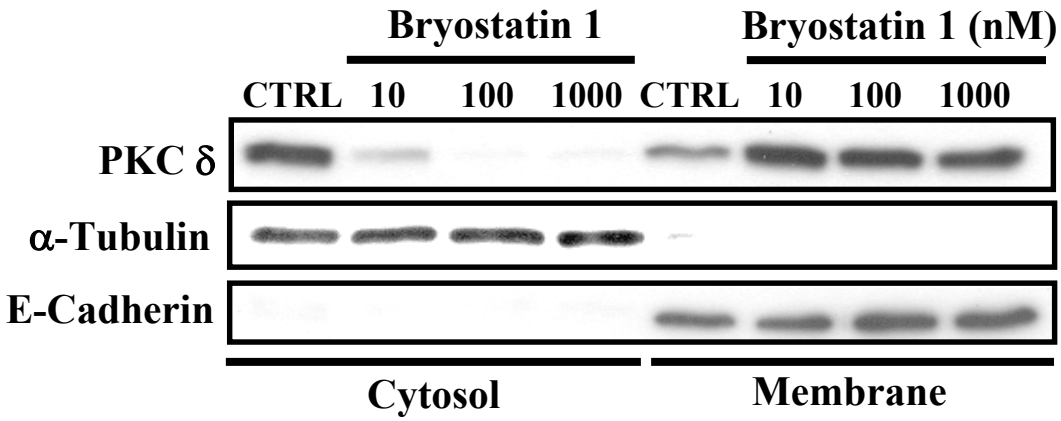


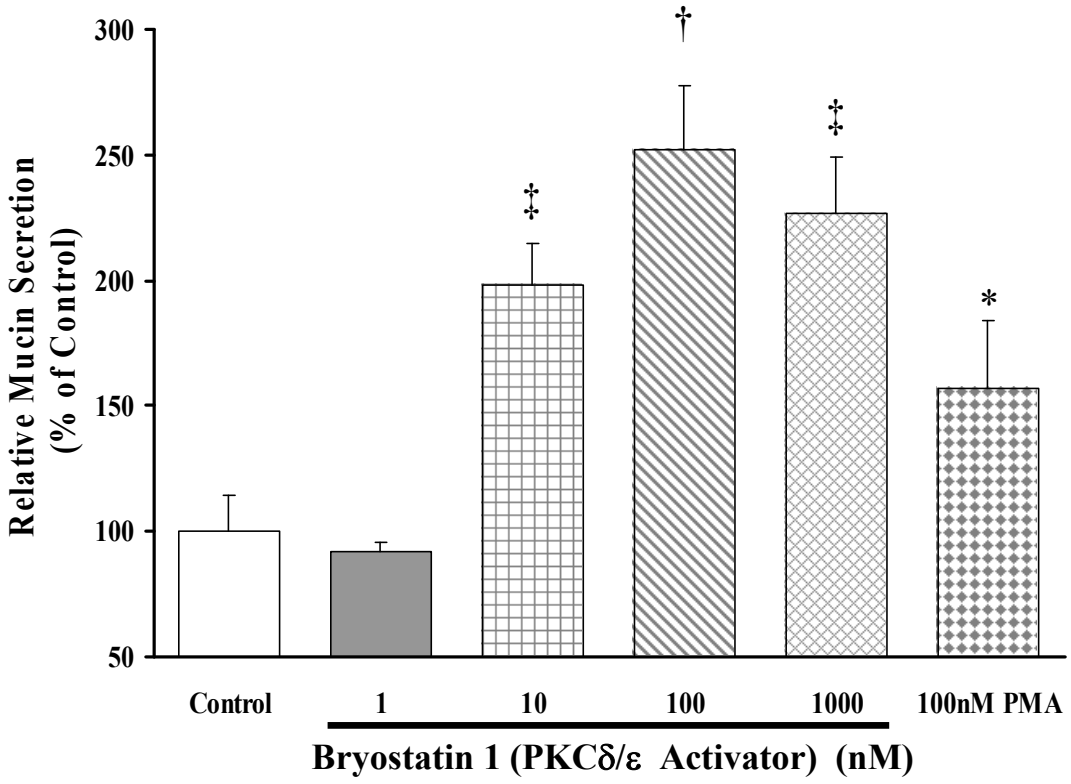
Figure 1. Rottlerin, a PKCδ specific inhibitor, attenuates PMA-induced mucin secretion and phosphorylation of MARCKS in well-differentiated NHBE cells

Figure 2. Effect of bryostatin 1, a PKCδ activator, on mucin secretion in well-differentiated NHBE cells

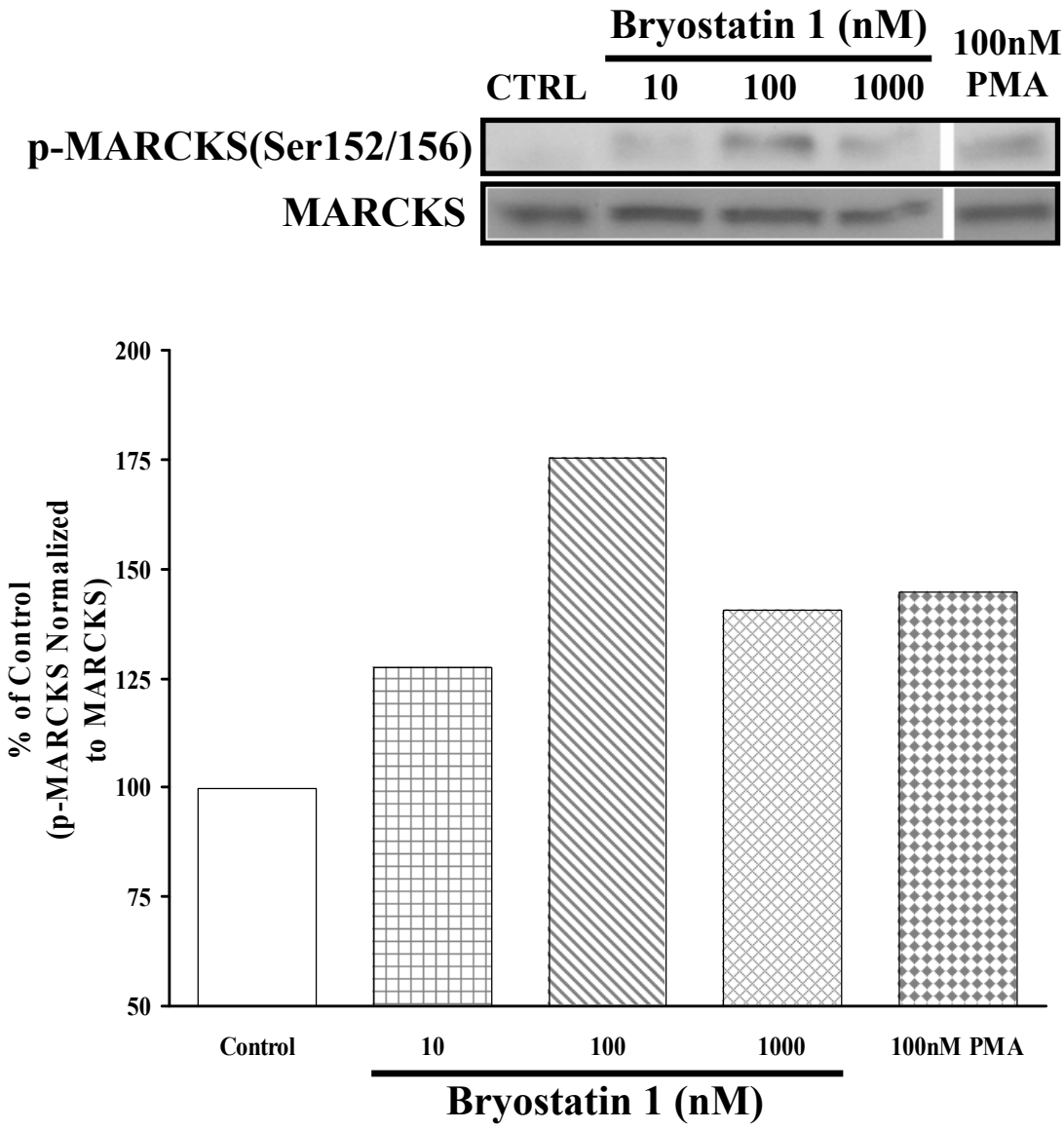
A


Bryostatin 1
Bryostatin 1 (nM)
CTRL 10 100 1000 CTRL 10 100 1000
PKC δ
α-Tubulin
E-Cadherin
Cytosol
Membrane

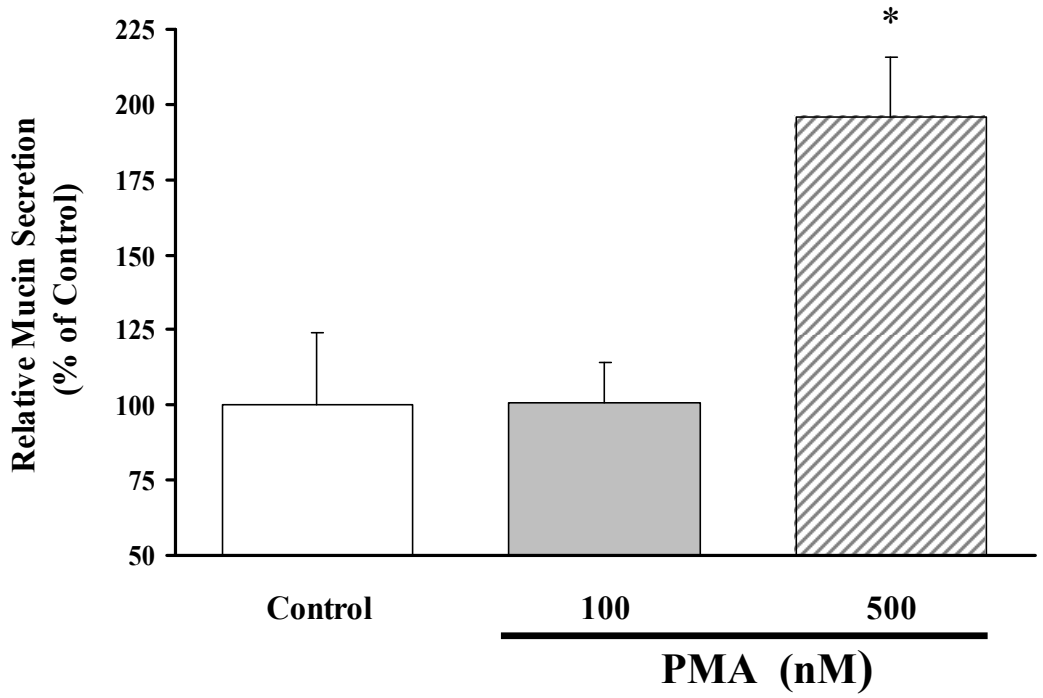
B


Relative Mucin Secretion (% of Control)
300
250
200
150
100
50
†
‡
‡
*
Control
1
10
100
1000
100nM PMA
Bryostatin 1 (PKCδ/ε Activator) (nM)

C


Bryostatin 1 (nM)
100nM
CTRL
10
100
1000
PMA
p-MARCKS(Ser152/156)
MARCKS
% of Control
(p-MARCKS Normalized
to MARCKS)
200
175
150
125
100
75
50
Control
10
100
1000
100nM PMA
Bryostatin 1 (nM)

A



B

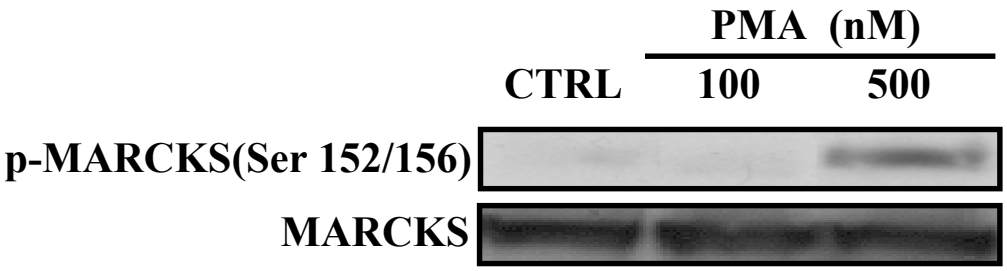
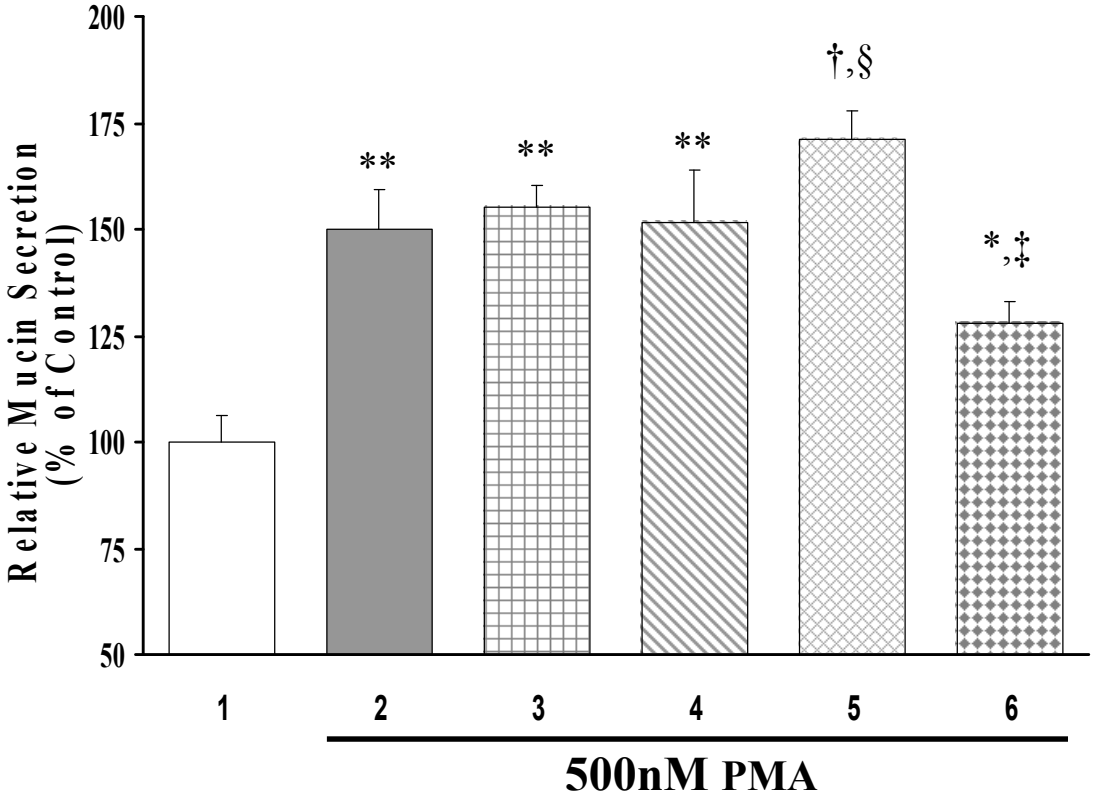
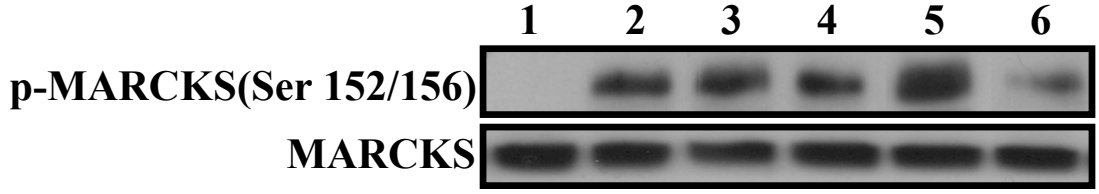


Figure 3. HBE-1 cells secrete mucin in response to PKC activation

A



B



For A and B
1. Media control without PMA
2. Media control with PMA
3. MOCK transfection (FuGene® 6) with PMA
4. 0.5µg empty vector (pEGFP-N1) with PMA
5. 0.5µg wtPKCδ (pEGFP-N1/PKCδ) with PMA
6. 0.5µg dnPKCδ (pEGFP-N1/PKCδ^{K376R}) with PMA

Figure 4. Transient transfection of HBE-1 cells with a dominant negative PKCδ construct results in reduction of mucin hypersecretion

OVERALL DISCUSSION AND POSSIBLE FUTURE DIRECTIONS

The studies presented in this dissertation investigated intracellular mechanisms of mucin secretion using well-differentiated normal human bronchial epithelial (NHBE) cells and a virally transformed human bronchial cell line (HBE-1) *in vitro*. Primary NHBE cells utilized in this dissertation differentiated into mucin secreting cells and ciliated cells, which mimics the *in vivo* condition (173). This *in vitro* approach allowed investigation of the intracellular mucin secretory pathway with various stimuli such as HNE, PMA, and bryostatin and inhibitors including calphostin C, bisindoylmaleimide 1, and rottlerin. Additionally, the functional role of PKCδ in mucin hypersecretion was investigated with the transient transfection of a dominant negative PKCδ in the HBE-1 cell line.

Five novel findings were demonstrated in manuscripts one and two:

1) HNE provokes a rapid and significant increase in release of mucin from NHBE cells.
2) Major secreted mucins, MUC5AC and MUC5B, are released in response to HNE in NHBE cells.
3) HNE-induced mucin secretion involves activation of PKCδ in NHBE cells.
4) Activation of the PKCδ isoform mediates mucin hypersecretion in airway epithelial cells *in vitro*.
5) Phosphorylation of MARCKS is involved in PKCδ-mediated mucin secretion of airway epithelial cells *in vitro*.

Novel findings 1, 2, and 3 are demonstrated in the first manuscript. Mucin hypersecretion and neutrophil-dominant inflammation are major pathophysiological features in COPD. A mechanistic link between neutrophil recruitment and mucin hypersecretion has been suggested (58, 59). In addition, the role of human neutrophil elastase (HNE) in secretion of activated azurophilic mucin granules has been demonstrated using *in vitro* and *in vivo* models (62, 78, 80, 82, 83). However, no direct evidence existed demonstrating the mechanism by which HNE mediates mucin secretion. We found that mucin secretion is rapidly increased in response to 0.01-1.0uM HNE, which is a far lower concentration than can be found in sputum from the patients with cystic fibrosis and chronic bronchitis (77-80). Furthermore, it was found that HNE induced secretion of the major gel-forming mucins, MUC5AC and MUC5B. MUC5AC and MUC5B are also the major mucin glycoproteins increased in the sputum of patients with chronic inflammatory airway disease. Therefore, our *in vitro* findings are relevant to clinical indications of chronic airway disease.

The specific mechanisms that release intracellular mucins via extracellular HNE exposure are largely unknown. Interestingly, HNE induces production of interlukin-8 (IL-8) and monocyte chemotractant protein-1 (MCP-1) via a protease activated receptor (PAR) -2-dependent pathway in HGF cells (174). PAR is a G-protein coupled receptor and is activated by cleavage of its N-terminal region by proteases such as elastase, trypsin, thrombin, and cathepsin G (175). Uehara et al. demonstrated that HNE- and PAR-2 agonist-mediated pro-inflammatory cytokine inductions are completely inhibited by the phospholipase C inhibitor (U73122), suggesting that activation of PLC is the downstream target of PAR-2. In general, PLC can hydrolyze phosphatidyl inositol 4, 5-biphosphate to form diacylglycerol and inositol 1, 4, 5-triphosphate which are the cofactors for the PKC activation. In addition, Kawaguchi et

al. provided direct evidence that HNE rapidly induces production of diacyl glycerol and inositol in smooth muscle cells (176). We can, therefore, suggest possible mechanisms in which HNE activates PKC via sequential activation of PAR-2 and PLC. It has been demonstrated that a PAR-2 agonist peptide elicits mucus secretion in injured gastrointestinal tract in a rat model (177). However, this peptide failed to increase mucin gene expression (MUC2 and MUC5AC) in airway epithelial cells (178). Production of pro-inflammatory cytokines is increased by HNE-mediated PAR-2 activation in non-epithelial cells (174), but Dulon and colleagues demonstrated that HNE inactivates PAR-2 in airway epithelial cells (179). These seemingly contradictory roles of HNE activation of PAR-2 are possibly due to cell or tissue specific differences; however these important differences have not been thoroughly investigated.

Interestingly, Suzuki and colleagues demonstrated that HNE, as well as a PAR-1 agonist peptide, induce DNA fragmentation after 4 hrs exposure in lung epithelial cells, while treatment with a selective inhibitor of PAR-1 (SCH79797) and transfection of PAR-1 siRNA significantly reduces HNE-mediated apoptosis (180). Their results highlight the effort to find possible HNE receptors on airway epithelial cells. Therefore, HNE may potentially activate PAR-1, rather than PAR-2, in the mucin secretory pathway of airway epithelial cells via PAR-2-mediated signaling pathways similar to those in other cell types.

We previously demonstrated that MARCKS is an important regulator of mucin secretion and both its phosphorylation and de-phosphorylation are involved in mucin hypersecretion in response to the PKC-activator, PMA (154). In the first manuscript, it was demonstrated that HNE mediated phosphorylation of MARCKS via activation of PKC and

PKCδ. To further support this finding, HNE-induced mucin secretion was attenuated by the inhibition of PKCδ activity using a specific chemical inhibitor, rottlerin.

Novel findings 4 and 5, demonstrated in the second manuscript, further clarify the mechanism by which PKCδ mediates mucin hypersecretion in our *in vitro* models. We found that two well known secretagogues, HNE and PMA, mediated the phosphorylation of MARCKS in a PKCδ-dependent manner. Both secretagogues activated PKCδ and phosphorylation of MARCKS in NHBE cells. Specific inhibition of PKCδ activity using rottlerin reduced both HNE- and PMA-mediated mucin secretion as well as MARCKS phosphorylation. Bryostatin 1, a PKCδ specific activator, provoked mucin hypersecretion and phosphorylation of MARCKS in NHBE cells *in vitro*. In addition, the transient transfection of catalytically inactive PKCδ into HBE-1 cells significantly reduced PMA-induced phosphorylation of MARCKS and (resultant) mucin secretion. We don't preclude the possibility that other isoforms of PKC could be involved in the secretagogue-mediated mucin secretory pathway. In the second manuscript, we transiently transfected a catalytically inactive PKCδ construct to suppress endogenous PKCδ activity, however, it may not completely exclude any possibility of inhibition of other PKC isoforms due to their structural similarity. Therefore, additional experiments using dominant negative constructs of other PKC isoforms, for example, PKCα or PKCε, would strengthen the conclusion of a specific role of PKCδ in airway mucin secretion. In addition, when assessing mucin secretion from the cells transfected with a construct having a deletion mutation of the phosphorylation site domain followed by PKCδ activation, if there is a decrease it would strongly support that phosphorylation of MARCKS mediated by PKCδ regulates mucin secretion in airway

epithelial cells. In our study, despite the low transfection efficiency of the catalytically inactive PKCδ construct into HBE-1 cells, mucin secretion as well as phosphorylation of MARCKS was reduced. This might be explained by exclusive localization of the dominant negative PKCδ^{K376R} in the membrane fraction (181), which leads to effective inhibition of endogenous PKCδ activity by enhanced availability of PKCδ^{K376R} to bind to MARCKS localized in the membrane fraction. Another possible reason is the heterogeneity of epithelial cell types (goblet and ciliated type cells) present in the transfected cells, though it remains to be verified.

Our novel findings have highlighted the importance of PKC-dependent phosphorylation in the mucin secretory pathway of bronchial epithelial cells. It is reasonable to assume that PKC-dependent phosphorylation of these proteins initiates exocytosis in many secretory cell types. Indeed, PKC activation with phorbol esters augments secretory responses in adrenal chromaffin cells, hippocampal neurons, and synaptosomes (reviewed in(182)). Interestingly, various cytoplasmic proteins found to be involved in exocytosis are phosphorylated by PKC (169). MARCKS, therefore, is not the only phosphorylation target of PKC during exocytosis. Upon termination of granule movement through the cytoplasm, the granule membrane fuses with the plasma membrane and releases its contents. Membrane fusion is mediated by the SNARE complex, which is controlled by protein-protein interactions. The affinity of these interactions appears to be dependent on the phosphorylation state of the SNARE assembly. Therefore, phosphorylation of various granule-associated proteins appears to regulate exocytosis. However, direct mechanisms

regulating the phosphorylation of the SNARE complex during exocytosis have not been elucidated. It is also unclear which PKC isoform(s), if any, are involved in this process.

Therefore, future studies will focus on:

1) Whether or not the activation of PAR-1 is involved in mucin secretion in response to HNE in NHBE cells. We will investigate the effect of a PAR-1 agonist peptide on mucin secretion in airway epithelial cells *in vitro* to further elucidate the mechanism of HNE-induced mucin secretion.

2) Whether or not PKCδ has the specific role in airway mucin secretion and phosphorylation of MARCKS. As an extension of the second manuscript, dominant negative constructs of other PKC isoforms will be transfected to exclude any possibility of non-specific action of dominant negative PKCδ within cells. In addition, a MARCKS construct having a truncated phosphorylation site domain will be transfected into HBE-1 cells in combination with PKCδ activation by bryostatin 1. These approaches will strengthen our conclusion in the second manuscript that PKCδ regulates mucin secretion via phosphorylation of MARCKS.

3) Whether or not PKCε is involved in the mucin secretory pathway. In contrast to HNE-induced mucin hypersecretion, which involves activation of PKCδ only, PMA also induces PKCε activation. This suggests that there could be different PKC isoforms activated by different stimuli in airway epithelial cells. A possibility is that PKCε may be a regulator of PMA-induced mucin secretion by modulating events upstream of PKCδ activation. However, PKCε knockout mice appear to demonstrate no difference from wild-type in antigen-induced mast cell degranulation in bone marrow despite evidence of PKCε

translocation seen in the wild-type mice after antigen stimulation (183). I plan to investigate the role of PKCε in the mucin secretory pathway in response to PMA by overexpressing a dominant negative construct of PKCε in HBE-1 cells and assessing the secretory response.

4) Whether or not PKCδ mediates phosphorylation of other cytosolic proteins involved in exocytosis of granules. There is evidence that PKCδ may be the potential kinase regulating phosphorylation of granule-associated proteins. Chung et al. demonstrated that PKCδ phosphorylates syntaxin, which is a member of the SNARE complex, *in vitro* (171) Additionally, they showed that thrombin-mediated platelet secretion and phosphorylation of syntaxin are reduced by a general PKC inhibitor, but not by a conventional PKC inhibitor, suggesting a novel PKC isoform plays a role in thrombin-mediated platelet secretion by altered phosphorylation of syntaxin. In addition, other granule-associated proteins (Munc18, SNAP-25, and Rab3A) shown to be phosphorylated by PKC *in vitro*, (168, 169) may be potential substrates for PKCδ during exocytosis. Therefore, PKCδ may have phosphorylation targets other than MARCKS, including a number of granule-associated, docking and fusion proteins, as part of the exocytotic process.

Overall, we have demonstrated intracellular mechanisms of mucin secretion in response to well known secretagogues in airway epithelial cells *in vitro*. Two overall conclusions are derived. The first is that the PKCδ isoform appears to be an important regulator of agonist-induced mucin secretion in airway epithelial cells *in vitro*. Secondly, phosphorylation of MARCKS, mediated by PKCδ, correlates with airway mucin secretion *in vitro*. These findings add additional major pieces to the "puzzle" regarding the precise mechanisms and stimulatory pathways whereby MARCKS acts as a key regulator of airway

mucin secretion. There is little question that this work is highly relevant to translational medicine, as the more we can dissect the mechanism, the more potential therapeutic molecular targets can be discovered to treat diseases characterized by excess mucus in the airways.

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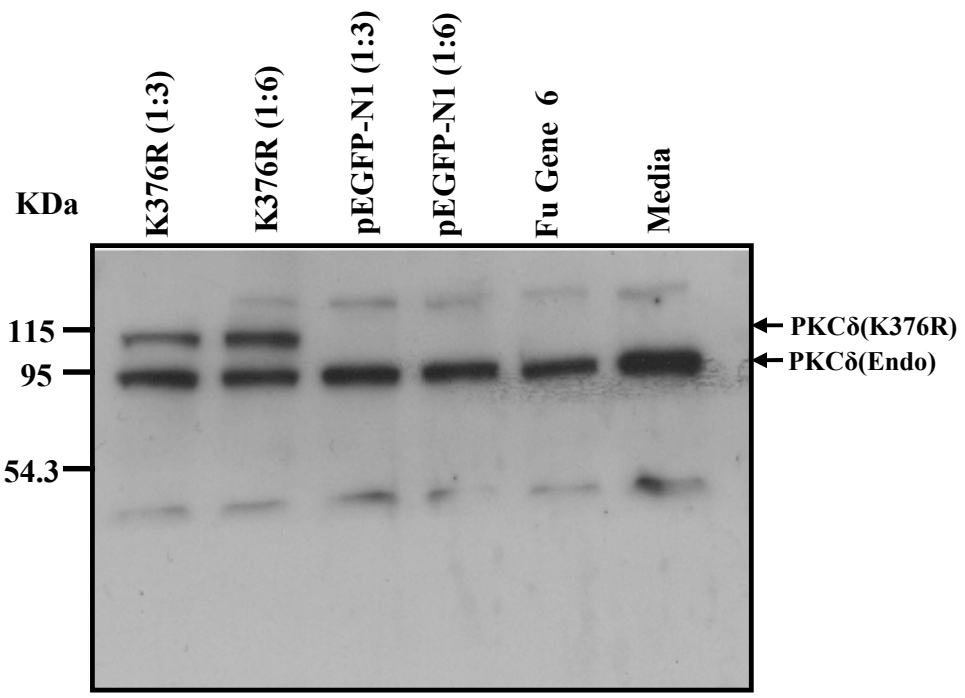
APPENDICES

A1. TRANSFECTION OF HBE-1 CELLS

A dominant negative PKCδ construct was overexpressed in HBE-1 cells to suppress endogenous PKCδ activity. As described in the second manuscript, HBE-1 cells were transiently transfected with the pEGFP-N1 construct expressing dominant negative PKCδ cDNA (K376R) using the FuGene 6® transfection reagent (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol. Different ratios of DNA to FuGene 6® reagent were tested, including 1:3 and 1:6 μg of DNA to μl of FuGene 6® reagent. Duration of transfection was determined by detecting overexpressed PKCδ at 24 hrs, 36hrs, and 48hrs post-transfection. Overexpressed PKCδ tagged with GFP protein was detected by Western blot analysis using monoclonal antibodies against PKCδ and GFP (Cell Signaling Technology, Inc., Danvers, MA). As negative controls, media alone, transfection reagent alone, and empty vector (pEGFP-N1) transfection were used. After testing various transfection conditions, one condition was picked for all future experiments. Optimally, a 1:6 ratio of DNA to FuGene 6® reagent and a 48 hr transfection duration resulted in highest transfection efficiency with lowest cytotoxicity. The amount of DNA used in the transfection was 0.5μg per 3.8 cm^2 cell surface area. As shown in Figure A1.a, the expression ratio of overexpressed PKCδ to endogenous PKCδ was 50:50 using optimal transfection conditions. PKCδ tagged with GFP protein was confirmed by fluorescence microscopy (Figure A1.b, A2). Transfection efficiency of the dominant negative construct was about 20%, as determined by quantification of GFP.

a)



b)

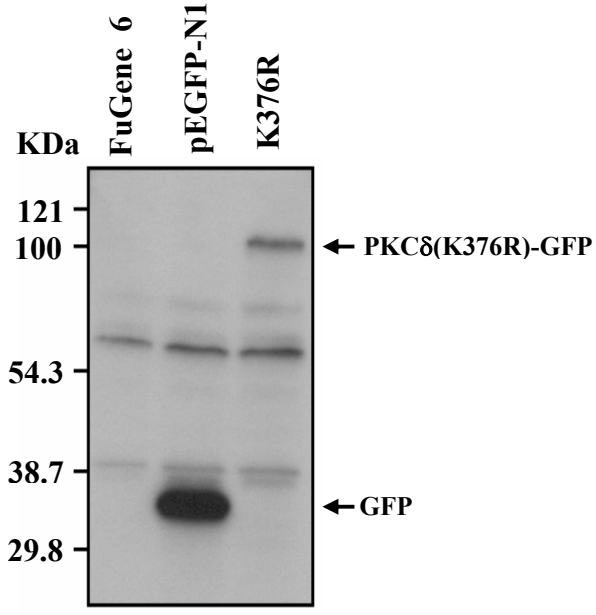
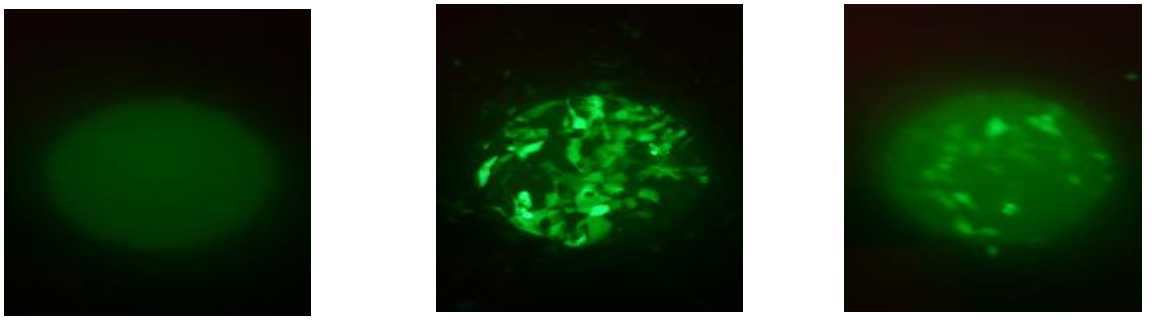


Figure A1-1. Transfection of overexpressed PKCδ tagged with GFP was detected by Western blot analysis



FuGene 6 pEGFP-N1 pEGFP-N1/PKCδ(K376R)

Figure A1-2. Transfection of overexpressed PKCδ tagged with GFP was detected by fluorescent microscopy

A2. EXPOSURE OF HBE-1 CELLS TO HNE

In the secod manuscript, mucin secretion was induced by 500nM PMA from HBE-1 cells after transfection with the dominant negative PKCδ construct. HNE was not appropriate to use as a secretagogue in the HBE-1 cells because HNE is able to stimulate mucin secretion and degrade secreted mucin in the epithelial cells at the same time (63). Unlike NHBE cells, exposure of HBE-1 cells to 500nM HNE appeared to cause degradation of the released mucin, as HBE-1 cells exhibit much lower amounts of secreted mucin. Exposure of various concentrations of HNE may be appropriate to determine the appropriate concentration of HNE to stimulate mucin secretion from HBE-1 cells by lowered enzymatic activity. However, enzymatic activity of HNE is still required for stimulation of mucin secretion from epithelial cells. Therefore, we chose PMA as an optimal secretagogue to induce mucin secretion from HBE-1 cells in the second manuscript.

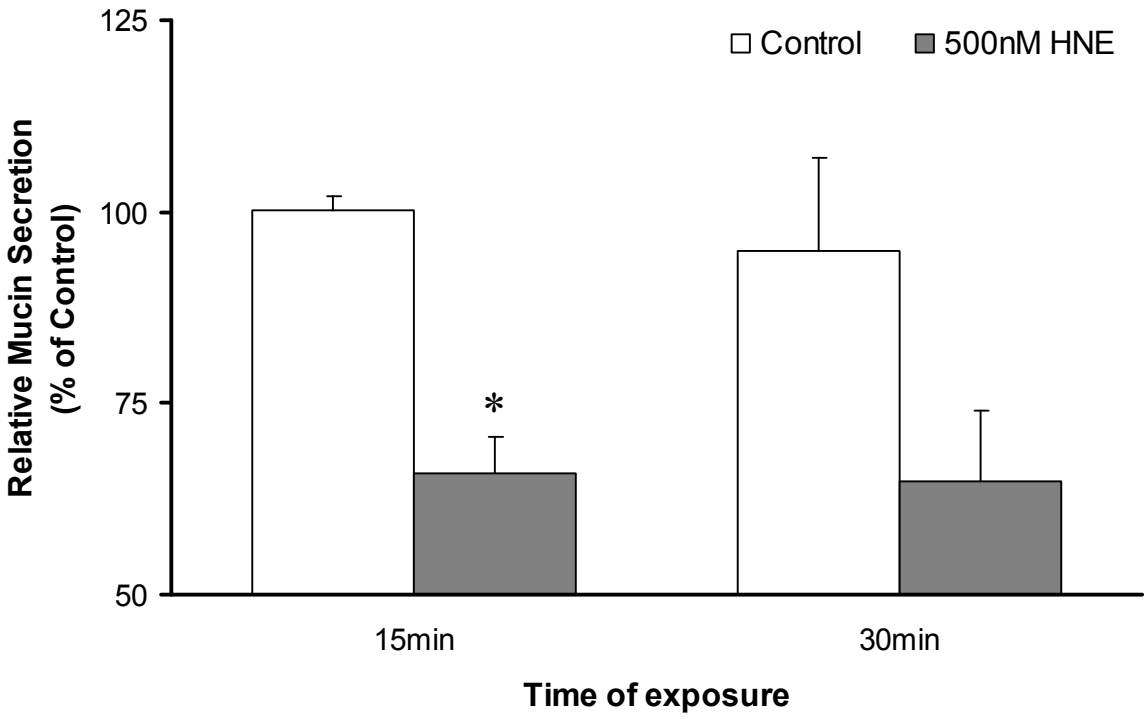


Figure A2. Effect of HNE on mucin secretion from HBE-1 cells

HBE-1 cells were exposed to 500nM HNE or vehicle control for 15min and 30min. At each time point, mucin secretion from HBE-1 cells treated with 500nM HNE was reduced compared to vehicle control. *, Significantly different from vehicle control ($P < 0.05$). Data are presented as mean ± SEM ($n = 3$).