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

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Mucin hypersecretion is a major pathological feature of many respiratory diseases, yet cellular mechanisms regulating secretion of mucin have not been fully elucidated. Previously, we reported that mucin hypersecretion induced by human neutrophil elastase involves activation of protein kinase C (PKC), specifically the δ -isoform (PKC δ). Here, we further investigated the role of PKC δ in mucin hypersecretion using both primary human bronchial epithelial cells and the human bronchial epithelial 1 cell line as *in vitro* model systems. Phorbol-12-myristate-13-acetate (PMA)-induced mucin hypersecretion was significantly attenuated by rottlerin, a PKC δ -selective inhibitor. Rottlerin also reduced PMA- or human neutrophil elastase-induced phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) protein in these cells. Both secretion and MARCKS phosphorylation were significantly enhanced by the PKC δ activator bryostatin 1. A dominant-negative PKC δ construct (pEGFP-N1/PKC δ ^{K376R}) transfected into human bronchial epithelial 1 cells significantly attenuated both PMA-induced mucin secretion and phosphorylation of MARCKS, whereas transfection of a wild-type construct increased PKC δ and enhanced mucin secretion and MARCKS phosphorylation. Similar transfections of a dominant-negative or wild-type PKC ϵ construct did not affect either mucin secretion or MARCKS phosphorylation. The results suggest that PKC δ plays an important role in mucin secretion by airway epithelium via regulation of MARCKS phosphorylation. (*Am J Pathol* 2007, 171:1822–1830; DOI: 10.2353/ajpath.2007.070318)

of the epithelial layer. Mucins, the glycoprotein component of mucus, constitute a family of large, highly glycosylated macromolecules that impart physical (aggregation, viscosity, viscoelasticity, and lubrication) and biological (protection) properties to mucus (reviewed in Ref. 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 chronic bronchitis, asthma, and cystic fibrosis, increasing morbidity and mortality in these patients (reviewed in Refs. 1 and 2). Mucin hypersecretion is potentiated by many pathophysiological mediators, such as bacterial proteinases and endotoxin, adenine and guanine nucleotides, cytokines, inflammatory mediators, and eicosanoids (reviewed in Ref. 3). 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,^{4,5} insulin,⁶ neurotransmitters,⁷ and platelet dense granules.⁸ Previously, we demonstrated that mucin secretion in airway epithelial cells is regulated by PKC via phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS).^{9,10} In addition, we demonstrated that mucin hypersecretion in human airway epithelial cells *in vitro* in response to human neutrophil elastase (HNE) appears to be mediated by the δ -isoform of PKC (PKC δ).¹¹ Not surprisingly, PKC δ , a novel PKC isoform, has a strong affinity for MARCKS and can phosphorylate MARCKS both *in vitro* and *in vivo*.^{12–14} Increasing evidence suggests that PKC δ mediates exocytotic secretion in several different cell types.^{4,8,15–17}

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Mucus produced by epithelium of respiratory, gastrointestinal, and reproductive tracts provides a barrier between the external environment and cellular components

Here, we further elucidate the role of PKC δ in the mucin secretory pathway in human airway epithelial cells *in vitro*. The mucin secretory response and phosphorylation of MARCKS were assessed after exposure of well differentiated normal human bronchial epithelial (NHBE) cells to phorbol-12-myristate-13-acetate (PMA), a general PKC activator, or bryostatin 1, a PKC δ/ϵ activator. In addition, we used the papilloma virus-transformed human bronchial epithelial 1 (HBE1) cell line for molecular manipulations. A dominant-negative PKC δ construct (K376R) transfected into HBE1 cells attenuated PMA-stimulated mucin secretion and MARCKS phosphorylation, whereas a similar dominant-negative PKC ϵ construct was without effect. The results indicate that PKC δ is a key isoform regulating airway mucin secretion, and the mechanism of its action appears to involve phosphorylation of MARCKS protein.

Materials and Methods

Culture of Bronchial Epithelial Cells

Primary NHBE cells purchased from Cambrex Bioscience (Walkersville, MD) were expanded and maintained in a humidified air/5% CO₂ incubator as described previously.^{9,11} Cells from at least three separate donors were used in these studies. Passage 31 or 32 of human papilloma virus-transformed human bronchial epithelial cells (HBE1)¹⁸ was seeded and maintained as described previously. Transient transfection of HBE1 cells was performed after 10 days of culture in air-liquid interface.

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 HBE1 cells were exposed to PMA (EMD Biosciences, La Jolla, CA) applied apically only. Cells were preincubated with the PKC δ -selective inhibitor, rottlerin (EMD Biosciences) for 20 minutes before PMA exposure. Initial stock solutions of rottlerin or PMA were prepared in dimethyl sulfoxide, kept at -20°C , and diluted in growth medium directly 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. 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, secreted mucin was collected as the baseline sample and quantified by sandwich enzyme-linked immunosorbent assay using the 17Q2 antibody (Covance Research Products, Berkeley, CA), a monoclonal antibody that reacts specifically with a carbohydrate epitope on human airway mucins.¹⁹ The 17Q2 an-

tibody was purified using an ImmunoPure(G) IgG purification kit (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol and then conjugated with alkaline phosphatase (EMD Biosciences). To account for variability between cultures and experiments, levels of mucin secretion were reported as percentage of the nontreated control. Actual values for mucin released by control cell cultures in these experiments ranged from 40 to 70 ng/ml in NHBE cells and 7 to 10 ng/ml in HBE1 cells.

Subcellular Localization of PKC Isoforms

Activation of PKC δ was assessed by subcellular fractionation following the protocol described by Kajstura et al²⁰ and subsequent Western blot analysis using a PKC δ -specific antibody (Cell Signaling Technology, Inc., Danvers, MA). Briefly, cells were washed with cold PBS and scraped into lysis buffer [20 mmol/L Tris-Cl (pH 7.5), 1 mmol/L ethylenediamine tetraacetic acid, 100 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L dithiothreitol, 1% (v/v) protease inhibitor cocktail, and phosphatase inhibitor cocktail (Sigma, St. Louis, MO)]. The lysate was then sonicated and pelleted at $20,000 \times g$ (Eppendorf 5417 centrifuge) for 40 minutes. The supernatant was collected and kept as the cytosolic fraction at -80°C until used. The remaining pellet was resuspended in lysis buffer containing 1% Triton X-100, sonicated, and centrifuged at $20,000 \times g$ for 40 minutes. The supernatant membrane fraction was stored at -80°C until analyzed by Western blot.

Western Blot Analysis

Total MARCKS, phosphorylated MARCKS, PKC δ , and PKC ϵ 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 $2\times$ SDS sample buffer [125 mmol/L Tris-Cl (pH 6.8), 25% glycerol, 4% SDS, 10% β -mercaptoethanol, and 0.04% bromophenol blue] for 10 minutes. Sample lysates (30 to 60 μg) were loaded on 10 or 12% SDS-polyacrylamide gels and then transferred to a polyvinylidene difluoride membrane (Schleicher & Schuell BioScience, Inc., Keene, NH) following electrophoresis. Polyvinylidene difluoride membranes were blocked with 5% nonfat milk and then 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 Health care Life Sciences, Piscataway, NJ) following the manufacturer's protocol. Amounts of specific proteins in bands were quantified 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 Biosciences, 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.).

After detection, the membrane was stripped in 62.5 mmol/L Tris-Cl (pH 6.5), 10% SDS, and 100 mmol/L β -mercaptoethanol for 10 minutes at room temperature and reprobed with a monoclonal antibody against total MARCKS protein (clone no. 2F12; Upstate, Charlottesville, VA) to verify equal loading.

Transient Transfection of PKC Constructs

Transient transfection of vectors overexpressing wild-type or dominant-negative PKC δ and PKC ϵ in HBE1 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 PKC δ cDNA²¹ and a dominant-negative PKC δ mutant cDNA [lysine (AAG) $\rightarrow$ arginine (AGG) mutation, position 376]²² were generously provided by Dr. Arti Shukla (University of Vermont, Burlington, VT) and Dr. Peter Blumberg (National Cancer Institute, Bethesda, MD). The K376 $\rightarrow$ R mutation in the ATP binding site of the catalytic domain has been demonstrated previously to inhibit PKC δ kinase activity.²³ Briefly, HBE1 cells grown in air/liquid interface were dissociated in versene solution (Invitrogen, Carlsbad, CA) and re-seeded in 12-well culture plates at a density of 1×10^5 cells/cm². 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 PKC δ cDNA (K376R). Isotype controls for the dominant-negative PKC δ consisted of transient transfection of both wild-type PKC ϵ as well as a catalytically inactive dominant-negative PKC ϵ (K437R) construct-tagged with hemagglutinin²⁴ (PKC ϵ constructs were kindly provided by Dr. Jae-Won Soh, University of Inha, Inha, Republic of Korea). Cells were subsequently cultured for 48 hours to allow for detectable protein expression. Transfection of PKC δ constructs was confirmed by fluorescent microscopy and assessment of expression of green fluorescence protein (GFP)-tagged PKC δ assessed by Western blot analysis using monoclonal antibodies against PKC δ or GFP (Cell Signaling Technology, Inc.). Transfection of PKC ϵ was confirmed by Western blot analysis using PKC ϵ and HA monoclonal antibodies (Covance Research Products).

Cytotoxicity Assay

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

Statistical Analysis

Data were expressed as the ratio of treatment to the corresponding vehicle (dimethyl sulfoxide or media) control. Results were evaluated using one-way analysis of

variance with Dunnett's test and a Bonferroni posttest 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 PKC δ is an important regulatory molecule in mucin secretion, we investigated the effect of rottlerin, a PKC δ -selective inhibitor, on PMA-induced mucin secretion in well differentiated NHBE cells. As illustrated in Figure 1A, 100 nmol/L PMA provoked translocation of PKC δ from cytosol to membrane in these cells. Inhibition of PKC δ activity by pretreatment of cells with rottlerin (1 to 10 μ mol/L) for 20 minutes significantly attenuated PMA-induced mucin secretion in a concentration-dependent manner (Figure 1B). Phosphorylation of MARCKS mediated by PMA in the presence or absence of 15 μ mol/L rottlerin was analyzed by Western blot. 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 Figure 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 PKC δ/ϵ activator, was used to investigate further the relationship between PKC δ and mucin secretion in NHBE cells. A naturally occurring marine invertebrate-derived cyclic lactone, Bryostatin 1, causes rapid activation of PKC δ and prolonged protection of PKC δ against ubiquitination and proteolysis.^{26,27} Bryostatin 1 interacts with the diacylglycerol binding site on PKC,²⁸ but its complete mode of action has not been fully elucidated, and it can affect PKCs in a cell-type specific manner.²⁹ As illustrated in Figure 2, exposure of NHBE cells to bryostatin 1 over a range of concentrations (10 to 1000 nmol/L) for 15 minutes resulted in translocation of PKC δ from cytosol to membrane in response to all concentrations tested (Figure 2A). Mucin secretion was also significantly increased by bryostatin 1, with maximal stimulation at 100 nmol/L (Figure 2B). As illustrated in Figure 2C, phosphorylation of MARCKS also was induced in these cells in response to bryostatin 1 (from 10 to 1000 nmol/L) with maximal phosphorylation at 100 nmol/L. None of these treatments induced cytotoxicity as measured by lactate dehydrogenase release assay (data not shown).

PKC Activation Stimulates Mucin Secretion in HBE1 Cells

To investigate further a role for PKC δ as a regulator of mucin secretion using molecular manipulation of PKC δ

Figure 1. Rottlerin, a selective PKC δ inhibitor, attenuates PMA-induced mucin secretion and phosphorylation of MARCKS in well differentiated NHBE cells. Cells were preincubated with 15 μ M/L rottlerin (or dimethyl sulfoxide solvent control) for 20 minutes before addition of 100 nmol/L PMA (**B** and **C**) or 500 nmol/L HNE (**C**). **A:** PKC δ translocates from cytosol to membrane in response to PMA (100 nmol/L) at 15 minutes. After detecting PKC δ , the polyvinylidene difluoride membrane was stripped and reprobbed 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 mucin secretion in a concentration-dependent manner. *Significantly different from vehicle control ($P < 0.05$); [†]significantly different from PMA alone ($P < 0.05$). Data are presented as mean $\pm$ SEM ($n = 4$). **C:** Rottlerin attenuates phosphorylation of MARCKS in NHBE cells exposed to two different mucin secretagogues, HNE (500 nmol/L) or PMA (100 nmol/L). Phosphorylated MARCKS was detected by Western blot using a specific antibody against MARCKS phosphorylated at serine 152/156. After detecting phosphorylated MARCKS, the polyvinylidene difluoride membrane was stripped and reprobbed with an antibody against total MARCKS protein as a loading control for each lane. Blots are representative of two replicate experiments. Densitometry measurements of the band intensities were quantified using LabWorks (BioImaging Systems, UVP, Inc., Cambridge, UK) and are shown below the blots.

activity, the HBE1 cell line was used. As illustrated in Figure 3, exposure of HBE1 cells to 500 nmol/L PMA for 15 minutes significantly increased mucin secretion (by $\sim$ 1.7-fold compared with medium vehicle control) and also induced phosphorylation of MARCKS in these cells.

Figure 2. Effect of bryostatin 1, a PKC δ activator, on mucin secretion in well differentiated NHBE cells. NHBE cells were exposed to bryostatin 1 over a range of concentrations from 1 to 1000 nmol/L for 15 minutes. **A:** PKC δ translocates from cytosol to membrane in response to bryostatin 1. α -Tubulin and E-cadherin were used as controls for the cytosolic and membrane fractions, respectively. Blots are representative of three replicate experiments. **B:** Bryostatin 1 provokes mucin secretion by NHBE cells in a concentration-dependent manner. Significantly different from vehicle control: * $P < 0.05$; [†] $P < 0.001$; [‡] $P < 0.005$. Data are presented as mean $\pm$ SEM ($n = 4$). **C:** Phosphorylation of MARCKS in NHBE cells is induced by bryostatin 1 in a concentration-dependent manner. Blots are representative of three replicate experiments.

PKC δ Appears to Regulate Mucin Secretion in Airway Epithelial Cells

Transient transfection of the PKC δ and PKC ϵ constructs into HBE1 cells was confirmed by fluorescent microscopy and Western blot analysis. After 48 hours of transfection, GFP expressed in the transfected cells was detected with a fluorescent microscope [Eclipse TE300 (Nikon, Tokyo,

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 500 nmol/L PMA for 15 minutes. Mucin secretion and phosphorylation of MARCKS were assessed by enzyme-linked immunosorbent assay and Western blot analysis, respectively. **A:** Mucin secretion is significantly enhanced by PMA at 500 nmol/L (but not 100 nmol/L) in HBE1 cells. *Significantly different from vehicle control ($P < 0.05$). Data are presented as mean $\pm$ SEM ($n = 4$). **B:** Phosphorylation of MARCKS is increased by exposure of HBE1 cells to 500 nmol/L PMA. Blots are representative of three replicate experiments.

Japan) or Axiovert 35 (Zeiss, Welwyn Garden City, UK)] before PMA exposure (data not shown). After exposure to PMA, cells were lysed to detect expression of PKC δ protein fused with GFP via Western blot analysis using anti-PKC δ and -GFP antibodies (data not shown). Transfection of PKC ϵ was confirmed by Western blot analysis using an antibody against PKC ϵ and an HA tag. Transfection efficiency of all constructs and controls was about 20 to 25% as determined by quantification of GFP and X-gal assay for PKC δ and PKC ϵ , respectively (data not shown).

As illustrated in Figure 4A, mucin secretion by transfected HBE1 cells was stimulated by exposure to 500 nmol/L PMA. Transfection of HBE1 cells with the dominant-negative PKC δ construct (pEGFP-N1/PKC δ^{K376R}) resulted in significant reduction of PMA-induced mucin secretion (~45%), whereas cells transfected with the wild-type PKC δ construct (pEGFP-N1/PKC δ) showed a significant enhancement of PMA-induced mucin secretion (~40%) compared with control cells transfected with no DNA or empty vector (pEGFP-N1). In additional control studies, the effects of Bryostatin 1 on both mucin secretion and phosphorylation of MARCKS were markedly attenuated in cells transfected with the dnPKC δ construct (data not shown). PKC ϵ constructs, either wild type or mutated, did not affect the secretory response to PMA. As shown in Figure 4B, phosphorylation of MARCKS in response to PMA was decreased in HBE1 cells transfected with the dominant-negative PKC δ construct (pEGFP-N1/PKC δ^{K376R}) but increased in cells transfected with the wild-type PKC δ construct (pEGFP-N1/PKC δ). PKC ϵ constructs, either wild type or mutated, had no effect on PMA-induced MARCKS phosphorylation when transfected into HBE1 cells (Figure 4C).

Discussion

Airway mucus is a heterogeneous mixture of water, mucins, enzymes and anti-enzymes, salts, endogenous and

Figure 4. Transient transfection of HBE1 cells with a dominant-negative PKC δ construct results in reduction of mucin hypersecretion. HBE1 cells were transiently transfected with empty vector (pEGFP-N1), a wild-type PKC δ construct (pEGFP-N1/PKC δ), or a dominant-negative construct (pEGFP-N1/PKC δ^{K376R}) using the FuGene 6 transfection reagent as described in Materials and Methods. As an additional control, a wild-type (pHACE/PKC ϵ) and a dominant-negative PKC ϵ construct (pHACE/PKC ϵ^{K437R}) were also transfected. **A:** After 48 hours transfection, cells were exposed to 500 nmol/L PMA (lanes 2 to 8) or vehicle control (lane 1) for 15 minutes, at which time media were collected and mucin secretion assessed by enzyme-linked immunosorbent assay. Data are significantly different from media control (* $P < 0.05$; ** $P < 0.001$); significantly different from cells transfected with no DNA or empty vector and exposed to PMA († $P < 0.05$, ‡ $P < 0.001$); and significantly different from cells transfected with the pEGFP-N1/PKC δ^{K376R} and exposed to PMA (§ $P < 0.05$). Data are presented as mean $\pm$ SEM ($n = 3-4$). After collecting media from transfected HBE1 cells exposed to PMA, cell lysates were analyzed by Western blot for MARCKS phosphorylated at Serine 152/156 residues (**B** and **C**). Blots are representative of three replicate experiments. **B:** Transfection of the dnPKC δ construct results in decreased phosphorylation of MARCKS in response to PMA, whereas overexpression of PKC δ via transfection of the wild-type construct enhances MARCKS phosphorylation after PMA exposure. **Lane 1**, control medium; **lane 2**, PMA plus control medium; **lane 3**, PMA plus mock transfection; **lane 4**, PMA plus empty vector (pEGFP-N1); **lane 5**, PMA plus wild-type PKC δ (pEGFP-N1/PKC δ); and **lane 6**, PMA plus dnPKC δ (pEGFP-N1/PKC δ^{K376R}). **C:** In a separate experiment, phosphorylation of MARCKS is attenuated by transfection of the dnPKC δ (pEGFP-N1/PKC δ^{K376R} , **lane 2**), whereas similar transfection of the dnPKC ϵ (pHACE/PKC ϵ^{K437R} , **lane 3**) does not appear to decrease PMA-induced phosphorylation of MARCKS.

exogenous bacterial agents, organic compounds, and cell debris.^{30,31} Airway injury or disease often leads to alterations in mucin production and/or mucus com-

position. Despite recognition of mucin hypersecretion as a major pathophysiological feature in many airway diseases,^{2,32–34} relatively little is known about actual intracellular mechanisms that regulate secretion under normal or pathological conditions.

Previously, we reported that mucin secretion and PKC δ activity are both increased in normal human airway epithelial cells in response to HNE. In addition, pretreatment of cells with a PKC δ -selective inhibitor resulted in attenuation of HNE-induced mucin secretion.¹¹ PKC is a serine/threonine kinase that has many roles in cell function. All PKCs contain both a regulatory and catalytic domain, each of which consists of four conserved 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. In addition, a pseudosubstrate or autoinhibitory domain is located in the amino terminus near the C1 domain.³⁵ Enzymatic activity of PKC is derived from its catalytic domain on the binding of the C3 and C4 regions to ATP and substrate, respectively.

PKC isoforms have been classified into three subfamilies depending on their mode of activation: conventional (α , β , and γ) PKCs, which are activated by phosphatidylserine, diacylglycerol, or phorbol esters; novel (δ , ϵ , η , θ , and μ) PKCs, which are phosphatidylserine, diacylglycerol, or 12-O-tetradecanoylphorbol-13-acetate/PMA dependent but Ca²⁺ independent; and atypical (ζ and ι/λ) PKCs, which are Ca²⁺, phosphatidylserine, diacylglycerol, and 12-O-tetradecanoylphorbol-13-acetate/PMA independent for their activation.^{35,36} Involvement of PKC activation during airway mucin secretion in response to various secretagogues has been reported.^{9,37–39} However, involvement of any specific isoform(s) has not been fully characterized.

Among the PKC isoforms, PKC δ has been shown to regulate many aspects of lung/airway epithelial cell biology and function. Lungs of mice exposed to asbestos show increased membrane translocation of PKC δ and enhanced PKC δ protein expression, indicating PKC δ activation.⁴⁰ Increased PKC δ in response to asbestos exposure is mainly detected in the membrane of proliferating cell nuclear antigen-positive cells, suggesting that asbestos fibers mediate proliferation of epithelial cells in a PKC δ -dependent manner. Interestingly, activation of PKC δ also is involved in asbestos-induced apoptosis in type 2-like epithelial cells (C10) via translocation of PKC δ to the mitochondria followed by activation of caspase-9 and -3 pathways.⁴¹ In the C5 human salivary epithelial cell line, PKC δ is also required for mitochondria-dependent apoptosis mediated by etoposide, UV radiation, or taxol.⁴² In that report, overexpression of wild-type PKC δ led to a robust induction of apoptosis (indicated by DNA fragmentation), whereas a dominant-negative PKC δ blocked apoptosis in response to diverse stimuli. Previous work has demonstrated that PKC δ is involved in regulating expression of inducible nitric oxide synthase, granulocyte colony stimulating factor, RANTES, and intercellular adhesion molecule-1 in airway epithelium.^{43,44}

PKC δ has been implicated in secretory pathways of various cell types. It has recently been demonstrated to regulate secretion of neurotensin from human endocrine (BON) cells via phosphorylation of MARCKS protein.⁴⁵ In human platelets, PKC δ is activated in response to protease-activated receptor agonist peptides (SFLLRN and AYPGKF), leading to dense granule release,⁸ a process attenuated by the PKC δ inhibitor, rottlerin, but not by Go6976 (a conventional PKC inhibitor). Ishikawa et al¹⁵ demonstrated that carbachol-stimulated insulin secretion in rat pancreatic islets is associated with translocation of PKC δ . 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 the process. Cho et al¹⁷ demonstrated that PKC δ activation is involved in antigen-induced mast cell degranulation, which could subsequently be inhibited by rottlerin or transfection of a dominant-negative mutant of PKC δ . In contrast, Leitges et al¹⁶ reported that PKC δ is a negative regulator of antigen-induced mast cell degranulation.

PKC δ also has been recognized as a regulator of mucin production and secretion in airway epithelial cells. Our previous report suggested that the PKC δ isoform serves as a regulatory molecule in HNE-induced airway mucin secretion from NHBE cells.¹¹ Specific involvement of PKC δ also has been shown in rat secretory cells (SPOC) in response to purinergic agonists (ATP γ S) and PMA.⁴ Recently, Wu et al⁴⁶ demonstrated that expression of a major gel-forming mucin, MUC5B, is regulated in a PKC δ -dependent manner in human airway epithelial cells (NHBE primary cells and the HBE1 and A549 cell lines).

Another isoform that potentially could be involved in mucin secretion is PKC ϵ . PKC ϵ translocates from cytosol to membrane in response to PMA stimulation of NHBE cells, similar to that observed for PKC δ .¹¹ Activation of PKC ϵ appears to contribute to cAMP-stimulated bicarbonate secretion from mouse duodenal mucosa *in vitro*,⁴⁷ and a role for PKC ϵ as a negative regulator has been suggested in epidermal growth factor- and carbachol-mediated Cl[−] secretion from human intestinal epithelial cells (T84).^{48,49} Recently, Suzuki et al⁵⁰ suggested that PKC ϵ plays a dominant role in glucagon-like peptide-1-induced insulin secretion by rat islet cells. In their studies, they observed that glucagon-like peptide-1 mediates translocation of both PKC α and PKC ϵ from cytosol to membrane and also, interestingly, translocation of MARCKS from the plasma membrane to the cytosol. However, a relationship between PKC ϵ activation and MARCKS translocation in their study was not clear, because a PKC ϵ inhibitor peptide attenuated glucagon-like peptide-1-mediated insulin secretion but did not affect MARCKS translocation.

Previously, we demonstrated that MARCKS protein is a key molecule in the mucin secretory pathway in airway epithelium.^{9,51} MARCKS is phosphorylated by PKC at serine residues 152, 156, and 163 located in its phosphorylated site domain.¹³ Although MARCKS is a prominent PKC substrate, its phosphorylation is differentially regulated depending on the specificity of each PKC isoform. Herget et al¹³ demonstrated the differential effi-

ciency of each PKC isoform to phosphorylate MARCKS by performing PKC phosphorylation assays *in vitro*. It was shown that PKCs α , $\beta 1$, $\beta 2$, γ , δ , and ϵ , but not ζ , can phosphorylate MARCKS, with PKC δ being the most potent isoform, followed by ϵ and $\beta 1$, as determined by $V_{\max}$ to K_m catalytic efficiency ratios.¹³ This finding was supported by results of studies by Fujise et al¹² showing that PKC δ has the highest affinity for MARCKS among the isoforms.

The results of the studies reported here indicate a relationship between airway mucin secretion and PKC δ . This is supported by several lines of evidence. First, we demonstrate that mucin secretion correlates well with PKC δ -mediated phosphorylation of MARCKS in both NHBE and HBE1 cells. Two mucin secretagogues (HNE and PMA) (Figure 1) and the PKC δ activator bryostatin 1 (Figure 2) induce mucin secretion and phosphorylation of MARCKS. In addition, pretreatment of cells with the selective PKC δ inhibitor rottlerin decreases phosphorylation of MARCKS in response to HNE or PMA and also attenuates mucin secretion (Figure 1).

Rottlerin is often referred to as a selective inhibitor of PKC δ , and it has been shown to inhibit activity of this isoform by competing with ATP for binding to the enzyme molecule with a median inhibitory concentration (IC_{50}) of 3 to 6 $\mu\text{mol/L}$.⁵² Gschwendt et al⁵³ showed that rottlerin has a specific inhibitory effect on PKC δ rather than on other PKC isoforms (IC_{50} : 30 to 42 $\mu\text{mol/L}$ for α , β , and γ and 80 to 100 $\mu\text{mol/L}$ for ϵ , η , and ζ) via *in vitro* experiments using purified proteins of each. Specific inhibition of PKC δ by rottlerin at 3 to 6 $\mu\text{mol/L}$ also has been confirmed by Keenan et al.⁵⁴ Rottlerin has been widely used for selective inhibition of PKC δ kinase activity in numerous *in vitro* systems.^{11,46,55–57}

However, the specificity and exact targets of many pharmacological and chemical inhibitors can be variable, and rottlerin also has been shown to possibly affect a number of other enzymes and molecules, including, for example, calmodulin-kinase III.⁵⁵ In a cell-free system, 20 $\mu\text{mol/L}$ rottlerin down-regulated activity of MAPKAP-K2, PRAK, and GSK β while being essentially ineffective against PKC δ .⁵⁸ However, other evidence seems to support an inhibitory effect of rottlerin on PKC δ activity in epithelial cells. Page et al⁴⁴ actually measured the effect of 2 $\mu\text{mol/L}$ rottlerin on recombinant PKC δ activity in a cell-free system and showed, via phosphorylation of MBP, that rottlerin substantially reduced PKC δ activity. Similarly, Song et al⁵⁹ demonstrated a direct inhibitory effect of 4 $\mu\text{mol/L}$ rottlerin on PKC δ activation by the nitric oxide donor NOR-1 in airway epithelial cells.

In a previous study, we showed that rottlerin at concentrations used here significantly decreased PKC activity in airway epithelial cells.¹¹ Similar to our findings reported here, the effects of rottlerin often parallel those of transfection of dnPKC δ constructs into epithelial cells.^{41,44} In addition, we show here that that rottlerin, at concentrations that attenuate mucin secretion in response to PMA, also attenuates phosphorylation of MARCKS in human airway epithelial cells (Figure 1). Because calmodulin-kinase III, MAPKAP-K2, PRAK, or GSK β does not phosphorylate MARCKS protein directly and because the time course of the

study (30 minutes) essentially precludes synthesis of new proteins, the evidence suggests that the effects of rottlerin seen here relate to attenuation of PKC δ activity. We do recognize, however, that there could be other nonspecific effects of rottlerin that might influence the results.

Transfection of HBE1 cells with a dominant-negative PKC δ allowed targeted investigation of PKC δ mediation of the mucin secretion process. Competition of endogenous PKC δ 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 PKC δ significantly enhanced both mucin secretion and phosphorylation of MARCKS in PMA-stimulated cells. Because PKC ϵ can also phosphorylate MARCKS, we transfected either a wild-type or a dominant-negative PKC ϵ construct (generated in the same manner as the PKC δ constructs) as an additional control. The results indicated that PKC ϵ was not involved in the MARCKS-mediated mucin secretion pathway (Figure 4).

Collectively, the results suggest that active PKC δ , via phosphorylation of MARCKS, appears to be a key regulatory molecule in the mucin secretion pathway in airway epithelial cells *in vitro*. PKC δ appears to be a highly important PKC isoform in airway epithelium, influencing cell growth and proliferation,⁴⁰ apoptosis,^{41,42} and inflammation.^{43,44} Our results indicate another important role for PKC δ in airway epithelial function, regulation of mucin secretion, and this seems to occur via a mechanism involving PKC δ -induced phosphorylation of MARCKS protein. These findings fit in well with our previous reports that MARCKS is a key molecule regulating mucin secretion, and the dynamics of its phosphorylation and dephosphorylation appear to be important in this function. The precise mechanism of how MARCKS acts to regulate the secretory pathway, as well as other proteins and molecules involved, remain to be determined.

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