Regulation of Airway Epithelial Cell NF-κB-Dependent Gene Expression by Protein Kinase C δ

Kristen Page, Jing Li, Limei Zhou, Svetlana Iasvoyskaia, Kevin C. Corbit, Jae-Won Soh, I. Bernard Weinstein, Allan R. Brasier, Anning Lin and Marc B. Hershenson

*J Immunol* 2003; 170:5681-5689; doi: 10.4049/jimmunol.170.11.5681
http://www.jimmunol.org/content/170/11/5681

References

This article cites 74 articles, 32 of which you can access for free at: http://www.jimmunol.org/content/170/11/5681.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts

Errata

An erratum has been published regarding this article. Please see next page or: /content/171/2/1109.full.pdf
Regulation of Airway Epithelial Cell NF-κB-Dependent Gene Expression by Protein Kinase Cδ

Kristen Page, Jing Li, Limei Zhou, Svetlana Iasvoyskaia, Kevin C. Corbit, Jae-Won Soh, I. Bernard Weinstein, Allan R. Brasier, Anning Lin, and Marc B. Hershenson

Airway epithelial cells synthesize proinflammatory molecules such as IL-8, GM-CSF, RANTES, and ICAM-1, the expression of which is increased in the airways of patients with asthma. We investigated the regulation of these NF-κB-dependent genes by the novel protein kinase C (PKC) isoform PKCδ in 16HBE14o- human airway epithelial cells, focusing on IL-8 expression. Transient transfection with the constitutively active catalytic subunit of PKCδ (PKCδ-CAT), and treatment with bryostatin 1, an activator of PKCδ, each increased transcription from the IL-8 promoter, whereas overexpression of PKCδ had minor effects. Expression of a dominant negative PKCδ mutant (PKCδ-KR) or pretreatment of cells with rottlerin, a chemical PKCδ inhibitor, attenuated TNF-α- and phorbol ester-induced transcription from the IL-8 promoter. Bryostatin 1 treatment increased IL-8 protein abundance in primary airway epithelial cells. Selective activation of PKCδ by bryostatin also activated NF-κB, as evidenced by p65 RelA and p50 NF-κB binding to DNA, NF-κB trans-activation, and IκB degradation. The sufficiency of PKCδ to induce NF-κB nuclear translocation and binding to DNA was confirmed in a 16HBE14o- cell line inducibly expressing PKCδ-CAT under the tet-off system. Deletion of the NF-κB response element severely attenuated PKCδ-induced IL-8 promoter activity. Finally, PKCδ-CAT induced transcription from the GM-CSF, RANTES, and ICAM-1 promoters. Together these data suggest that PKCδ plays a key role in the regulation of airway epithelial cell NF-κB-dependent gene expression.


The respiratory epithelium plays a key role in the asthmatic process, as it is the point of first contact between the lung and viruses, aeroallergens, and irritants. In response to these stimuli, airway epithelial cells produce inflammatory chemokines and express adhesion molecules on their cell surface. These molecules, which include IL-8, GM-CSF, RANTES, and ICAM-1 (1–4), attract and promote the survival and attachment of neutrophils and eosinophils, fostering airway inflammation and the remodeling that follows. Airway epithelial cell expression of the neutrophil chemoattractant IL-8 may be particularly important in severe or acute asthma. Neutrophils and IL-8 are increased in patients with severe persistent asthma (5), status asthmaticus requiring mechanical ventilation (6), and sudden-onset fatal asthma (7).

Recent reports support a role for protein kinase C (PKC) in the regulation of airway epithelial cell responses, including gene expression. Treatment with phorbol esters stimulates phospholipase C activation (8), TNF-α receptor shedding (9), mucin release (10), NaCl-K cotransport (11), and NF-κB trans-activation (12). Treatment with PMA has been shown to induce GM-CSF expression in A549 cells (13) and human bronchial epithelial cells (14). In addition, several studies have demonstrated airway epithelial cell TNF-α responses to be at least partially sensitive to PKC inhibitors. In bovine bronchial epithelial cells, TNF-α-induced migration is blocked by the pan-specific PKC inhibitors calphostin C and H-7 (15). In guinea pig airway epithelial cells, TNF-α-induced mucin release is blocked by the pan-specific PKC inhibitors calphostin C, bisindolylmaleimide, and Ro31-8220 (16). In human bronchial epithelial cells, cigarette smoke-induced, C5a-mediated IL-8 expression (17) and TNF-α-induced ICAM-1 expression (18) are blocked by calphostin C, and TNF-α-induced GM-CSF expression is inhibited by the pan-PKC blocker staurosporine (14). 12-O-tetradecanoylphorbol-13-acetate up-regulates bronchial epithelial cell ICAM-1 expression through the presence of a NF-κB target sequence (19). Although these studies did not pinpoint the PKC isoforms responsible for phorbol ester-induced responses, they are consistent with the idea that PKC isoforms may regulate airway epithelial cell gene expression in an NF-κB-dependent manner.

PKC is a complex family including three types of isoenzymes. The classical isoforms (α, β1, β2, and γ) are activated by calcium, phorbol esters, and phosphatidyserine, whereas the novel isoforms (δ, ε, η, θ, and μ) are calcium insensitive and are activated by phorbol esters and phosphatidyserine. The atypical isoforms (ζ and ηλ) are calcium and phorbol ester insensitive and are activated by phosphatidyserine. PKCα, -β1, -β2, -δ, -ε, and -ζ, but not PKCγ, are expressed in human tracheal epithelial cells (11, 20). Recently, specific atypical and novel PKC isoforms have been noted to activate signaling through the NF-κB pathway. PKCζ has been demonstrated to directly activate IkB kinase-β (IKKβ) in vitro, suggesting that this PKC isoenzyme may function as an IKK

Copyright © 2003 by The American Association of Immunologists, Inc.

0022-1767/03/$02.00
kinase (21). PKCζ also regulates the phosphorylation and transcriptional activity of the NF-κB family member RelA (22, 23). Overexpression of another atypical isoform, PKCφ, is sufficient to activate NF-κB in PC12 neuronal cells (24). NF-κB activation induced by TCR/CD28 costimulation is mediated by the novel PKC isoform PKCθ (25). Activation of PKCε elicits NF-κB DNA binding activity in cardiac myocytes (26). It has recently been shown that PKCδ regulates ICAM-1 expression via NF-κB activation in HUVEC (27). However, a precise PKC isoform involved in this process has not been determined (31). The mega (Madison, WI). Recombinant PKCα (Beverly, CA). Luciferase assay buffer and transcription factor construction were obtained from Upstate Biotechnology (Lake Placid, NY).

In this study we investigated the role of the novel PKC isoform PKCδ in the regulation of human airway epithelial cell gene expression, focusing on IL-8. Activation of PKCδ, but not PKCε, increased IL-8 expression, while inhibition of PKCδ attenuated transcription from the IL-8 promoter. PKCδ activation increased NF-κB binding and trans-activation, whereas deletion of the NF-κB response element severely attenuated PKCδ-induced IL-8 promoter activity. Finally, PKCδ also induced transcription from the GM-CSF, RANTES, and ICAM-1 promoters. Together these data suggest that PKCδ plays a key role in the regulation of airway epithelial cell NF-κB-dependent gene expression.

Materials and Methods

Materials
Phorbol 12,13-dibutyrate (PDBu); PMA; peroxidase-linked, goat anti-rabbit IgG; anisomycin; and rottlerin were obtained from Sigma-Aldrich (St. Louis, MO). [γ-32P]ATP and an ECL kit were obtained from DuPont/NEN (Wilmington, DE). Human TNF-α was purchased from R&D Systems (Minneapolis, MN). Bryostatin 1 was purchased from Biomol (Plymouth Meeting, PA). Go 6976 was purchased from Calbiochem (La Jolla, CA). Abs against PKCδ, p65 (Rel A), p50 (NF-κB1), Rel B, c-Rel, and IKKγ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An Ab against IkBα was obtained from Alexis Biochemicals (San Diego, CA). An anti-hemagglutinin (anti-HA) mAb (HA.11) was purchased from Babco (Beverly, CA). Luciferase assay buffer and transcription factor consensus oligonucleotides for NF-κB and AP-2 were purchased from Promega (Madison, WI). Recombinant PKCδ was obtained from Upstate Biotechnology (Lake Placid, NY).

Cell culture

A derivative of 16HBE14o- human bronchial epithelial cells, provided by S. White (University of Chicago, Chicago, IL), was studied. Cell lines were originally established from bronchial epithelial tissue by transfection with pSVSV40, which contains the origin-defective SV40 genome (28). Unlike the parental line, these cells do not grow in distinct clusters and demonstrate improved transfection efficiency. Cultures show specific immunostaining with pan-cytokeratin c11 Ab (Santa Cruz Biotechnology), bind galactose or galectosamine-specific lectins particular to basal epithelial cells (29), and express β1, α5, α6, and αv integrin subunits on their cell surface (30). Cells were grown on coated plates (fibronectin, 10 μg/ml; collagen, 30 μg/ml; BSA, 100 μg/ml) in MEM with 10% FBS, 1% penicillin-streptomycin, and 200 mg/l γ-glutamine.

Normal human bronchial epithelial cells were purchased from Clonetics (Walkersville, MD). These cells were grown in bronchial/traacheal epithelial cell basal medium (BioWhittaker, Walkersville, MD) to which bovine pituitary extract (0.5 μg/ml), hydrocortisone (0.5 μg/ml), human recombinant epidermal growth factor (0.5 μg/ml), epinephrine (0.5 μg/ml), transferrin (10 μg/ml), insulin (5 μg/ml), retinoic acid (0.1 μg/ml), triiodothyronine (6.5 μg/ml), gentamicin (50 μg/ml), and amphotericin B (50 μg/ml) were added.

Plasmid vectors
pHACE, a mammalian expression vector that contains a CMV promoter, Kozak translation initiation sequence, ATG start codon, N-terminal HA epitope tag, EcoRI cloning site, and stop codon, was used to generate PKC mutants with an N-terminal HA tag. Plasmid DNAs encoding dominant negative forms of PKCζ-KR and PKCε-KR and constitutively active forms of PKCζ-CAT, PKCε-CAT, and PKCδ-CAT were generated as previously described (31). The −162/+44 fragment of the full-length human IL-8 promoter was subcloned into a luciferase reporter plasmid (−162/+44 HIL-8/Luc) (32). The reporter activities of this fragment have been shown to be identical with the full-length promoter in response to respiratory syncytial virus (RSV) infection (32), and this fragment contains the NF-κB nuclear factor for IL-6 (NF-κB6) and AP-1 binding sites required for maximal TNF-α responses (33). Site-directed mutagenesis of the NF-κB site in the context of the −162/+44 HIL-8 was introduced by PCR with mutagenic primers (32) to obtain ANF-κB162/44 HIL-8/Luc. GST-IkBα-s4 was purified on glutathione-agarose as described previously (34). Construction of a cDNA encoding a dominant negative IKKβ (IKKβ-AA, in which Ser177 and Ser181 were replaced by alanines) has been described previously (34). The NF-κB and serum response element (SRE) reporter plasmids, NF-κB-TATA/Luc and SRE-TATA/Luc, were purchased from Stratagene. A cDNA encoding the −620 bp proximal functional promoter region of the human GM-CSF promoter subcloned into luciferase was provided by P. Cockerill (Hanson Center for Cancer Research, Adelaide, Australia) (35). A cDNA encoding the full-length ICAM-1 promoter subcloned into luciferase (36) was provided by J. Soloway (University of Chicago). A cDNA encoding −884 to +64 of human RANTES promoter subcloned into luciferase was provided by R. Schleimer (The Johns Hopkins Asthma and Allergy Center, Baltimore, MD) (37). Murine sarcoma virus and AP-2/Luc reporter plasmids were provided by J. Soloway (University of Chicago). pCMV-β-galactosidase was provided by M. Rosner (University of Chicago).

Immunoblotting

Cell lysates were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose by semidry transfer ( Hoefer, San Francisco, CA). After incubation with Ab, signals were amplified and visualized by ECL.

Measurement of endogenous PKCδ activity

16HBE14o- cells were grown to near confluence, and deprived of serum for 24 h. Selected cells were treated with TNF-α, PDBu, or brystatin 1 for 15 min at 37°C. Cells were washed twice with PBS and incubated in a lysis buffer consisting of 30 mM Tris-HCl (pH 7.5), 1% Nonidet P-40, 150 mM NaCl, 10 mM NaF, 50 μg/ml aprotinin, 10 μg/ml leupeptin, 50 μg/ml pepstatin, 0.4 mM sodium pyrophosphate, 400 μM Na3VO4, and 500 μM PMFS (30 min at 4°C). Insoluble materials were removed by centrifugation (13,000 rpm for 10 min at 4°C). Cell lysates were then incubated overnight with 30 μl of protein A-Sepharose beads precoupled for 2 h with the PKCδ Ab. Immunoprecipitates were washed three times with high salt buffer (0.5 M Tris-HCl (pH 7.4), 0.5 M NaCl, and 1% bovine serum albumin), three times with lysis buffer (without protease inhibitors), and twice with kinase buffer containing 25 mM HEPES (pH 7.4), 20 mM MgCl2, 20 mM β-phosphoglycerate, 2 mM DTT, 20 μM Na3VO4, and 20 mM 3-nitrophenyl phosphate. Immune complexes were resuspended in a final volume of 30 μl of kinase buffer and incubated (20 min at 30°C) with 5 μCi of [γ-32P]ATP and 0.25 μg/ml myelin basic protein (MBP). Reactions were terminated by adding Laemmli buffer and boiling. Samples were resolved on a 10% SDS gel, and the proteins were transferred to a nitrocellulose membrane by semidry transfer. After Ponceau staining, the membrane was exposed to film, and substrate phosphorylation was assessed by optical scanning (Jandel Scientific, San Rafael, CA). Equal loading of PKCδ protein was confirmed by immunoblotting using an anti-PKCδ Ab.

Measurement of cell-free recombinant PKCδ activity

To assess the direct effect of the chemical inhibitor rottlerin on PKCδ activation, recombinant PKCδ was resuspended in PKC enzyme dilution buffer (Upstate Biotechnology; 10 ng/μl/assay) and added to a reaction mixture consisting of 20 μl of kinase buffer (20 mM MOPS (pH 7.2), 25 mM β-glycerophosphate, 1 mM Na3VO4, and 1 mM DTT), 10 μl of lipid activator (0.5 mg/ml phosphatidylycerine and 0.05 mg/ml diglycerides; Upstate Biotechnology), 5 μl of [γ-32P]ATP, and 0.25 mg/ml MBP (10 min at 30°C). Selected samples contained rottlerin (2 μM). Reactions were terminated by adding Laemmli buffer and boiling. Samples were resolved on a 10% SDS gel, and the proteins were transferred to a nitrocellulose membrane by semidry transfer. After Ponceau staining, the membrane was exposed to film.

Measurement of endogenous IKK activity

Endogenous IKK activity was determined by immunoprecipitation with an anti-IKKγ Ab, followed by in vitro phosphorylation assay using recombinant IκBα as a substrate. The activity of the immune complex was assayed in 30 μl of kinase buffer in the presence of 10 μM ATP, 5 μCi of [γ-32P]ATP, and GST-IκBα (3 μg/sample) as a substrate (30°C for 15 min). As for PKCδ, immunoprecipitates were resolved by 10% SDS-PAGE and transferred to nitrocellulose, and phosphorylation was assessed by optical scanning. Equal expression of IKKγ was confirmed by immunoblotting.
Measurement of IL-8 protein
Supernatant IL-8 protein levels were measured by ELISA (Amersham Pharmacia Biotech, Arlington Heights, IL). Piscia-ry bronchial epithelial cells were deprived of bovine pituitary extract and epidermal growth factor for 24 h and then treated with TNF-α (10 ng/ml), PDBU (200 ng/ml), or bryostatin 1 (10 nM).

Transient transfection of human airway epithelial cells
Expression vectors were cotransfected with cDNA encoding the IL-8R plasmid using a liposome-mediated technique as previously described (38). 16HBE14o- cells were grown to 50% confluence, washed in OptiMEM (Life Technologies, Ghaisburg, MD), and incubated with a solution of plasmid DNA (~0.5 μg total DNA/35-mm dish). Lipofectamine (Life Technologies; 4 μl/dish) and OptiMEM. After 4 h, the liposome solution was replaced with 10% FBS/MEM. After incubation for 24 h, cells were treated with human TNF-α (10 ng/ml), PDBU (200 ng/ml), or bryostatin 1 (10 nM). In selected experiments cells were pretreated with Go 6976 (10 nM) or rotterlin (2 μM). Sixteen hours after treatment, cells were harvested and analyzed for luciferase activity as previously described (38). Luciferase activity was measured at room temperature using a luminometer (Turner Designs, Sunnyvale, CA). Luciferase content was assessed by measuring the light emitted during the initial 30 s of the reaction, and the values were expressed in arbitrary units. The background activity from cell extracts is typically <0.02 U, compared with signal on the order of 102–103 units. β-Galactosidase activity was assessed by colorimetric assay using o-nitrophenyl-β-D-galactoside as a substrate (39).

Preparation of nuclear extracts for EMSAs
Nuclear extracts were prepared by the method of Dignam et al. (40) with some modifications. Primary bronchial epithelial cell cultures were trypsinized, rinsed twice with PBS (0.1 M sodium phosphate, pH 7.5), and incubated on ice for 10 min with 4 vol of buffer A, which consisted of 10 mM HEPES buffer (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM PMSF, and 0.5 mM DTT. After centrifugation (1000 rpm for 3 min at 4°C), cells were resuspended in 1.5 original packed cell volume of buffer A. After centrifugation (10,000 × g for 20 min at 4°C), cells were suspended in 1.5 packed cell volume of extraction buffer C (20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 40 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT). After centrifugation (1000 rpm for 3 min at 4°C), cells were resuspended in 1.5 original packed cell volume of buffer A. After centrifugation (10,000 × g for 20 min at 4°C), cells were suspended in 1.5 packed cell volume of extraction buffer C (20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 40 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT). After centrifugation (10,000 × g for 20 min at 4°C), supernatants were dialyzed for 1 h against 20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 40 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.5 mM DTT. Following dialysis, nuclear extracts were clarified by centrifugation at 14,000 rpm for 20 min. Protease inhibitors (leupeptin, antipain, chymostatin, and pepstatin A; 1 μg/ml each) were added, and aliquots were stored at −80°C.

EMSAs
EMSAs were performed using nuclear extracts (4 μg) and binding buffer containing 5 mM Tris-HCl (pH 7.5), 37.5 mM KCl, 0.5 mM EDTA, 2% Ficoll, 50 μg/ml poly(dI-dC), and 30–100,000 cpm of γ-32P-labeled probe and incubated on ice for 15 min. Nuclear extracts were added, and the mixture was incubated at room temperature for 10 min. In some instances Abs against p65 (Rel A), p50 (NF-κB1), Rel B, or c-Rel were added (10 μg/ml at room temperature). An oligonucleotide probe encoding the consensus sequences of NF-κB was purchased from Promega. The protein-DNA complexes were analyzed by electrophoresis through a 5% polyacrylamide gel. The gels were dried and exposed to radiographic film.

Establishment of PKCδ-CAT-inducible cell line and cell culture
To establish PKCδ-CAT tet-off inducible cell lines, 16HBE14o- cells were initially transfected with the pTet-Off plasmid (Clontech, Palo Alto, CA), which expresses (TATA regulator proteins. Colonies resistant to G418 (100 μg/ml for selection, followed by 200 μg/ml for maintenance) were selected and amplified. Clones were tested for inducibility under the tet-off system by transient transfection with a tetracycline response element promoter (pTRE) luciferase reporter (Clontech). Next, cells expressing α-TATA proteins were subjected to a second round of transfection with the pTRE- HA-PKCδ-CAT construct, where the truncated PKCδ gene was inserted into two BamHI sites of the pTRE plasmid. Orientation was checked by restriction analysis. The cells transfected with pTRE-PKCδ-CAT plasmid were selected by puromycin (2 μg/ml, followed by 0.5 μg/ml) and puromycin-resistant colonies were collected for measurement of PKCδ activity under the tet-off system, as assessed by in vitro kinase assay (above). To induce the expression of PKCδ-CAT, cells incubated in G418, puromycin, and doxycycline (5 μg/ml) were washed four times and placed in doxycycline-free medium for 48 h, then in antibiotic-free, serum-free medium for another 24 h.

Statistical analysis
Data are reported as the mean ± SEM. Differences between groups were identified by one-way ANOVA. Differences identified by ANOVA were pinpointed by Student-Newman-Keuls multiple range test.

Results
Expression of PKCδ in cultured bronchial epithelial cells
We examined the expression of selected PKC isoforms in primary human bronchial epithelial cells. Cellular proteins were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with selected anti-PKC isoform-specific Abs. As demonstrated previously (11, 20), bronchial epithelial cells expressed classical (α), novel (δ, ε, θ, and atypical (ζ) PKC isoforms (Fig. 1A).

To confirm the expression of HA-tagged dominant-negative or active PKCδ isoforms in transfected 16HBE14o- cells, proteins from transfected cells were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with an anti-HA Ab (Fig. 1B).

Activation of PKCδ in cultured bronchial epithelial cells
16HBE14o- human bronchial epithelial cells were grown to near confluence and treated with either TNF-α (10 ng/ml) or bryostatin 1 (10 nM) for 10 min before harvest. Bryostatin 1 binds to and activates PKC, but is not a complete tumor promoter. It induces only a subset of the responses to phorbol esters and blocks those that it does not induce. It is particularly potent for activating PKCδ and PKCε (41, 42). Both TNF-α and bryostatin 1 significantly increased PKCδ activation, as determined by an endogenous PKCδ in vitro phosphorylation assay using MBP as a substrate (Fig. 2).

PKCδ regulates IL-8 expression
Primary human bronchial epithelial cells were treated with TNF-α (10 ng/ml), PDBU (200 ng/ml), or bryostatin 1 (10 nM). After overnight incubation, IL-8 protein abundance in the cell supernatants was measured by ELISA. Treatment with bryostatin 1 induced levels of IL-8 protein expression similar to those obtained with either TNF-α or PDBU (Fig. 3A), suggesting that activation of novel PKCs, particularly PKCδ, is sufficient for IL-8 protein expression.

FIGURE 1. Anti-PKC immunoblots. A, Cellular proteins from primary human airway epithelial cells (AEC) were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with Abs against PKCα, PKCδ, PKCε, PKCδ, or PKCζ (BD Transduction Laboratories, Lexington, KY). Controls were rat brain lysate. B, Anti-HA immunoblots demonstrating the expression of epitope-tagged, dominant negative (PKCδ-KR) and active (PKCδ-CAT) forms of PKCδ in 16HBE14o- human bronchial epithelial cells.
PKCβ AND AIRWAY EPITHELIAL CELL GENE EXPRESSION

FIGURE 2. Activation of PKCβ by TNF-α and bryostatin. 16HBE14o-cells were treated with TNF-α (10 ng/ml) or bryostatin (10 nM) for 10 min before lysis. Cell lysates were incubated overnight with 30 μl of protein A-Sepharose beads precoupled for 2 h with the PKCβ Ab. Upper panel, PKCβ activity was determined by an in vitro kinase assay using MBP as substrate. Lower panel, Levels of endogenous PKCβ in immunoprecipitates were determined by Western blot analysis. These data are representative of three separate experiments.

To examine transcription from the IL-8 promoter, 16HBE14o-human bronchial epithelial cells were transfected with cDNAs encoding the −162/+44 fragment of the full-length human IL-8 promoter and treated with either TNF-α or PDBU. Selected cultures were pretreated with Go 6976 (10 nM) or rottlerin (2 μM). Go 6976 is a selective inhibitor of PKC that inhibits calcium-dependent (classical) PKC isoenzymes, but not novel or atypical forms. Rottlerin is a PKC inhibitor that exhibits greater selectivity for PKCβ (43). Preliminary studies confirmed that rottlerin inhibits PKCβ in airway cells (not shown). TNF-α, phorbol ester, and bryostatin 1 each induced transcription from the IL-8 promoter. Inhibition of classical PKC isoforms with Go 6976 had no effect on TNF-α- and PDBU-induced IL-8 promoter activity, whereas rottlerin significantly reduced responses (Fig. 3, B and C).

To investigate the precise role of PKCβ in human airway epithelial cell gene expression, we transiently transfected cells with either the dominant negative (PKCβ-κKR) or constitutively active (PKCβ-CAT) forms of PKCβ. Overexpression of PKCβ-κKR attenuated phorbol ester- and TNF-α-induced IL-8 promoter activity (Fig. 3D). PKC-δKR also inhibited bryostatin 1-induced transcription from the IL-8 promoter, whereas a dominant negative form of PKCε, PKCε-KR, did not (Fig. 3E). On the other hand, overexpression of PKCε-KR attenuated PMA-induced SRE transcriptional activity, while PKCδ-KR did not (Fig. 3F). Finally, overexpression of active PKCβ significantly increased transcription from the IL-8 promoter, whereas selective activation of PKCζ or PKCe had minimal effects (Fig. 3D).

To confirm the reported inhibitory effects of rottlerin on PKCβ activity, we measured the effect of rottlerin on recombinant PKCζ kinase activity in a cell-free system. Addition of 2 μM rottlerin substantially reduced PKCζ activity, as evidenced by MBP phosphorylation (Fig. 4).

FIGURE 3. PKCβ regulates IL-8 expression. A, Primary human airway epithelial cells were deprived of growth factors for 8 h and then treated with TNF-α (10 ng/ml), phorbol ester (200 ng/ml), or bryostatin (10 nM) overnight. Supernatants were collected, and IL-8 protein levels were measured by ELISA. The addition of TNF-α, phorbol ester, or bryostatin significantly increased IL-8 protein abundance (n = 3). *, p < 0.05 (by ANOVA/Student-Newman-Keuls multiple range test). B and C, 16HBE14o-human bronchial epithelial cells were transfected with cDNAs encoding the −162/+44 fragment of the full-length human IL-8 promoter and treated with either TNF-α or PDBU. Selected cultures were pretreated with Go 6976 (10 nM; B) or rottlerin (2 μM; C). Inhibition of classical PKC isoforms with Go 6976 had no effect on TNF-α- and PDBU-induced IL-8 promoter activity, whereas rottlerin significantly reduced responses (n = 3–5). *, p < 0.05 (by ANOVA). D, 16HBE14o-cells were transiently cotransfected with −162/+44 hIL-8/Luc and either dominant negative (PKCζ-KR) or constitutively active forms of PKCζ (PKCζ-CAT), PKCε (PKCε-CAT), and PKCε (PKCe-CAT). Overexpression of PKCζ-KR attenuated phorbol ester- and TNF-α-induced IL-8 promoter activity (n = 5). *, p < 0.05 (by ANOVA). Overexpression of active PKCζ significantly increased transcription from the IL-8 promoter, while selective activation of PKCζ or PKCe had minimal effects (n = 4–8). ***, p < 0.05 (by ANOVA). E and F, 16HBE14o-cells were transiently cotransfected with −162/+44 hIL-8/Luc and either PKCζ-KR or PKCe-KR. Cells were treated with either bryostatin 1 (E) or PMA (F). Overexpression of PKCζ-KR or PKCe-KR substantially reduced PMA-induced responses (n = 4). *, p < 0.05 (by ANOVA).
Activation of PKCδ is sufficient for DNA binding and trans-activation of NF-κB

Since the IL-8 promoter is regulated in part by NF-κB-responsive sequences, we tested the role of PKCδ in NF-κB activation. Primary human bronchial epithelial cells were treated with either bryostatin 1 or TNF-α, and nuclear extracts were harvested for EMSAs. Both stimuli increased the binding of nuclear proteins to an oligonucleotide encoding the NF-κB consensus binding sequence (Fig. 5A). Further, coincubation of nuclear extracts from bryostatin-treated cells with Abs against p65 Rel A and p50 NF-κB1 each induced supershift of the DNA binding complex, demonstrating the presence of these NF-κB family transcription factors. Incubation with anti-c-Rel or Rel B had no effect on protein-DNA complexes. These data suggest that PKC isoforms induce activation of the NF-κB signaling pathway. As would be expected with NF-κB activation, treatment of 16HBE14o- cells with TNF-α and bryostatin 1 each induced degradation of IκBα (Fig. 5B).

We transfected 16HBE14o- cells with a cDNA encoding NF-κB responsive promoter elements subcloned into luciferase (NF-κB-TATA-Luc). Treatment with phorbol ester and bryostatin 1 each increased NF-κB trans-activation to a level analogous to that induced by TNF-α (Fig. 5C). Overexpression of the catalytic subunit of PKCδ (PKCδ-CAT) also significantly increased NF-κB-mediated gene expression.

To determine the importance of the IL-8 promoter NF-κB site for PKCδ-induced responses, we transfected 16HBE14o- cells with either the full-length IL-8 promoter subcloned into luciferase or an IL-8 construct in which the NF-κB site was mutated. Mutation of the NF-κB site abolished responsiveness to PKCδ-CAT (Fig. 5D), demonstrating that PKCδ-induced IL-8 expression requires NF-κB trans-activation.

To confirm that selective activation of PKCδ induces NF-κB activity, we generated PKCδ-CAT tet-off-inducible cell lines from 16HBE14o- cells using the tet-off system. To induce the expression of PKCδ-CAT, cells incubated in G418, puromycin, and doxycycline (5 μg/ml) were washed four times and placed in doxycycline-free medium for 48 h, then in antibiotic-free, serum-free medium for another 24 h. Induction of PKCδ activation was assessed by in vitro kinase assay using MBP as a substrate (Fig. 6A). To test whether PKCδ activation was sufficient for NF-κB activation, nuclear extracts were harvested for EMSAs. Withdrawal of doxycycline was associated with binding of nuclear proteins to the NF-κB oligonucleotide (Fig. 6B). Again, coincubation of nuclear extracts with Abs against p65 Rel A and p50 NF-κB1 each induced supershift of the DNA binding complex, demonstrating the presence of these NF-κB family transcription factors.

IKK activation is required for PKCδ-induced gene expression

To test whether IKK is activated following PKCδ activation, cells were treated with bryostatin 1, and lysates were immunoprecipitated
with an Ab against IKKγ. Immunoprecipitates were incubated with [\textsuperscript{32}P]ATP and recombinant IkBα. Bryostatin 1 treatment induced IkBα phosphorylation, indicative of IKK activation (Fig. 7A). To test whether IKKβ activation is required for PKCδ-mediated responses, 16HBE14o- cells were cotransfected with either NF-κB-TATA/Luc or -162/+44 hIL-8/Luc and either empty vector or cDNA encoding a dominant-negative mutant of IKKβ (IKKβ-AA). Selected cultures were treated with bryostatin 1 or cotransfected with PKCδ-CAT or empty vector. Overexpression of IKKβ-AA attenuated bryostatin 1- and PKCδ-CAT-induced NF-κB trans-activation (Fig. 7B), as well as transcription from the IL-8 promoter (Fig. 7C). These data suggest that PKCδ stimulates NF-κB activation at least in part via the classic IKK pathway.

**PKCδ is required and sufficient for transcription from the GM-CSF, RANTES, and ICAM-1 promoters**

To determine the sufficiency of PKCδ activation for transcription from the promoters of other proinflammatory genes, 16HBE14o-cells were cotransfected with cDNAs encoding either empty vector or PKCδ-CAT and the appropriate luciferase-tagged promoter. Selected cultures were also treated with TNF-α or bryostatin 1. Active PKCδ and bryostatin 1 each induced transcription from the GM-CSF, RANTES, and ICAM-1 promoters, but had insignificant effects on irrelevant promoters (Fig. 8).

**Discussion**

We have found that stimulation of human bronchial epithelial cells induces PKCδ kinase activity. Overexpression of active PKCδ increases transcription from IL-8, GM-CSF, RANTES, and ICAM-1 promoters, whereas inhibition of PKCδ with the chemical inhibitor rottlerin or expression of a dominant negative mutant attenuated IL-8 promoter activity. Activation of PKCδ by bryostatin 1 and expression of a constitutively active catalytic fragment of PKCδ was associated with the binding of nuclear proteins to an oligonucleotide encoding NF-κB binding sequences and with NF-κB trans-activation. Deletion of the IL-8 NF-κB response element severely attenuated PKCδ-induced promoter activity. Together these data suggest that PKCδ regulates airway epithelial cell NF-κB-dependent gene expression.

Recent studies have suggested that the transcription factor complex NF-κB plays a key role in the regulation of pulmonary epithelial cell cytokine expression. In A549 type II pulmonary epithelial cells, deletion of NF-κB promoter sequences has been demonstrated to reduce RSV- and rhinovirus-mediated transcription from the IL-8 (32, 44–48) and ICAM-1 promoters (49, 50). TNF-α induction of IL-8 (51, 52) and RANTES (53) is also dependent on NF-κB in these cells. LPS induces NF-κB activation and ICAM-1 expression in A549 cells (54), and particulate matter stimulates NF-κB activation and ICAM-1 expression in cultured BEAS-2B cells (55). In primary human tracheobronchial epithelial cell cultures, RSV infection is associated with NF-κB translocation and DNA binding (56).

Stimulation of bronchial explants with TNF-α ex vivo increases the expression of NF-κB, IL-8, and GM-CSF in the epithelium (57). Finally, the airway epithelium of patients with asthma demonstrates increased translocation and DNA binding of p65 relative to control subjects (58).

The basic NF-κB complex is a dimer of two members of the Rel family of proteins, p50 (NF-κB1) and p65 (Rel A). Both subunits contact DNA, but only RelA contains a trans-activation domain near its C terminus that directly interacts with the basal transcription apparatus. In unstimulated cells, NF-κB is sequestered in the cytoplasm by IκB family proteins, the best characterized of which is IκBα. Phosphorylation of IκBα, with subsequent polyubiquitination and degradation, allows unmasking of the NF-κB nuclear localization sequence, leading to its translocation to the nucleus, where it may regulate gene transcription. In the present study we found that activation of PKCδ was associated with the binding of nuclear proteins to NF-κB oligonucleotide binding sequences as well as the trans-activation of an NF-κB reporter plasmid. We also identified the presence of p65 RelA and p50 NF-κB1 in the DNA binding complex. Treatment with bryostatin 1 induced degradation of IκBα. Finally, mutation of the IL-8 promoter NF-κB site abolished PKCδ-induced IL-8 transcription. Together, these data demonstrate the importance of the IL-8 promoter NF-κB site for PKCδ-induced responses. As noted above, it has been shown that PKCδ regulates ICAM-1 expression via NF-κB activation in HUVEC (27), and we have recently demonstrated that PKCδ negatively regulates airway smooth muscle cyclin D1 expression in part by activation of NF-κB (59).

For most stimuli, phosphorylation and degradation of IκB are mediated by IKK. IKK consists of two catalytic subunits (IKKα and IKKβ) and a regulatory subunit (IKKγ). While IKKα and IKKβ contain similar kinase domains with essentially identical activation loops (60), they are functionally distinct. Recent studies suggest that IKKβ serves as the target for proinflammatory signals,
whereas IKKα plays a critical role in development. Replacement of the IKKβ activation loop phosphoaccepting serine residues with alanines prevents TNF-α-induced NF-κB translocation, whereas replacement of the IKKα activation loop serines does not (61).

Results from knockout studies confirm that IKKα is not required for IKK activation by TNF-α (62). On the other hand, IKKα appears to be critical for morphogenetic events, including limb and skeletal patterning, proliferation and differentiation of epidermal keratinocytes, and mammary gland development (62, 63). Although IKK is the most likely point of convergence for NF-κB signaling pathways, recent studies suggest that IKK may not be required for NF-κB signaling in some instances. UV-C irradiation (64, 65), hepatitis B protein X (66), and p21-activated kinase (67) have been demonstrated to activate NF-κB via as yet unidentified IKK-independent mechanisms.

PKCζ has been demonstrated to directly activate IKKβ in vitro, suggesting that PKC isoenzymes may function as an IKK kinase (21). Subsequent studies demonstrated that an atypical PKC binding protein, p62, bridges PKCζ to receptor-interacting protein (68), which is involved in signaling by the TNF receptor superfamily. Further, thrombin-induced NF-κB activation is inhibited by a kinase-inactive PKCζ, rottlerin, and a kinase-inactive mutant of IKKβ, suggesting that IKKβ is a downstream target of PKCζ. In the present study we also examined the effects of an IKKβ mutant on PKCζ responses. Bryostatin 1 treatment induced IKK activation. In addition, the expression of an IKKβ-AA attenuated PKCζ-induced transcription from the IL-8 promoter and, to a lesser extent, NF-κB trans-activation. These data suggest that PKCζ stimulates NF-κB activation at least in part via the classic IKK pathway. Further studies will be needed to determine whether PKCζ activates IKK directly or via the activation of an IKK kinase. In addition, since expression of IKKβ-AA did not completely attenuate bryostatin 1- or PKCζ-CAT-induced NF-κB trans-activation, it is conceivable that PKCζ also activates IKKβ-independent pathways.

Selective activation of PKCζ not only increased IL-8 promoter activity, but also increased transcription from the GM-CSF, RANTES, and ICAM-1 promoters. Consistent with the activation of NF-κB by PKCζ, the promoters of IL-8, GM-CSF, RANTES, and ICAM-1 each contain NF-κB-responsive elements (32, 48, 49, 53, 69). Each of these promoters also contains AP-1-responsive elements (35, 48, 52, 70–73). PKCζ has been demonstrated to activate extracellular signal-regulated kinase (74), an upstream activator of AP-1. Future studies will therefore address whether PKCζ regulates airway epithelial cell chemokine expression via AP-1 as well as NF-κB cis-acting promoter elements.

It is important to consider the potential limitations of the present study. Since the specificity of all chemical and genetic inhibitors may be questioned, we cannot rule out the possibility that other PKC isoforms besides PKCζ may be involved in the airway epithelial cell response to TNF-α. For example, bryostatin 1 may activate PKCe as well as PKCζ (41, 42). Also, a previous report surveying the specificity of commonly used protein kinase inhibitors showed that 20 μM rottlerin had no effect on PKCζ activity, but instead inhibited a number of other kinases (75). However, in our study the expression of a dominant negative PKCe, although
capable of blocking PMA-induced SRE transcriptional activity, failed to attenuate TNF-α-induced transcription from the IL-8 promoter, suggesting that PKCε is not required for the observed response. Also, our finding that PKCδ-ε-KR did not inhibit SRE transactivation suggests that the PKC mutants we used behave isoform-specifically in our cell system. Further, we found in a cell-free system that 2 μM rottlerin substantially attenuates PKCδ activity. Finally, in each set of experiments we found similar results with chemical and genetic inhibitors/activators. Taken together, these data suggest that PKCδ indeed regulates NF-κB activation in our system.

The airway epithelial cell products IL-8, GM-CSF, RANTES, and ICAM-1 each attract and promote the survival and attachment of neutrophils and eosinophils, thereby fostering airway inflammation and remodeling in asthma. We have shown that PKCδ regulates the expression of these NF-κB-dependent genes. Together, these data suggest that PKCδ may be a key mediator of airway inflammation and a promising target for therapeutic intervention.

Acknowledgments
We thank Peter Cockerill (Hanson Center For Cancer Research), Robert Schleimer (The Johns Hopkins Asthma and Allergy Center), Julian Solway (University of Chicago), and Marsha Rosner (University of Chicago) for their gifts of plasmid vectors.

References


CORRECTION

Kristen Page, Jing Li, Limei Zhou, Svetlana Iasvovskaia, Kevin C. Corbit, Jae-Won Soh, I. Bernard Weinstein, Allan R. Brasier, Anning Lin, and Marc B. Hershenson. Regulation of Airway Epithelial Cell NF-κB-Dependent Gene Expression by Protein Kinase Cδ. The Journal of Immunology 2003;170:5681–5689.

In the footnote section, Kristen Page and Marc B. Hershenson’s addresses are reversed. Correspondence and reprint requests go to Dr. Marc B. Hershenson, University of Michigan, 1500 East Medical Center Drive, L2221 Women’s Hospital, Box 0212, Ann Arbor, MI 48109-0212. E-mail address: mhershen@umich.edu.

Kristen Page’s current address is Children’s Hospital, 3333 Burnet Avenue, ML 2005, Cincinnati, OH 45229.

In addition, the fourth author’s last name was misspelled. The correct spelling is Svetlana Iasvovskaia.