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PMA-induced up-regulation of MMP-9 is regulated by a PKCα-NF-κB cascade in human lung epithelial cells

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Abbreviations: DAG, diacylglycerol; ECM, extracellular matrix; MMP, matrix metalloproteinase; PDTC, pyrrolidine dithiocarbamate; PKC, protein kinase C

Abstract

Expression of matrix metalloproteinase-9 (MMP-9) is associated with airway remodeling and tissue injury in asthma. However, little is known about how MMP-9 is up-regulated in airway epithelial cells. In this study, we show that phorbol myristate acetate (PMA) induces MMP-9 expression *via* a protein kinase Cα (PKCα)-dependent signaling cascade in BEAS-2B human lung epithelial cells. Pretreatment with either GF109203X, a general PKC inhibitor, or Gö6976, a PKCα/β isozyme inhibitor, inhibited PMA-induced activation of the MMP-9 promoter, as did transient transfection with PKCα antisense oligonuclotides. PMA activated NF-κB by phosphorylating IκB in these cells and this was also inhibited by GF109203X and Gö6976, suggesting that PKCα acts as an upstream regulator of NF-κB in PMA-induced MMP-9 induction. Our results indicate that a "PKCα-NF-κB"-dependent cascade is involved in the signaling leading to PMA-induced MMP-9 expression in the lung epithelium.

Keywords: asthma; bronchial hyperreactivity; epithelial cells; matrix metalloproteinase 9; NF-κB; protein kinase C-α

Introduction

Airway inflammation, remodeling and hyper-responsiveness are key features of bronchial asthma (Pascual and Peters, 2005). Many inflammatory cells including eosinophils, lymphocytes and mast cells infiltrate from the circulation to specific sites within asthmatic tissue (Hamid *et al.*, 2003). This leads to structural changes in the airway wall involving smooth muscle hypertrophy, subepithelial deposition of ECM proteins and loss of epithelium (Vliagoftis *et al.*, 2000). Although many studies have focused on the key mediators contributing to these structural changes, much of the detailed mechanism is unknown.

The matrix metalloproteinase (MMP) family is a group of structurally-related proteins that degrade ECM and basement membrane in a zinc-dependent manner at physiological pH (Visse and Nagase, 2003). It comprises at least 26 secreted and membrane-tethered endopeptidases, classified according to their structures and substrate specificities (Nagase *et al.*, 2006). Members of the family include: collagenases (MMP-1, -8, -13), gelatinases (MMP-2, -9), stromelysins (MMP-3, -7, -10, -11, -12), and membrane-type-MMPs (MT1-MMP to MT6-MMP). These enzymes have been shown to play key roles in tumor cell invasion, metastasis and angiogenesis by promoting ECM degradation and the processing of cytokines, growth factors, hormones and cell receptors (John and Tuszynski, 2001; Nguyen *et al.*, 2001). MMPs are produced not only by structural cells including fibroblasts, endothelial cells and epithelial cells (Bove *et al.*, 2007) but also by inflammatory cells such as macrophages (Mautino *et al.*, 1997; Lee *et al.*, 2006), lymphocytes (Owen *et al.*, 2003), neutrophils (Delclaux *et al.*, 1996) and eosinophils (Fujisawa *et al.*, 1999). By degrading components of the ECM, MMPs are thought to play a role in tissue remodeling and the accumulation of inflammatory cells associated with asthma (Kelly and Jarjour, 2003). Among the MMP family, MMP-9 is believed to be the major MMP in asthma because its level is increased in the sputum (Vignola *et al.*, 1998) and broncoalveolar lavage (BAL) fluid (Kelly *et al.*, 2000; Atkinson and Senior, 2003; Ohbayashi and Shimokata, 2005) of patients with bronchial asthma.

Previous work has suggested that the induction of MMP-9 is affected by various factors such as pro-inflammatory cytokines, TGF-β, EGF, and certain

oncogenes (Sehgal and Thompson, 1999; Han *et al.*, 2001; Nee *et al.*, 2004; Wu *et al.*, 2004; Chou *et al.*, 2006). Phorbol myristate acetate (PMA), a phorbol ester, is known to substitute for DAG as a high affinity ligand for conventional protein kinase C (PKC) and novel PKC isoforms (Easom *et al.*, 1989). It has been shown to strongly stimulate MMP-9 expression via PKC in several systems (Juarez *et al.*, 1993; Simon *et al.*, 1998; Genersch *et al.*, 2000; Park *et al.*, 2000; 2003). The MMP-9 promoter contains binding sites for transcription factors AP-1, NF-κB, SP1, AP-2 and Ets (PEA3) (Sato and Seiki, 1993; Gum *et al.*, 1996; Chakraborti *et al.*, 2003). Some PKC isoforms activate NF-κB and AP-1 (Dominguez *et al.*, 1993; Lallena *et al.*, 1999), while the atypical PKC isoform ζ regulates IKKβ activity *in vitro* (Lallena *et al.*, 1999) and PKCε modulates NF-κB and AP-1 in adult rabbit cardiomyocytes (Li *et al.*, 2000). The novel PKC isoform θ is responsible for NF-κB activation in response to TCR/CD28 costimulation (Coudronniere *et al.*, 2000), and the atypical PKC isoform, PKCζ, phosphorylates and inactivates IκBα *in vitro* (Diaz-Meco *et al.*, 1994; Esteve *et al.*, 2002).

Although PKC is important for MMP-9 expression in a number of cell types, little is known about the signaling pathways involved in PKC-mediated MMP-9 expression in human lung epithelial cells. In the present study, we focused on the involvement of the different PKC isoforms in regulating MMP-9 expression in BEAS-2B human bronchial epithelium cells. We report that activation of PKCα by PMA activates NF-κB and induces MMP-9 expression in these cells.

Materials and Methods

Chemicals and plasmids

PMA, gelatin, dimethyl sulfoxide (DMSO) and pyrrolidine dithiocarbamate (PDTC) were purchased from Sigma-Aldrich (St. Louis, MO). Antibodies to MMP-9, IκB-α, phospho IκB-α, tubulin and various PKC isoforms were from Cell Signaling Technology (Beverly, MA). The following inhibitors were obtained from Calbiochem (La Jolla, CA): GF109203X, a general PKC inhibitor (IC_{50} = 0.2 μM); Gö6976, a potent inhibitor of Ca^{2+}-dependent PKC isozymes (IC_{50} = 6.2 nM); rottlerin, a PKCδ inhibitor with (IC_{50} = 3-6 μM). The reporter gene pMMP-9-luc containing a 673 bp upstream sequence (-670~+3) of the MMP-9 promoter was a gift from Dr. A.S. Jeong (KAIST, Taejon, Korea). All other chemicals were from standard sources and were of molecular biology grade or higher.

Cell culture

BEAS-2B human bronchial epithelial cells (American Type Culture Collection, ATCC CRL 9609), were grown in RPMI1640 supplemented with 10% FBS, 0.1 mM non-essential amino acids, penicillin (50 U/ml) and streptomycin (50 μg/ml) at 37°C in a humidified 95%/5% (v/v) mixture of air and CO_2.

Gelatin zymography

MMP-9 activity was assayed by gelatin zymography as described previously (Woo *et al.*, 2004). 80% confluent BEAS-2B cells were incubated in serum-free medium for 6 h before PMA treatment. After 12 h of PMA stimulation, the culture medium was harvested and stored at -70°C. 40 μl aliquots of thawed culture medium was mixed with non-reducing Laemmli's sample buffer and run on an 8% SDS-PAGE gel containing 0.2% gelatin. After electrophoresis, the gel was washed three times in 2.5% Triton X-100 at room temperature for 90 min to remove SDS, and incubated overnight in a buffer containing 50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 5 mM $CaCl_2$ and 200 μg/ml Brij-35 at 37°C. It was then stained with Coomassie Brilliant Blue, and gelatinolytic activities were visualized as a clear band against a blue-stained background.

Western blot analysis

Cells were washed twice with ice-cold PBS, and the whole-cell lysates were obtained using lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.1% Triton X-100, 0.02% NaN_3, 5 mM EDTA, 1 mM NaF, 1 mM PMSF, 1 μg/ml leupeptin and 2 μg/ml pepstatin A). The protein content was quantified using Bradford reagent (Bio-Rad, Hercules, CA), and then 50 μg of total protein was subjected to SDS-PAGE on 10% acrylamide gels, followed by transfer to polyvinylidine difluoride (PVDF) membranes (Amersham Pharmacia Biotech, Ltd, UK) using a wet transfer unit (NOVEX, San Diego CA; for 1 h at 100 V). The membranes were blocked for 1 h with TBS-T [TBS (150 mM NaCl, 25 mM Tris-Cl, pH 7.4) with 0.05% Tween-20] containing 5% (w/v) nonfat dry milk, washed three times with TBS-T for 5 min each, and then incubated with primary antibody in TBS-T containing 2% (w/v) nonfat dry milk overnight at 4°C, and then with HRP-conjugated secondary anti-rabbit antibody for 1 h. The bands were developed using enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech, Ltd, UK).

Transient transfection and luciferase assay

BEAS-2B cells were seeded in 35 mm dishes at a concentration of 10^5 cells per dish 24 h prior to

transfection. The pMMP-9-Luc reporter was introduced into the cells using Lipofectamine-Plus (Invitrogen, Calsbad, CA). Transfection efficiencies were normalized by co-transfection with 0.4 μg of pSV-βGAL, a eukaryotic expression vector containing the *E. coli* β-galactosidase (*lac Z*) structural gene under the control of the SV40 promoter. The cells were incubated with the DNA mixture for 3 h following which the culture medium was changed to complete RPMI 1640 medium. 24 h after transfection, the cells were preincubated (where desired) with inhibitors for 30 min prior to stimulation with PMA for 6 h, and then lysed in 0.15 ml of lysis solution (0.2 M Tris pH 7.6, 0.1% Triton X-100). The resulting extracts were spun for 5 min and the supernatants were assayed for protein and β-galactosidase activity. Luciferase activity was assayed in 10 μl samples of extract and luciferase luminescence was measured in a Turner TD 20/20 luminometer (Turner Designs, Sunnyrale, CA) and normalized with the co-transfected β-galactosidase activity, as described (Yoo *et al.*, 2001). Transfection experiments were performed in triplicate with two independently isolated sets of cells and the results were averaged.

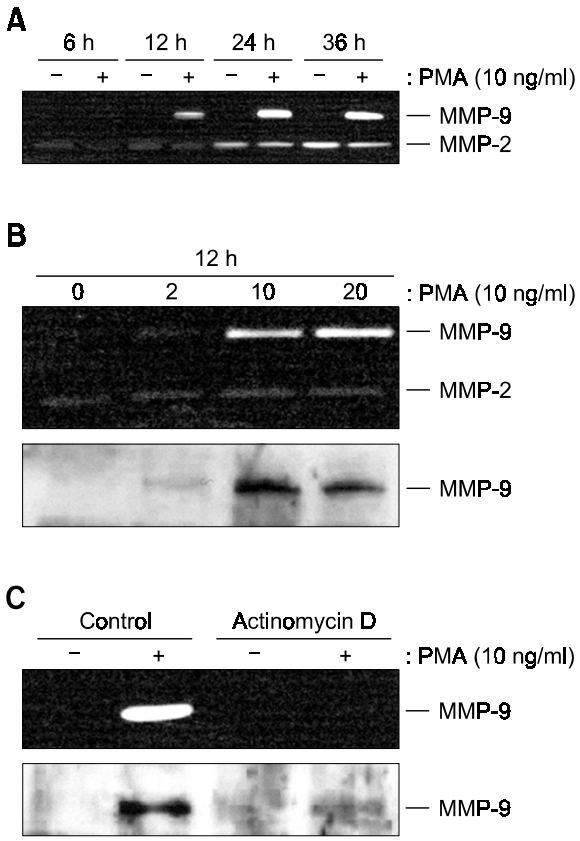
Transfection with antisense PKCα oligonucleotides

Phosphorothioate derivatives of antisense and sense oligonucleotides of PKCα were designed against the translation start site of the PKCα isoform (Shih *et al.*, 1999) and obtained from GenoTech (Taejon, Korea). For transient transfection with the antisense oligonucleotides, about 1×10^5 BEAS-2B cells were plated in 35 mm dishes for 24 h. The Lipofectamine Plus/DNA complex was then added and after 3 h incubation the cells were rinsed with PBS and incubated for 24 h in fresh RPMI 1640 supplemented with 10% FBS.

Results

PMA induces MMP-9 expression in BEAS-2B cells

We first examined the effect of PMA on MMP-9 activity in BEAS-2B cells. After starvation in serum-free RPMI 1640 for 6 h the cells were stimulated



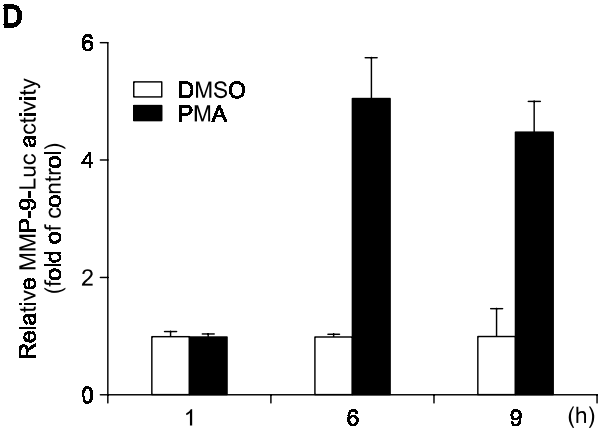


Figure 1. PMA induces MMP-9 expression in BEAS-2B cells. (A) Time-dependent activation of MMP-9 by PMA. BEAS-2B cells were grown for the indicated periods in serum-free medium alone or with PMA (10 ng/ml). Conditioned media were subjected to 8% SDS-PAGE with 0.2% gelatin and the gelatinolytic activities of MMP-9 and MMP-2 were determined by zymography as described in Materials and Methods. (B) Concentration-dependent induction of MMP-9 by PMA. BEAS-2B cells were grown with the indicated concentrations of PMA for 12 h. Conditioned media and whole cell lysates were analyzed for MMP-9 and MMP-2 activity as before (upper panel), and for MMP-9 expression by Western blotting (lower panel). (C) Inhibition of PMA-induced MMP-9 induction by actinomycin D. BEAS-2B cells were pretreated with actinomycin D (2 μg/ml) for 30 min before challenge with PMA (10 ng/ml) for 12 h and the gelatinolytic activity and expression of MMP-9 were assayed by zymography (upper panel) and Western blotting (lower panel), respectively. (D) PMA induces MMP-9 promoter activity. BEAS-2B cells were transfected with an MMP-9-responsive luciferase reporter gene (pMMP-9-Luc). The transfectants were incubated with DMSO or PMA (10 ng/ml) for the indicated times, and their relative luciferase activities were measured. Bars depict the means ± SD of three independent experiments, and the data are expressed as fold increases relative to the control value.

with PMA (10 ng/ml) and conditioned media were collected at successive times point. MMP-9 activity was found to increase progressively up to 24 h (Figure 1A) while MMP-2 activity did not greatly change. The optimal concentration of PMA was 10-20 ng/ml (Figure 1B). To see whether MMP-9 expression is regulated transcriptionally, we examined the effect of the transcription inhibitor, actinomycin D, on MMP-9 expression. When the BEAS-2B cells were incubated with actinomycin D (2 μg/ml), PMA-induced MMP-9 expression was dramatically reduced (Figure 1C). We also examined activation of the MMP-9 promoter by means of luciferase reporter gene assays and found that PMA stimulation (6 h and 9 h) increased promoter activity about 5-fold. These results indicate that PMA induces MMP-9 expression at the transcriptional level (Figure 1D).

PMA-induced MMP-9 up-regulation is mediated by a PKC-dependent cascade

Phorbol esters such as PMA activate PKC by serving as hydrophobic anchors to recruit PKC to the membrane. Prolonged stimulation with phorbol esters results in down-regulation of some PKC isoforms (Yaney *et al.*, 2002). When BEAS-2B cells were pre-incubated with PMA (50 ng/ml) for 18 h and then stimulated with PMA, MMP-9 induction was significantly reduced (Figure 2A). To identify the PKC

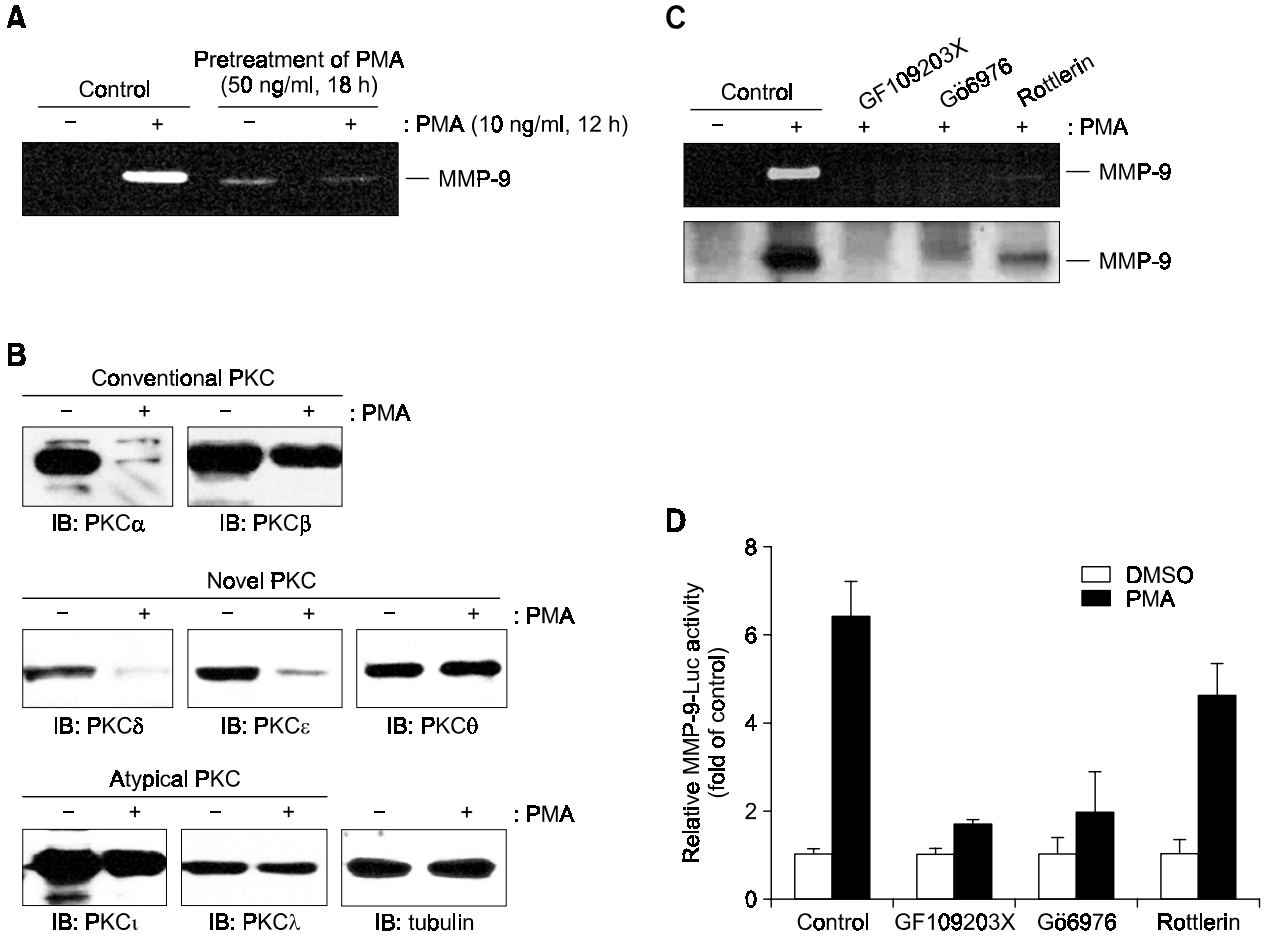


Figure 2. PKC is involved in PMA-induced MMP-9 up-regulation. (A) BEAS-2B cells were pretreated with PMA (50 ng/ml) for 18 h and the culture medium was replaced to remove the PMA. After 12 h, the cells were re-stimulated with PMA (10 ng/ml) and the gelatinolytic activity of MMP-9 was measured. (B) BEAS-2B cells were exposed to DMSO or PMA (50 ng/ml) for 18 h and whole cell lysates were analyzed for down-regulation of PKC isoforms by Western blotting using antibodies against specific PKC isoforms. The blot was also probed with anti-tubulin antibody as a loading control. (C) BEAS-2B cells were pretreated with control buffer, GF109203X (100 nM), Gö6976 (100 nM) or rottlerin (5 μM) for 30 min before challenge with PMA (10 ng/ml) for 12 h. The activity of MMP-9 was analyzed (upper panel) and MMP-9 expression was measured by Western blotting (lower panel). (D) After transfection with the pMMP-9-Luc reporter plasmid, cells were pretreated with buffer, GF109203X (100 nM), Gö6976 (100 nM) or rottlerin (5 μM) for 30 min before challenge with PMA (10 ng/ml) for 12 hr, and their relative luciferase activities were measured. Bars depict the means ± SD of three independent experiments, and the data are expressed as fold increases relative to the control value.

isoforms involved in MMP-9 induction we examined the levels of different PKC isoforms after prolonged (18 h) PMA treatment and found that PMA selectively down regulated PKCα, δ, and ε while not affecting PKCβ, θ, ι, and λ (Figure 2B). Zymography and Western blotting analysis revealed that MMP-9 expression in response to PMA was markedly decreased by the general PKC inhibitor (GF109203X, 100 nM) and the conventional PKC inhibitor Gö6976 (100 nM) (Figure 2C), and the same was true of MMP-9 promoter activity (Figure 2D). However, the PKCδ specific inhibitor, rottlerin, only partially suppressed MMP-9 induction. These results suggest that activation of a conventional PKC, probably PKCα, plays an important role in regulating MMP-9 activity in these cells.

PKCα and NF-κB are involved in the PMA-induced MMP-9 expression

To investigate the role of PKCα in MMP-9 expression in the BEAS-2B cells, we transfected the cells with PKCα antisense oligonucleotides and noted that MMP-9 secretion in response to PMA was greatly reduced (Figure 3A). A similar result was obtained in MMP-9 promoter assays (Figure 3B). The MMP-9 promoter contains binding sites for the transcription factors AP-1, NF-κB, SP1, AP-2 and Ets (PEA3) (Chakraborti *et al.*, 2003), and PKC isoforms have been reported to activate NF-κB and AP-1 (Dominguez *et al.*, 1993; Lallena *et al.*, 1999; Chakraborti *et al.*, 2003). To determine which transcription factor(s) are involved in PMA-induced MMP-9 expression, we transiently transfected BEAS-2B cells with NF-κB-luciferase or AP-1-luciferase plasmids, and stimulated them with PMA (10 ng/ml). PMA significantly activated NF-κB, but not AP-1 (data not shown). To see whether the NF-κB activation is related to PMA-induced MMP-9 expression, we tested the effects of the NF-κB inhibitor, pyrrolidine dithio carbamate (PDTC), on MMP-9 promoter activity. Pretreating BEAS-2B cells for 30 min with the NF-κB inhibitor, PDTC (50 μM) dramatically reduced PMA-induced MMP-9 promoter activation (Figure 4A), and lowered PMA-induced MMP-9 activity in a dose-dependent manner (Figure 4B, upper panel). Heterodimers of NF-κB consist of p50 and p65 subunits and exists in the cytoplasm in inactive form bound to the inhibitory protein IκB through p65 (Ghosh and Karin, 2002; Luo *et al.*, 2004). Phosphorylation of IκB and its subsequent degradation are critical steps in NF-κB activation. We found that PMA induced both phosphorylation and degradation of IκB-α in BEAS-2B cells within several minutes (Figure 4B, lower panel). We conclude that PMA-induced NF-κB activation results from phosphorylation and degradation of IκB-α and that the induction of MMP-9 is due to this NF-κB activation. To investigate whether PKCα is the upstream regulator of NF-κB activation during PMA-induced MMP-9 production, we examined the effect of the PKCα inhibitors on PMA-induced phosphorylation and degradation of IκBα. Western blotting revealed that NF-κB activation was markedly reduced by GF109203X and Gö6976, suggesting that a PKCα-NF-κB cascade is responsible for PMA-induced MMP-9 production (Figure 4C).

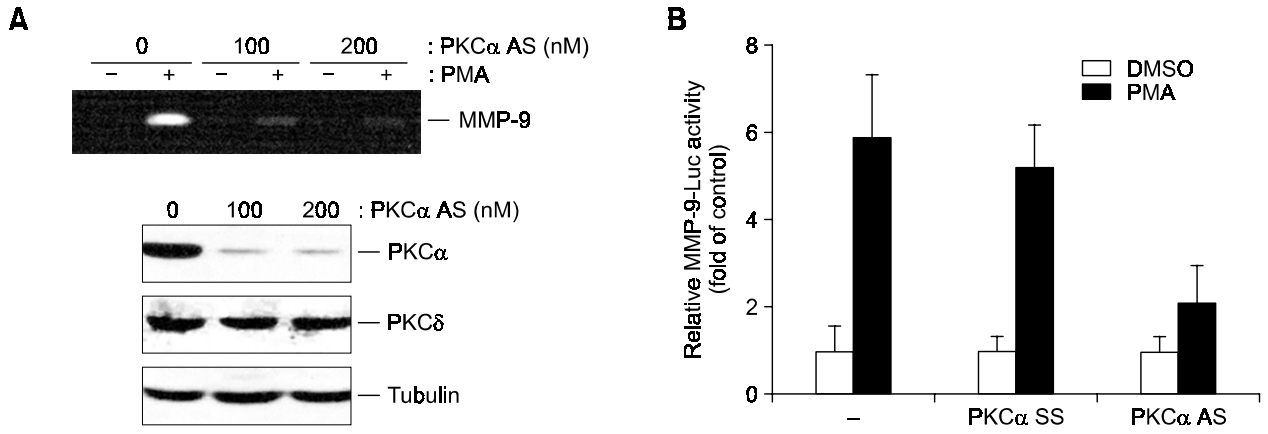


Figure 3. PKCα mediates PMA-induced MMP-9 up-regulation in BEAS-2B cells. (A) BEAS-2B cells were transfected with antisense PKCα oligonucleotides (PKCα AS) and transfectants and control cells were incubated for 12 h in the presence or absence of PMA (10 ng/ml). The conditioned media were assayed for gelatinolytic activity (upper panel) and whole cell lysates were analyzed for PKCα by Western blotting (lower panels). (B) BEAS-2B cells were co-tranfected with the pMMP-9-Luc reporter plasmid and antisense or sense PKCα oligonucleotides (PKCα SS), incubated with DMSO or PMA (10 ng/ml) for 6 h, and their relative luciferase activities were determined. Bars depict the means ± SD of three independent experiments, and the data are expressed as fold increases relative to the control.

Discussion

In this study we investigated the signaling pathways leading to the production of MMP-9 in BEAS-2B human bronchial epithelial cells. Our results demonstrate that a 'PKCα-NF-κB'-cascade is responsible for the up-regulation of MMP-9 in response to PMA.

MMPs are a family of proteases that are important in turnover of the extracellular matrix and in cell migration (Naito and Yoshikawa, 2005). Increased production of MMP-9 from infiltrating immune cells or from the respiratory epithelium occurs in inflammatory lung diseases, and abnormal expression and activation of MMP-9 tend to accompany tissue injury and airway remodeling (Okamoto *et al.*, 2002; Kim *et al.*, 2004; Dik *et al.*, 2005). Cataldo *et al.* showed that deletion of the MMP-9 gene reduces peribronchial inflammation, airway lymphocyte accumulation, airway IL-13 production and the development of bronchial hyperresponsiveness (2002). The detailed mechanisms controlling MMP-9 activation in the airway microenvironment during the development of asthma remain to be determined, but there could be cross-talk between the epithelium and inflammatory cells.

Our data are compatible with the recent observations that up-regulation of PKCα induces secretion of MMP-9 in capillary endothelial cells (Park *et al.*, 2003), that the PKC-dependent NF-κB activation is absolute for MMP-9 induction in hepatocellular carcinoma cells (Hah and Lee, 2003), and that inhibition of NF-κB activity by synthetic compounds inhibits MMP-9 secretion (Ko *et al.*, 2005). Different isoforms of PKC has been implicated in MMP-9 expression in various cell types. For example, PKCζ was shown to be involved in the regulation of MMP-9 expression in C6 glioma cells (Esteve *et al.*, 2002); PKCδ was involved in TPA-induced MMP-9 expression in breast cancer MCF-7 cells (Liu *et al.*, 2002); and PKCβ was involved in PMA-induced MMP-9 gene expression in human HL-60 myeloid leukemia cells (Xie *et al.*, 1998).

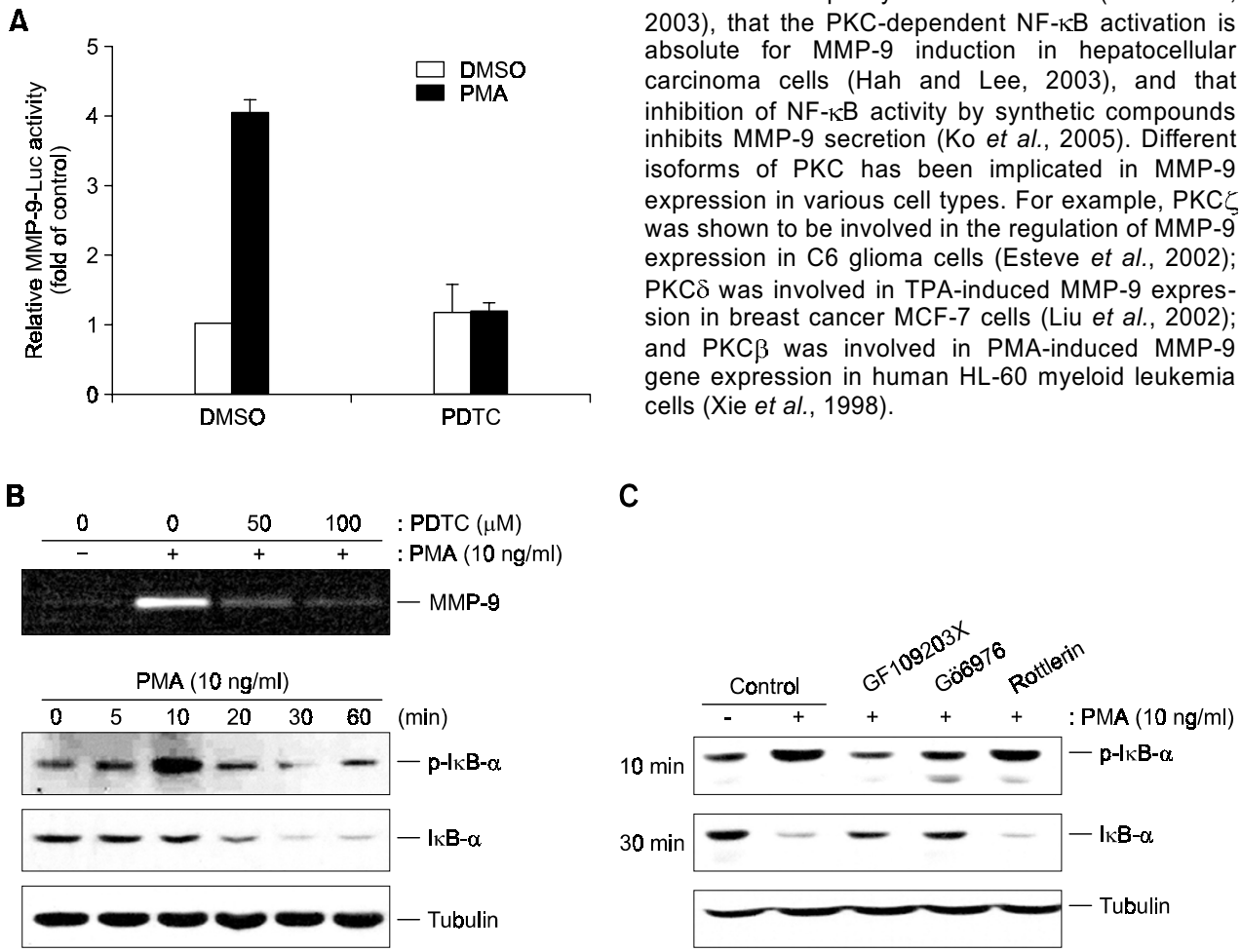


Figure 4. PMA induces NF-κB-dependent up-regulation of MMP-9 in BEAS-2B cells. (A) BEAS-2B cells were transfected with the pMMP-9-Luc reporter plasmid. The transfected cells were incubated with DMSO or PMA (10 ng/ml) in the presence or absence of PDTC (100 μM), an NF-κB inhibitor, and their relative luciferase activities were measured. Bars depict the means ± SD of three independent experiments, and the data are expressed as fold increases relative to the control value. (B) BEAS-2B cells were pretreated with the indicated concentrations of PDTC for 30 min and exposed to PMA (10 ng/ml) for 12 h. MMP-9 activity was determined by zymography (upper panel). In addition BEAS-2B cells were exposed to PMA (10 ng/ml) for the indicated times and phosphorylation and degradation of IκB-α were analyzed by Western blotting (lower panels). The results shown are representative of three independent experiments. (C) BEAS-2B cells were pretreated with buffer, GF109203X (100 nM), Gö6976 (100 nM) or rottlerin (5 μM) for 30 min and then exposed to PMA (10 ng/ml) for the indicated times. Phosphorylation and degradation of IκB-α were analyzed by Western blotting. The results shown are representative of three independent experiments.

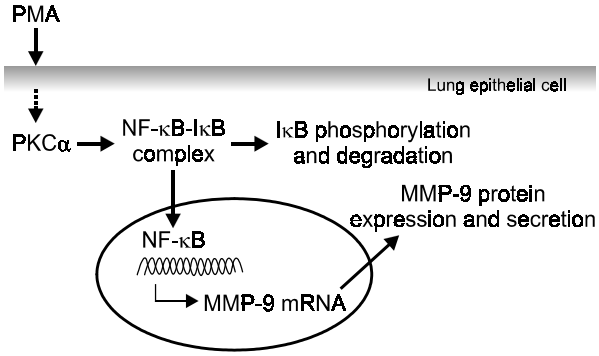


Figure 5. Schematic model of MMP-9 induction by PMA. PMA triggers activation of PKCα which then activates NF-κB as a result of the phosphorylation and subsequent degradation of IκB-α which binds to NF-κB and anchors it in the cytoplasm. The freed NF-κB translocates to the nucleus and induces MMP-9 transcription.

Although further studies are needed to examine the possible roles of PKCδ and PKCε in MMP-9 regulation, we have shown that at least a PKCα- NF-κB-linked cascade is important in the case of human lung epithelial cells, as summarized in the schematic model in Figure 5. We therefore suggest that PKCα inhibitors could be effective therapeutic agents against airway remodeling-related diseases such as asthma.

Acknowledgement

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