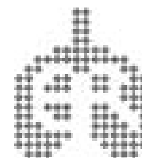
Eur Respir J 2008; 31: 725–735
DOI: 10.1183/09031936.00103507
Copyright©ERS Journals Ltd 2008



Differential regulation of *Moraxella catarrhalis*-induced interleukin-8 response by protein kinase C isoforms

H. Slevogt*, L. Maqami*, K. Vardarowa*, W. Beermann*, A.C. Hocke*, J. Eitel*, B. Schmeck*, A. Weimann#, B. Opitz*, S. Hippenstiel*, N. Suttorp* and P.D. N'Guessan*

ABSTRACT: *Moraxella catarrhalis* is a major cause of infectious exacerbations of chronic obstructive lung disease. In pulmonary epithelial cells, *M. catarrhalis* induces release of the pro-inflammatory cytokine interleukin (IL)-8, which plays a pivotal role in orchestrating airway inflammation.

The present study demonstrated that protein kinase (PK)C was activated by Moraxella infection and positively regulated *M. catarrhalis*-triggered nuclear factor (NF)-κB activation and subsequent IL-8 release. Activation of the PKC/NF-κB signalling pathway was found to be dependent on expression of the Moraxella-specific ubiquitous surface protein A2. In addition, it was shown that specific isoforms of PKC play differential roles in the fine-tuning of the *M. catarrhalis*-induced NF-κB-dependent gene expression through controlling *il8* promoter activity. Inhibition of PKCα and ε with chemical inhibitors or using short interfering RNA-mediated gene silencing significantly suppressed, whereas inhibition of PKCθ increased, the *M. catarrhalis*-induced IL-8 transcription and cytokine release.

In conclusion, it was shown that *Moraxella catarrhalis* infection activates protein kinase C and its isoforms α, ε and θ, which differentially regulate interleukin-8 transcription in human pulmonary epithelial cells.

KEYWORDS: Cytokine response, *Moraxella catarrhalis*, protein kinase C isoforms, pulmonary epithelial cells, ubiquitous surface protein A2

Chronic obstructive pulmonary disease (COPD) is one of the most common causes of morbidity and mortality worldwide with increasing prevalence [1]. In COPD patients, *Moraxella catarrhalis* continues to emerge as a leading human mucosal pathogen [2]. This Gram-negative diplococcus colonises the lower respiratory tract of up to 32% of adults with COPD and is associated with recurrent and persistent lower respiratory tract infections that cause ~10% of all COPD exacerbations [3]. Among the various putative virulence factors of this pathogen that have been identified to date, several proteinaceous antigens have been shown to protrude from the outer membrane of *M. catarrhalis*, including the ubiquitous surface protein (Usp)A1 and UspA2 proteins, which are expressed in most clinical isolates [2, 4, 5]. *In vitro* mutant analysis indicates that UspA1 is involved in adherence to epithelial cells, whereas UspA2 is essential for serum resistance [6]. However, little is known about the role of these proteins in the inflammatory immune responses of pulmonary epithelial cells. Inflammation in COPD is characterised by increased infiltration of neutrophils, lymphocytes and macrophages into the airways [7]. In addition, bronchial epithelium also directly contributes to immune responses by secreting bioactive substances when exposed to pathogens or stimulation by inflammatory mediators [8]. Among them, interleukin (IL)-8, a member of the CXC chemokine family, plays a pivotal role in regulating neutrophil and monocyte chemotaxis toward sites of infection, and in inducing airway inflammation [9, 10]. The presence of microorganisms such as *M. catarrhalis* in the lower respiratory tract of COPD patients has been found to increase IL-8 release in bronchoalveolar lavage of COPD patients, which is associated with disease progression [11–13]. Transcriptional regulation of IL-8 is controlled by a tight regulatory signalling network, most importantly involving the nuclear factor (NF)-κB. NF-κB comprises a family of Rel proteins that are normally retained in

AFFILIATIONS
*Dept of Internal Medicine/Infectious Diseases and Pulmonary Medicine, and
#Institute of Laboratory Medicine and Biochemistry, Charité – Universitätsmedizin Berlin, Berlin, Germany.

CORRESPONDENCE
H. Slevogt
Dept of Internal Medicine/Infectious Diseases and Respiratory Medicine
Charité – Universitätsmedizin Berlin
Augustenburger Platz 1
13353 Berlin
Germany
Fax: 49 30450553906
E-mail: hortense.slevogt@charite.de

Received:
August 09 2007
Accepted after revision:
December 11 2007

SUPPORT STATEMENT
This work was supported in part by grants from the Rahel-Hirsch Stipendium of the Charité-Universitätsmedizin Berlin, Germany (H. Slevogt), the Bundesministerium für Bildung und Forschung to S. Hippenstiel (PROGRESS C1) and N. Suttorp (PROGRESS B2), the Deutsche Gesellschaft für Pneumologie (S. Hippenstiel and B. Opitz), and the Deutsche Forschungsgemeinschaft to S. Hippenstiel (DFG HI789/6-1).

STATEMENT OF INTEREST
None declared.

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

the cytoplasm by binding to NF-κB inhibitors (IκBs) [9, 14]. Following cellular activation, phosphorylation of IκBα results in its ubiquitination and proteolysis, which lead to nuclear translocation and binding to the IL-8 κB-binding site within the *il8* promoter [14]. Subsequently, binding of the RNA polymerase II to the promoter determines the start of the IL-8 gene transcription [15, 16]. Recently, the current authors demonstrated that *M. catarrhalis*-induced NF-κB activation results in IL-8 release by pulmonary epithelial cells [17]. There are, however, considerable data to suggest that a number of additional signal transduction pathways are required for fine-tuning the NF-κB-dependent cytokine transcription [18]. Protein kinase (PK)C has been recently implicated in the control of IL-8 release by IL-1β or tumour necrosis factor (TNF)-α [19, 20]. Members of the PKC family are serine/threonine kinases that play ubiquitous roles in intracellular signal transduction [18]. The PKCs comprise 12 closely related isoforms (isozymes), which are classified into three subfamilies on the basis of their domain structure and their ability to respond to Ca^{2+} and diacylglycerol (DAG). Classical PKC isoforms (α, βI, βII and γ) require calcium, DAG and phosphatidylserine for activation. Novel PKC isoforms, including PKCδ, ε and θ, require DAG and phosphatidylserine. Atypical PKC isoforms (ζ, ι and λ) and PKCμ require phosphatidylserine only and, in contrast to conventional and novel PKCs, do not respond to phorbol esters [21]. The classical PKCs, PKCα and PKCβ, the novel PKCs, PKCδ, PKCε and PKCθ, and the atypical PKCζ have been shown to be expressed in the pulmonary epithelium [22–24]. Individual PKC isozymes are activated by diverse stimuli and have been implicated to play distinct and, in some cases, opposing roles in the transduction of intracellular signals, often being over-expressed in disease states [19]. However, the role of PKC and its isoforms in regulating epithelial NF-κB activation and IL-8 expression following *M. catarrhalis* infection has not yet been investigated. Therefore, the present work studied the involvement of Moraxella UspA1 and UspA2 and different PKC isoforms expressed in pulmonary epithelial cells in *M. catarrhalis*-induced IL-8 production. The present data demonstrate that expression of the Moraxella UspA2 was important for activating the PKC/NF-κB signalling pathway leading to IL-8 release. Moreover, it was found that the PKC isoforms PKCα, PKCε and PKCθ are involved in the *M. catarrhalis*-induced IL-8 release by differentially regulating the transcription at the *il8* promoter.

MATERIAL AND METHODS

Bacterial strains

The *M. catarrhalis* strain 25238 (serotype A) was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). *M. catarrhalis* wild-type strain O35E (Serotype A) and the UspA1- and UspA2-deficient mutant strains O35E.1 and O35E.2 were kindly provided by E. Hansen (University of Texas Southwestern Medical Center, Dallas, TX, USA). Antimicrobial supplementation for the *M. catarrhalis* mutant O35E.1 and O35E.2 involved kanamycin (15 μg·mL^{-1}). *M. catarrhalis* was grown overnight at 37°C on brain–heart infusion (BHI) agar (Dibco Laboratories, BD, Heidelberg, Germany) supplemented with 5% heated sheep blood as previously described [17, 25, 26]. For infection experiments, single colonies of bacterial overnight cultures were expanded by resuspension in BHI broth and incubation at 37°C for 2–3 h to mid-log phase (absorbance at 405 nm 0.4–0.6). Subsequently, bacteria were harvested by centrifugation, resuspended in cell culture medium without antibiotics and adjusted to an optical density (OD) of 0.3 (equivalent 1×10^6 colony-forming units (cfu)·mL^{-1}) at 405 nm and used for infecting epithelial cells at the indicated doses. Cells were infected with *M. catarrhalis* at doses 0.1–10 cfu·mL^{-1}. To investigate inactivated *M. catarrhalis*, bacterial suspension were treated by heat (95°C, 30 min) and subsequent viability was ruled out by plating the suspension on BHI agar for 12 h.

Cell lines

Human primary bronchial epithelial cells (PBECs) were obtained by endobronchial brushing during bronchoscopy of normal human volunteers. The study was approved by the local ethics committee (Charité – Universitätsmedizin Berlin, Berlin, Germany). PBECs were plated in bronchial epithelial cell basal medium supplemented with human epidermal growth factor (0.5 ng·mL^{-1}), insulin (5 μg·mL^{-1}), transferrin (10 μg·mL^{-1}), epinephrine (0.5 μg·mL^{-1}), triiodothyronine (6.5 ng·mL^{-1}), gentamicin (50 μg·mL^{-1}), amphotericin B (50 ng·mL^{-1}), bovine pituitary extract (52 μg·mL^{-1}), retinoic acid (0.1 ng·mL^{-1}) and cultured on tissue culture plates coated with human collagen (Vitrogen 100; Cohesion Technologies, Palo Alto, CA, USA). All experiments were performed with cells in passage 2. The bronchial epithelial cell line BEAS-2B was a kind gift from C. Harris (National Institutes of Health, Bethesda, MD, USA). BEAS-2B cells were grown in Keratinocyte-SFM (Gibco BRL Life Technologies, Paisley, UK) supplemented with 2 mM L-glutamine, penicillin (100 U·mL^{-1}) and streptomycin (100 μg·mL^{-1}), as previously described [17]. The cells were used between passages 10–35. A549 epithelial cells (type-II alveolar cells) were obtained from the ATCC and cultured in Dulbecco's modified Eagle medium (DMEM; Gibco BRL Life Technologies) supplemented with 10% foetal calf serum (FCS), 200 mM L-glutamine and streptomycin (100 μg·mL^{-1}), as previously described. The NF-κB-dependent reporter cell line, A549 6Btkluc, was a kind gift of R. Newton (University of Warwick, Coventry, UK). These cells contain a stably integrated plasmid with three tandem repeats of the sequence 5′-AGCTTACAAGGGATTTCCGCTGGGGACTTTC CAGGGA-3′, which contains two copies of the decameric NF-κB binding site upstream of a minimal thymidine kinase promoter (-105–51) driving a luciferase gene [27]. Cells were grown in medium without antibiotic supplements 12 h prior to the experiment. To assess the multiplicity of infection (MOI) per epithelial cell infected with *M. catarrhalis*, cell counts of confluent BEAS-2B layers per well of a 24-well plate were performed after trypsination in a Neubauer cytometer (Hecht Assistant, Sondheim, Germany). Cell counts were $\sim 5 \times 10^5$ cells·well^{-1}. Counting of PBEC and A549 cells·well^{-1} gave similar results. Experiments were performed in 500 μL volume per well. Therefore, the infection doses of 10^6 cfu·mL^{-1} *M. catarrhalis* corresponded with an MOI of 1.

Materials

DMEM, FCS, trypsin-EDTA-solution, CA-650 and antibiotics were obtained from Life Technologies (Karlsruhe, Germany). Protease inhibitors, Triton X-100 and Tween-20 were purchased from Sigma (Munich, Germany), and calphostin C, Gö6976, PKC inhibitor 20-28, PKCβ inhibitor, Rottlerin, PKCε

inhibitor peptide, PKCθ myristoylated pseudosubstrate inhibitor and PKCζ pseudosubstrate inhibitor were purchased from Calbiochem-Merck (Darmstadt, Germany) and dissolved in dimethylsulphoxide. Phorbol-12-myristate-13-acetate (PMA) was obtained from Calbiochem-Merck (Darmstadt, Germany). All other chemicals used were of analytical grade and obtained from commercial sources.

IL-8 ELISA

IL-8 secreted by PBECs, BEAS-B and A549 cells was measured using a commercially available sandwich-ELISA kit, according to the manufacturer's protocol (R&D Systems, Wiesbaden, Germany), as described previously [17].

RT-PCR analysis

RNA was extracted using RNeasy Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. All primers were purchased from TIB MOLBIOL (Berlin, Germany). PCR products were analysed on 1.5% agarose gels, stained with ethidium bromide and subsequently visualised. To confirm equal loading, PCR for glyceraldehyde-3-phoshate dehydrogenase was performed in parallel.

PKC assay

The PKC assay StressXpress (Stressgen Bioreagents Corp., Victoria, BC, Canada) was used to detect PKC activity, as described previously [28]. BEAS-2B and A549 cells were stimulated with *M. catarrhalis*. A cell extract containing activated PKC was collected and processed for PKC activity by ELISA according to manufacturer's instructions.

Western blot

Transfection efficiency of short interfering (si)RNA for PKCα, PKCε and PKCθ, the phosphorylation of myristoylated, alanine-rich C-kinase substrate (MARCKS), and PKC isotype translocation to membranes was assessed by Western blot analysis. Briefly, BEAS-2B cells were infected as indicated and lysed in buffer containing Triton X-100, subjected to SDS-PAGE and blotted on Hybond-ECL membrane (Amersham Biosciences, Freiburg, Germany). Immunodetection of target proteins was carried out with specific antibodies. In all experiments, actin or extracellular signal-regulated kinase 2 was determined simultaneously on the same membrane by using Odyssey Infrared Imaging System (LI-COR Biosciences GmbH, Bad Homburg, Germany) to confirm equal protein load as described previously [17, 28].

RNA interference in A549 cells and bacterial infection

Control nonsilencing siRNA (sense UUCUCCGAACGUGU CACGUtt, antisense ACGUGACACGUUCGGAGAAtt), siRNA targeting PKCα (sense UAGUUGAUCUCGCGGACGAtt, antisense UCGUCCGCGAGAUCAACUAtt), siRNA targeting PKCε (sense CGAUUCCAUCACUAUCCAUtt, antisense AUGGAU AGUGAUGGAAUCGtt) and PKCθ-siRNA (sense AAACCAC CGTGGAGCTCTACTtt, antisense AAGAGCCCGACCTTCTGT GAAtt) were purchased from MWG (Ebersberg, Germany). A549 cells were transfected by using Amaxa Nucleofector^{TM} (Amaxa, Köln, Germany) according to the manufacturer's protocol, (Nucleofector^{TM} Solution V, Nucleofector^{TM} program G-16) with 2 μg siRNA per 10^6 cells.

Chromatin immunoprecipitation

BEAS-2B cells were stimulated, culture medium was removed, and 1% formaldehyde was added as previously described [17]. After 1 min, the cells were washed in ice-cold 0.125 M glycin in PBS, then rapidly collected in ice-cold PBS, centrifuged and washed twice with ice-cold PBS. The cells were lysed in chromatin immunoprecipitation (ChIP) radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1% desoxycholic acid, 0.1% SDS, 1 mM EDTA, 1% aprotinin), and chromatin was sheared by sonication. The samples were cleared by centrifugation and the supernatants were stored in aliquots at -80°C until further use. NF-κB p65 (C-20) antibodies and polymerase II (N-20) antibodies were purchased from Santa Cruz Biotechnology Inc. (Heidelberg, Germany). Immunoprecipitations from soluble chromatin were carried out overnight at 4°C. Immune complexes were collected with protein A/G-agarose (Santa Cruz Biotechnology Inc.) for 60 min and washed thoroughly with RIPA buffer and high salt buffer (2 M NaCl, 10 mM Tris, pH 7.5, 1% Nonidet P-40, 0.5% desoxycholic acid, 1 mM EDTA). Immune complexes were extracted in elution buffer by shaking the samples for 15 min at $180\times g$ at 30°C. They were then digested with RNase for 30 min at 37°C. After proteinase K digestion for 6 h at 37°C and 6 h at 65°C, DNA was extracted using a PCR purification kit (Qiagen). *il8* promoter DNA was amplified by PCR using Hot Star Taq (Qiagen) DNA polymerase. PCR products were separated by agarose gel electrophoresis and detected by ethidium bromide staining. Equal amounts of input DNA was controlled by gel electrophoresis. The following promoter-specific primers for *il8* were used: sense, 5′-AAG AAA ACT TTC GTC ATA CTC CG-3′; antisense, 5′-TGG CTT TTT ATA TCA TCA CCC TAC-3′.

Statistical analysis

Data in figures 1–7 are shown as mean±SEM of at least three independent experiments, as indicated. Main effects were then compared *via* Newman–Keul's post-test. Stimulatory effects of *M. catarrhalis* as well as inhibitory effects of siRNA used were statistically evaluated employing a paired t-test.

RESULTS

M. catarrhalis-*induced IL-8 transcription and release in bronchial epithelial cells*

To study the host–pathogen interaction between *M. catarrhalis* and pulmonary epithelium *in vitro*, the present study used the SV-40 T-antigen-transformed bronchial epithelial cell line BEAS-2B as cell culture model, as well as PBECs closely resembling their *in vivo* state and thus offering an important tool for the study of PBEC activation *in vitro*. Moreover, BEAS-2B cells have been shown to behave similarly to the primary culture of PBEC cells in many aspects of their cellular functions [17, 26, 29]. Incubation of BEAS-2B cells and PBECs with *M. catarrhalis* strain O35E (10^7 cfu·mL^{-1} for 60, 120 and 240 min) produced a time-dependent increase in IL-8 transcription (fig. 1a and b). Infection of BEAS-2B cells with *M. catarrhalis* strain ATCC 25238 induced an equally strong IL-8 protein release when compared with strain O35E (fig. 1c). A dose-dependent IL-8 release could also be demonstrated by PBECs infected with Moraxella strain O35E (fig. 1d). In order to investigate the influence of bacterial viability on IL-8 release, *M. catarrhalis* strain O35E (1×10^7 cfu·mL^{-1}) was heat-inactivated

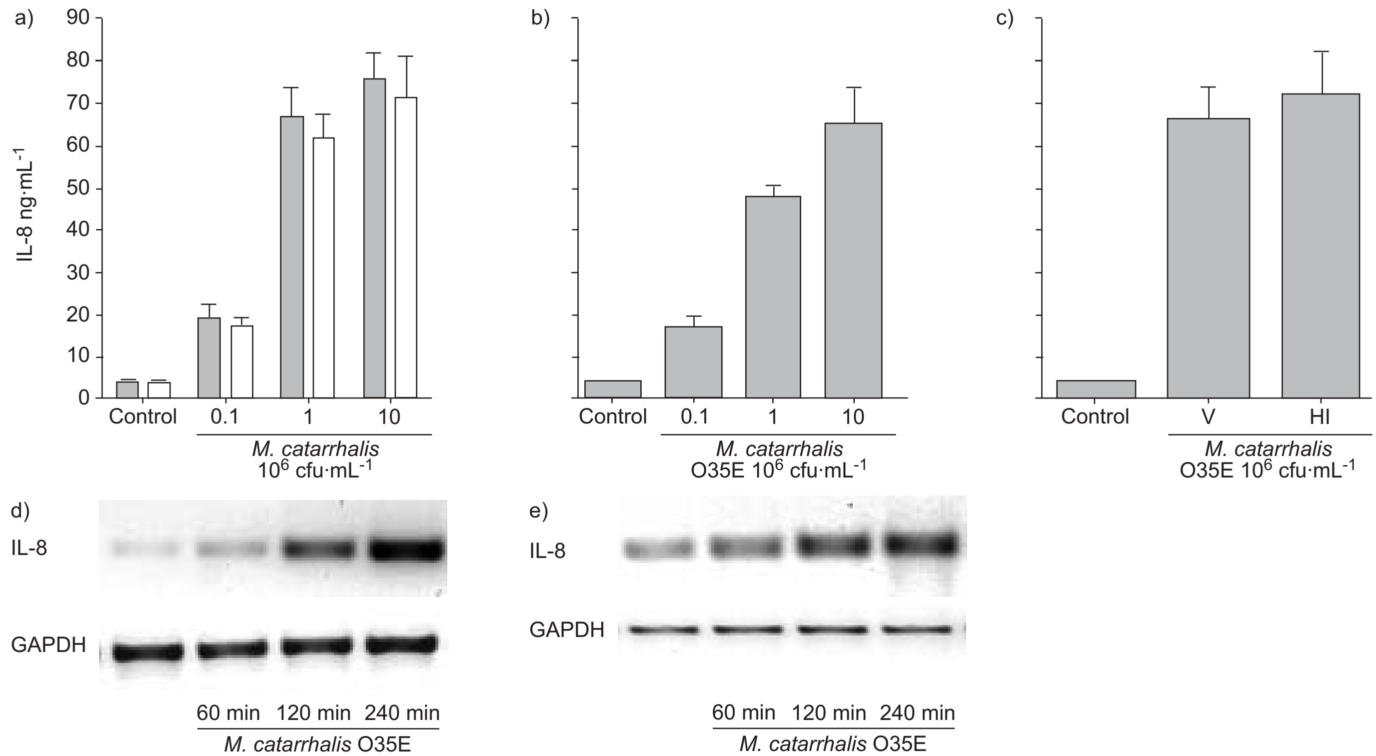


FIGURE 1. Determination of interleukin (IL)-8 production in pulmonary epithelial cells. BEAS-2B cells (a) and primary bronchial epithelial cells (PBECs; b) were infected with Moraxella strain O35E at the indicated doses and incubated for 12 h (■). IL-8 release in the supernatants of the cells was measured by ELISA. In addition, IL-8 release was measured in the supernatants of BEAS-2B cells after infection with *M. catarrhalis* strain American Type Culture Collection 25238 (a; □). c) BEAS-2B monolayer were incubated with viable (V) *M. catarrhalis* or heat-inactivated (HI) bacteria of strain O35E (10^7 colony forming units (cfu)·mL^{-1}) and IL-8 release was measured by ELISA as described previously. Data are presented as mean±SEM of three separate experiments (a–c). Representative gels out of three independent experiments are shown: d) BEAS-2B cells and e) PBECs were infected for 60, 120 and 240 min with *M. catarrhalis* strain O35E and IL-8 mRNA was detected by RT-PCR. GAPDH: glyceraldehyde-3-phoshate dehydrogenase.

prior to infection of BEAS-2B cells. No significant difference was found in the IL-8 release of BEAS-2B cells induced by inactivated bacteria when compared with infection with viable Moraxella at the same doses (fig. 1e).

M. catarrhalis *increased PKC activity in bronchial epithelial cells*

To further explore whether activation of PKC might play a crucial role in *M. catarrhalis*-induced cell activation, BEAS-2B cells were infected for 60, 120 and 240 min with *M. catarrhalis* (10^7 cfu·mL^{-1}) and a two- to four-fold increase in PKC activity was found (fig. 2a). A 2-h stimulation of the cells with strain O35E resulted in an increase of PKC activity that was comparable to that observed after infection with strain ATCC 25238 or a 1-h stimulation of the cells with PMA (160 nM), a strong inducer of PKC activity (fig. 2a). *M. catarrhalis*-induced PKC activation was confirmed by immunoblot showing a time-dependent increase of phophorylation of MARCKS, one major substrate of PKC in strain O35E-infected cells (fig. 2b) [30].

M. catarrhalis*-induced IL-8 release and PKC activation in bronchial epithelium is dependent on UspA2 but not on UspA1 expression*

In order to investigate the role of *M. catarrhalis* proteins UspA1 and UspA2 for the IL-8 release of pulmonary epithelial cells, BEAS-2B cells were infected with either the *M. catarrhalis* wild-type strain O35E, the UspA1-deficient mutant strain O35E.1 or the UspA2 deficient mutant strain O35E.2 for 16 h. As shown in figure 3a, IL-8 protein release induced by the strain O35E.1 did not differ from IL-8 secretion induced by the wild-type strain O35E. In contrast, strain O35E.2 induced significantly lower IL-8 secretion suggesting that UspA2-epithelial cell interaction is important for the *M. catarrhalis*-induced IL-8 release. Next, the impact of UspA1 and UspA2 expression on the activation of PKC was investigated by incubating the cells with either the O35E wild-type strain, the UspA1-deficient mutant strain O35E.1 or the UspA2-deficient mutant strain O35E.2. It was found that Moraxella expression of UspA1 was not involved in PKC activation of BEAS-2B cells, whereas PKC activity seemed to be dependent on UspA2 (fig. 3b). To exclude possible discrepancies in growth rates of the used Moraxella strains, the growth of the wild-type and mutant strains of *M. catarrhalis* was compared by incubating the bacterial suspensions at an initial OD of 0.01 (at 405 nm) in BHI broth and at 37°C with shaking. Growth was followed by means of OD measurements. Data were verified by different cfu countings at different OD of *M. catarrhalis* suspensions. Analogous to the results of AEBI *et al.* [31] no differences were found between the strains tested (data not shown). Therefore, these data suggest that UspA2 but not UspA1 is important for *M. catarrhalis*-induced PKC activation and IL-8 release in pulmonary epithelial cells.

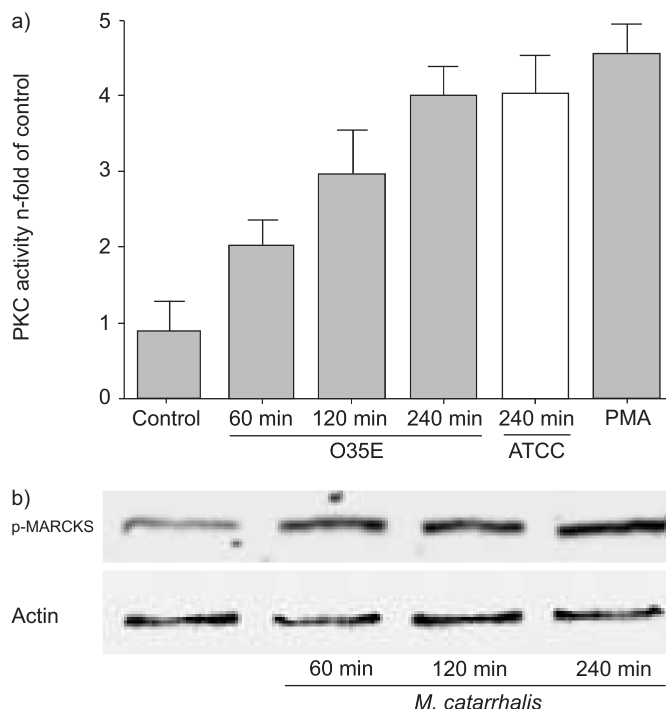


FIGURE 2. *Moraxella catarrhalis* induces protein kinase (PK)C activity in pulmonary epithelial cells. a) BEAS-2B cells were incubated with *M. catarrhalis* strain O35E (10^6 colony forming units·mL^{-1}) and PKC activity was detected by PKC activity assay. Phorbol-12-myristate-13-acetate (PMA; 160 nM, 60 min) was used as positive control. b) In BEAS-2B cells a time-dependent *M. catarrhalis*-induced phosphorylation of myristoylated, alanine-rich C-kinase substrate (MARCKS), one major substrate of PKC, was detected by Western blot. □: *M. catarrhalis* strain American Type Culture Collection (ATCC) 25238.

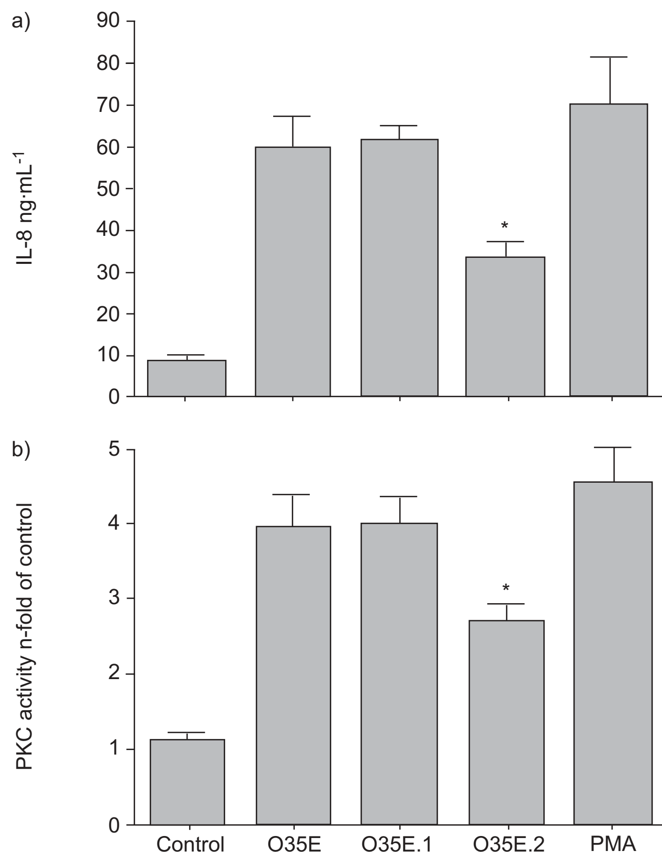


FIGURE 3. Moraxella-specific ubiquitous surface protein (Usp)A2 is important for interleukin (IL)-8 release and protein kinase (PK)C activation in pulmonary epithelial cells. a) BEAS-2B cells were incubated with *M. catarrhalis* wild-type strain O35E, the UspA1-deficient mutant O35E.1, the UspA2-deficient mutant strain O35E.2 (10^5 colony forming units·mL^{-1}) or phorbol-12-myristate-13-acetate (PMA; 160 nM) and incubated for 12 h. IL-8 secretion in the supernatants of the cells was measured by ELISA. b) BEAS-2B cells were incubated with wild-type Moraxella or the mutant strains O35E.1, O35E.2 or PMA for 2 h and PKC activity was assessed by PKC activity assay. Data presented are mean±SEM of three separate experiments. *: $p<0.05$ *versus* infected control.

M. catarrhalis*-induced IL-8 release is differentially regulated by the PKC isoforms PKCα, PKCε and PKCθ*

To further investigate the contribution of PKC and its different isoforms to *M. catarrhalis*-induced IL-8 release, BEAS-2B cells were pre-incubated with the pan-PKC inhibitors calphostin C or staurosporin for 1 h, and subsequently infected with Moraxella strain O35E. Induction of IL-8 release was assessed by ELISA. As shown in figure 4a, both inhibitors lead to a reduction of IL-8 release, suggesting an involvement of PKC in the IL-8 production in response to Moraxella infection. Next, the role of different PKC isoforms known to be expressed in pulmonary epithelium was studied. Therefore, confluent BEAS-2B cells were pre-incubated with chemical PKC isoform inhibitors blocking PKCαβ (Gö6976, PKC 20/28), PKCβ (PKCβ inhibitor), PKCδ (rottlerin), PKCε (PKCε-translocation inhibitor peptide), PKCθ (PKCθ pseudosubstrate inhibitor, myristoylated) or PKCζ (PKCζ pseudosubstrate inhibitor, myristoylated) before infecting the cells with *M. catarrhalis* for 16 h. As shown in figure 4b, blocking of PKCα and PKCε reduced *M. catarrhalis*-induced IL-8 production. In contrast, inhibiton of PKCθ led to a significant increase of the IL-8 response. Inhibition of other PKC isotypes did not affect IL-8 release as exemplarily shown for PKCβ and PKCζ (fig. 4b and data not shown). The chemical inhibitors used in these experiments did not reduce epithelial cell numbers, induce morphological signs of cytotoxicity, or alter bacterial growth within the time-frame tested (data not shown).

Next, the relevance of PKCα, PKCε and PKCθ for IL-8 expression was analysed in more detail using siRNA-mediated gene silencing. However, viability of BEAS-2B cells was significantly affected during the gene knock-out procedure (data not shown). Therefore, the pulmonary epithelial cell line A549 was used, which the current authors utilised previously with success for efficient target gene silencing [26, 32]. At first, involvement of the PKC isoforms PKCα, PKCε and PKCθ in Moraxella-induced IL-8 release was confirmed in this cell line by making use of the chemical inhibitors described previously in this article (fig. 5a). Next, siRNA was tested for ability to reduce expression of the target proteins and found that the PKCα-, PKCε- and PKCθ-specific siRNA substantially reduced protein levels of the corresponding kinase isotypes (fig. 5b–d). Moreover, knock-down of PKCα and PKCε by siRNA decreased the IL-8 production induced by O35E compared with cells transfected with control siRNA (fig. 5e and f). In contrast, transfection of PKCθ siRNA increased the Moraxella-induced IL-8 response in A549 cells (fig. 5g). Therefore, the

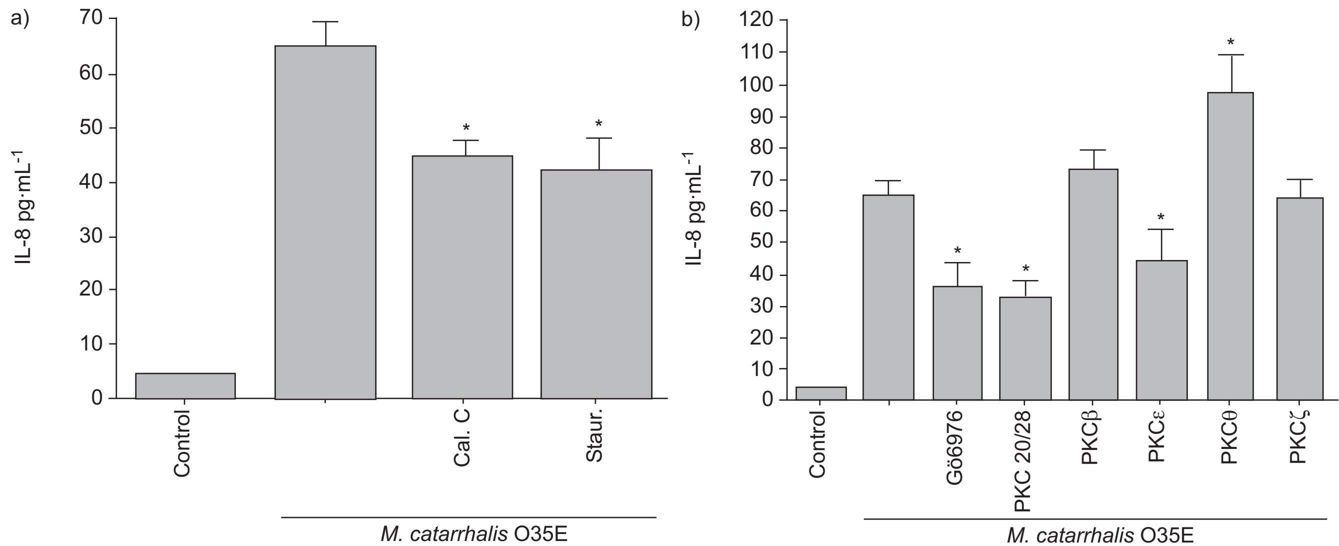


FIGURE 4. *Moraxella catarrhalis*-induced interleukin (IL)-8 release in BEAS-2B cells is differentially regulated by protein kinase (PK)C isoforms. a) BEAS-2B cells were pre-incubated (60 min) with the broad spectrum PKC inhibitors calphostin C (Cal. C; 10 μM) or staurosporin (Staur.; 50 nM) and infected with *M. catarrhalis* (10^6 colony forming units·mL^{-1}) for 12 h. b) BEAS-2B cells were pre-incubated for 1 h with inhibitors targeting the PKC isoforms PKCα and β (Gö6976 and PKC 20/28), PKCβ (PKCβ inhibitor), PKCε (PKCε translocation inhibitor peptide), PKCθ or PKCζ inhibitor (PKCθ or ζ pseudosubstrate inhibitor) and infected with *M. catarrhalis* for 12 h. IL-8 release was assessed by ELISA. ELISA data presented are mean±SEM of four separate experiments. *: $p<0.05$ *versus* stimulated control.

results confirm the different regulatory functions of these PKC isoforms for the *M. catarrhalis*-induced IL-8 release.

M. catarrhalis activates PKCα, PKCε and PKCθ activation in pulmonary epithelial cells

The translocation of kinases from cytosol to the cellular membrane is known to be an indicator for activation of these PKC isoforms [33, 34]. In order to confirm activation of the PKC isotypes PKCα, PKCε and PKCθ, translocated PKC isotypes were detected in the cell membrane following *M. catarrhalis* infection by Western blotting. As shown in figure 5a, by using a pan-specific PKC antibody, PKC strongly translocated from the cytosol to the cell membrane of infected cells. Similarly, translocation to the cell membrane could also be shown for PKCα, PKCε and PKCθ isoforms indicating activation of these isoforms by following Moraxella infection in pulmonary epithelium (fig. 5b–d).

PKC and UspA2 participate in M. catarrhalis-induced NF-κB activation

Recently, the current authors have demonstrated that *M. catarrhalis*-induced IL-8 release is dependent on the activation and translocation of NF-κB and its binding to the *il8* promoter [17]. Previous studies have demonstrated that activation of PKC by PMA and TNF-α were required for NF-κB activation in various cell types [35]. To assess the importance of PKC for *M. catarrhalis*-induced NF-κB activation, the NF-κB-dependent reporter cell line, A549 6Btkluc, was infected with *M. catarrhalis* for different time periods. Prior to the infection, cells were incubated with the pan-PKC inhibitor calphostin C. As shown in figure 7a, inhibition of PKC significantly reduced NF-κB activation measured by NF-κB luciferase activity. Moreover, infection of the cells with the UspA2-deficient mutant strain O35E.2 also resulted in a decreased NF-κB activation when compared with the wild-type strain (fig. 7a) and to the UspA1-deficient mutant strain O35E.1 (data not shown).

The PKC isoforms PKCα, PKCε and PKCθ differentially control IL-8 expression in M. catarrhalis-infected pulmonary epithelial cells

Following activation, NF-κB translocates from the cytosol to the nucleus, to bind the IL-8/κB-binding site of the *il8* promoter and induce transcription [14]. To characterise the role of the different PKC isoforms for the *M. catarrhalis*-induced transcription at the *il8* promoter, ChIP studies were employed. It was investigated whether *M. catarrhalis*-induced NF-κB activation in pulmonary epithelial cells led to an increased binding of RNA polymerase II (Pol II) at the *il8* promoter to indicate start of gene transcription [36]. As shown in figure 7b, infection of the BEAS-2B cells with *M. catarrhalis* resulted in an increased binding of Pol II to the *il8* promoter, confirming previous results of an increased IL-8 transcription induced by this pathogen (fig. 7b) [17]. To investigate the role of PKCα, PKCε and PKCθ in this process, BEAS-2B cells were pre-incubated for 60 min with the specific inhibitors of PKCα (Gö6976), PKCε (PKCε-translocation inhibitor peptide) or PKCθ (PKCθ pseudosubstrate inhibitor) before infection with *M. catarrhalis* (10^7 cfu·mL^{-1}) for an additional 120 min. Pre-incubation of the cells with PKCζ inhibitor did not influence the *M. catarrhalis*-induced IL-8 release, and was used as internal control. The present results clearly show that the *M. catarrhalis*-induced recruitment of the Pol II to the *il8* promoter was significantly reduced after inhibition of PKCα and PKCε (fig. 7b). In contrast, inhibition of PKCθ resulted in an increased binding of Pol II to the promoter in response to Moraxella infection, suggesting that *M. catarrhalis*-induced IL-8 transcription is negatively regulated by this PKC isoform

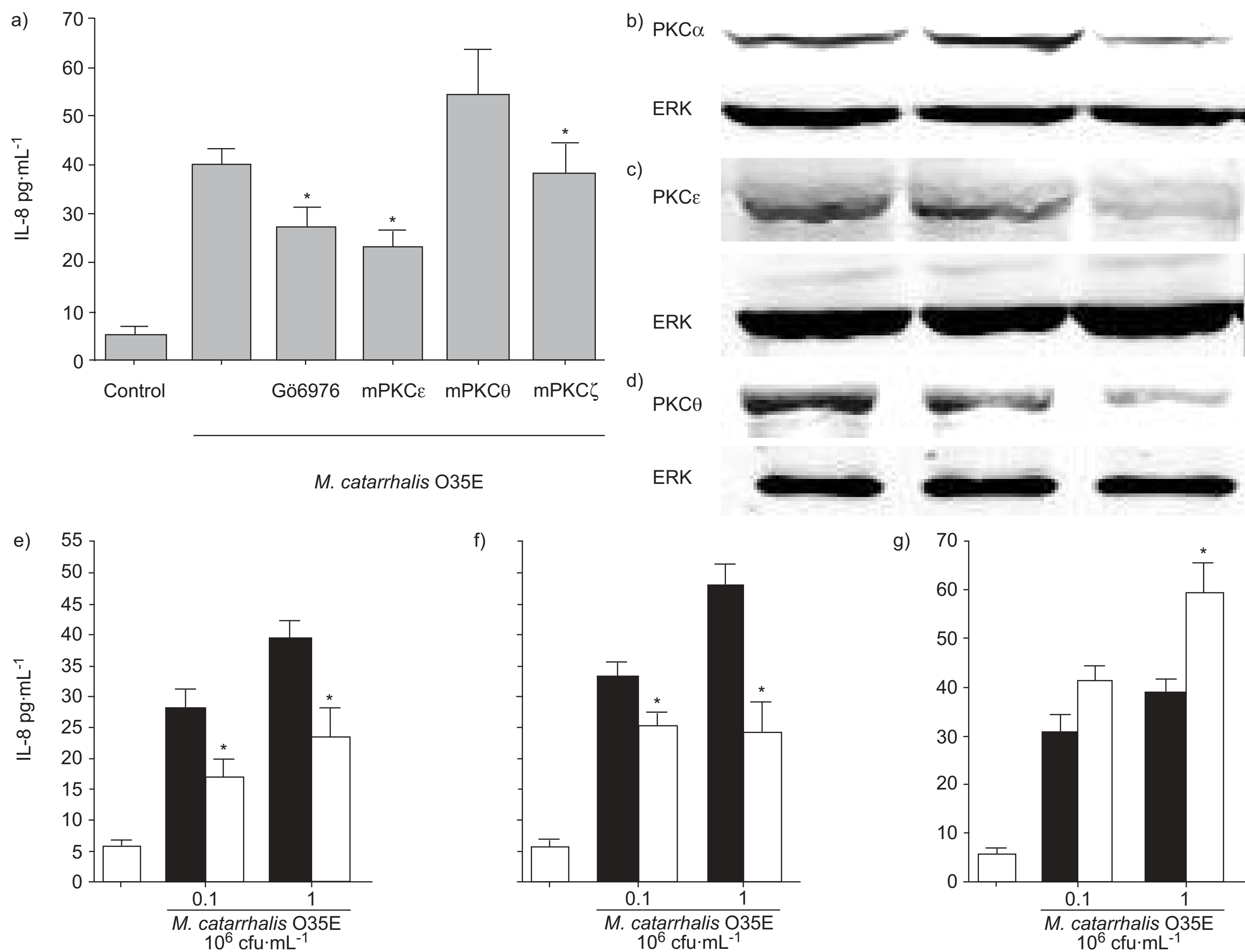


FIGURE 5. Gene silencing of the protein kinase (PK)C isoforms PKCα, PKCε and PKCθ confirmed differential regulation of interleukin (IL)-8 production in pulmonary epithelial cells. a) A549 cells were pre-incubated for 1 h with inhibitors targeting the PKC isoforms PKCα and β (Gö6976), PKCε (PKCε translocation inhibitor peptide), PKCθ (PKCθ pseudosubstrate inhibitor) or PKCζ (PKCζ pseudosubstrate inhibitor, myristoylated) or left untreated and infected with *Moraxella catarrhalis* for 12 h. IL-8 release was assessed by ELISA. A549 cells were left untreated, or were transfected with control nonsilencing siRNA (c-siRNA; middle lane) or siRNA targeting PKCα (b), PKCε (c) or PKCθ (d; all left-hand lane). After 72 h, cells were lysed and Western blots using anti-PKCα, PKCε- or PKCθ antibodies were performed. Membranes were simultaneously probed with anti-extracellular signal-regulated kinase 2 antibodies to confirm equal protein loading. Next, A549 cells were transfected with c-siRNA (■) or siRNA (□) targeting PKCα (e), PKCε (f) or PKCθ (g). Cells were either left untreated or stimulated with *M. catarrhalis* strain O35E for 16 h at the doses indicated and IL-8 in the cell supernatants were measured by ELISA. One representative experiment out of three independent experiments is shown (a, e–g), with each transfection performed in duplicate. *: $p<0.05$ *versus* wild-type strain O35E. ERK: extracellular signal-regulated kinase. cfu: colony forming units.

(fig. 7b). Inhibition of PKCζ did not change the binding pattern of Pol II when compared with the untreated but Moraxella-infected cells.

DISCUSSION

M. catarrhalis plays a pathological role in COPD exacerbations by stimulating the secretion of pro-inflammatory mediators, such as IL-8 [17, 26]. In the present study, it was demonstrated that the *M. catarrhalis*-induced NF-κB-dependent IL-8 release is controlled, at least in part, by activation of PKC. In addition, it was found that several PKC isoforms expressed in pulmonary epithelium were differentially involved in *M. catarrhalis*-induced IL-8 secretion [24]. Chemical inhibition, as well as siRNA-mediated gene silencing of PKCα and ε, significantly suppressed the *M. catarrhalis*-induced IL-8 release, whereas inhibition of PKCθ increased this release, suggesting an opposing effect of these isoforms. The activating effect of PKCα and ε on *M. catarrhalis*-induced IL-8 release, as well as the inhibiting effect of PKCθ, was accompanied by a translocation of these PKC isoforms from the cytosol to the cell membrane fraction. Other PKC isoforms expressed in pulmonary epithelium revealed no effects on *M. catarrhalis*-induced IL-8 production. Furthermore, it was demonstrated that the differential regulation of the PKC isoforms α, ε and θ was mediated through controlling the binding of the polymerase II to the *il8* promoter of respiratory epithelial cells. PKC activation was found to be dependent on the expression of the *M. catarrhalis*-specific outer membrane protein UspA2. Cells

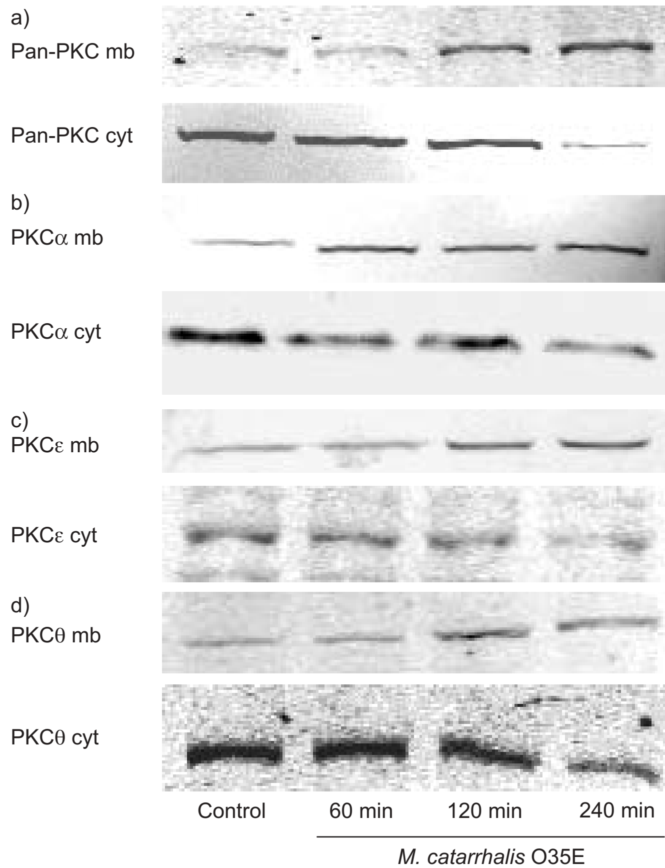


FIGURE 6. Cellular translocation studies of a) protein kinase (PK)C and its isoforms b) PKCα, c) PKCε and d) PKCθ in pulmonary epithelial cells. Translocation of PKCs from cytosol (cyt) to membrane compartments provides confirmation of their activation. To assess translocation of PKC and its isoforms PKCα, PKCε and PKCθ by Western blot, BEAS-2B epithelial cells were infected with *Moraxella catarrhalis* strain O35E (10^6 colony forming units·mL^{-1}) for 60, 120 and 240 min. Translocated PKC, as well as its isoforms PKCα, PKCε and PKCθ, could be detected after 60–120 min in the cellular membrane fraction (mb).

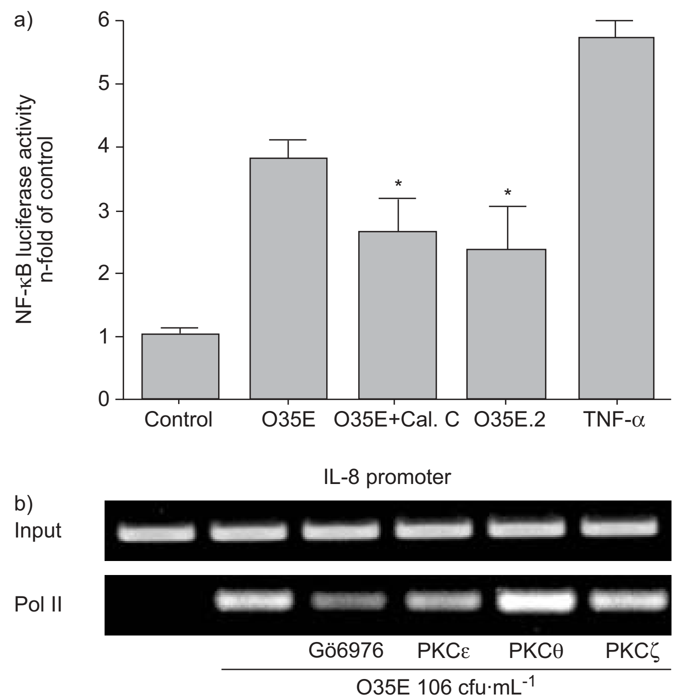


FIGURE 7. *Moraxella catarrhalis*-induced nuclear factor (NF)-κB-dependent interleukin (IL)-8 transcription is differentially regulated by the protein kinase (PK)C isoforms α, ε and θ in pulmonary epithelial cells. a) The NF-κB-dependent reporter cell line, A549 6Btkluc, was either left untreated or was pre-incubated with calphostin C (Cal. C; 10 μM) and infected with *M. catarrhalis* strain O35E or strain O35E.2 for 6 h. NF-κB luciferase activity was detected using luciferase assay. b) BEAS-2B cells were infected with *M. catarrhalis* for 2 h and the binding pattern of polymerase (Pol)II to *il8* promoter was analysed by chromatin immunoprecipitation (ChIP). BEAS-2B were either left untreated or were pre-incubated with Gö6976, PKCε-translocation inhibitor peptide and PKCθ inhibitor before infection with Moraxella and processed by ChIP. PKCζ inhibitor, which is not involved in *M. catarrhalis*-induced IL-8 secretion, was used as a control. a) Data are mean±SEM of four separate experiments; b) representative experiments out of three are shown. TNF: tumour necrosis factor; cfu: colony forming units. *: $p<0.05$ *versus* wild-type strain O35E.

infected with the UspA2-deficient *M. catarrhalis* mutant strain O35E.2 showed a decreased PKC activity resulting in a decreased NF-κB activation and IL-8 release when compared with infection with the wild-type strain or with the UspA1-deficient mutant strain O35E.1. The UspA2 protein is a putative autotransporter macromolecule that forms relatively short, filamentous projections on the surface of *M. catarrhalis* cells and is a target for biologically active antibodies [6, 37]. In contrast to UspA1, it is not functioning as adhesin but is directly involved in the expression of serum resistance found in UspA2-positive Moraxella strains [5, 38, 39]. In addition, the results of the present study suggest that the UspA2-mediated activation of PKC controlling the NF-κB-dependent IL-8 release may represent a new virulence mechanism of Moraxella UspA2 in respiratory epithelial cells. The main limitation of the present investigation is that it is an *in vitro* study making use of human bronchial epithelial cells. However, studying the mechanisms of the pathogenesis of this strictly human-specific pathogen is difficult because of the lack of simple animal models that mimic the pathogen–host interactions seen in humans. Therefore, as documented in several publications, *in vitro* cell culture methods provide a useful alternative for the investigation of the interactions between Moraxella, or other potentially pathogenic microorganisms, and the host pulmonary epithelium that occur during infection [13, 26, 29].

Recent reports support a role for PKC in the regulation of pro-inflammatory gene expression in airway epithelial cells. PKC activation by PMA has been shown to stimulate NF-κB activation and to induce granulocyte-macrophage colony-stimulating factor (GM-CSF) expression in A549 cells and human bronchial epithelial cells [40, 41]. In addition, several studies have demonstrated airway epithelial cell TNF-α responses to be at least partially sensitive to PKC inhibitors. In human bronchial epithelial cells, cigarette smoke-induced, as well as C5a-mediated, IL-8 expression [42] and TNF-α-induced intercellular adhesion molecule-1 expression [43] were blocked by calphostin C, and TNF-α-induced GM-CSF expression was inhibited by the pan-PKC blocker staurosporine [44]. In addition, HEWSON *et al.* [45] reported that PMA-induced expression of the airway mucins MUC5B and MUC5AC by

airway epithelial cells were attenuated after treatment of the cells with the pan-PKC inhibitor calphostin C. Although these studies did not determine the PKC isoenzymes responsible for phorbol ester-induced responses, they are consistent with the findings of the present study, that PKC isoforms may regulate airway epithelial cell gene expression in an NF-κB-dependent manner. The PKC family has long been known to play pivotal roles in controlling inflammation, cell growth, differentiation and apoptosis by regulating the activity of transcription factors such as NF-κB [19, 46]. However, their biological effects exerted by the activation of PKC may be stimulus- as well as cell- or tissue-specific. In epithelial cells, little is known concerning pathogen-induced specific regulation of PKC, and in particular PKC isoforms, and with regard to NF-κB-dependent pro-inflammatory cytokine transcription. *Candida albicans* was found to upregulate cyclooxygenase (COX)-2 through activation of PKC in epithelial cells [47]. Moreover, pathogen-related expression of COX-2 in *Helicobacter pylori*- or *Staphylococcus aureus*-infected epithelial cells was shown to be mediated by PKC activation [48, 49]. The current authors recently demonstrated that COX-2 expression and subsequent PGE_2-synthesis induced by *Legionella neumophila* infection of the alveolar epithelial cell line A549 was reliant on PKCα and NF-κB activation in lung epithelial cells [28]. As shown in the present study, infection of pulmonary epithelial cells with *M. catarrhalis* activated PKC, which triggered NF-κB activation resulting in IL-8 transcription and release. Moreover, the current authors found that the PKC isoforms PKCα, PKCε and PKCθ were involved in the *M. catarrhalis*-induced IL-8 release by differentially regulating IL-8 transcription. Therefore, the results suggest a specific regulation of PKC isoforms with regard to IL-8 induction in Moraxella-infected pulmonary epithelial cells.

The levels and different compositions of the protein kinase C isoforms represent a tissue-dependent balance between expression and degradation, which may be altered in settings of cellular stress, such as acute or chronic inflammation or smoking [50]. Changes in the expression of protein kinase C isoforms may further influence the inflammatory immune response following *Moraxella catarrhalis* infection. However, limited information exists about protein kinase C in human pulmonary epithelial cells and further studies are needed to analyse the composition of protein kinase C isoforms in normal and diseased pulmonary epithelium. A better understanding of the mechanisms of how individual protein kinase C isozymes contribute to the pathogenesis of bacterial infections in chronic obstructive pulmonary disease patients may provide a new therapeutic target to combat this chronic inflammatory disease.

ACKNOWLEDGEMENTS

We greatly appreciate the excellent technical assistance of J. Hellwig, F. Schreiber and D. Stoll (Charité – Universitätsmedizin Berlin). Part of this work will be included in the doctoral thesis of L. Maqami (Charité – Universitätsmedizin Berlin).

REFERENCES

1 Lopez AD, Mathers CD. Measuring the global burden of disease and epidemiological transitions: 2002–2030. *Ann Trop Med Parasitol* 2006; 100: 481–499.
2 Verduin CM, Hol C, Fleer A, van Dijk H, van Belkum A. *Moraxella catarrhalis*: from emerging to established pathogen. *Clin Microbiol Rev* 2002; 15: 125–144.
3 Murphy TF, Brauer AL, Grant BJ, Sethi S. *Moraxella catarrhalis* in chronic obstructive pulmonary disease: burden of disease and immune response. *Am J Respir Crit Care Med* 2005; 172: 195–199.
4 McMichael JC. Vaccines for *Moraxella catarrhalis*. *Vaccine* 2000; 19: Suppl. 1, S101–S107.
5 Meier PS, Troller R, Grivea IN, Syrogiannopoulos GA, Aebi C. The outer membrane proteins UspA1 and UspA2 of *Moraxella catarrhalis* are highly conserved in nasopharyngeal isolates from young children. *Vaccine* 2002; 20: 1754–1760.
6 Attia AS, Hansen EJ. A conserved tetranucleotide repeat is necessary for wild-type expression of the *Moraxella catarrhalis* UspA2 protein. *J Bacteriol* 2006; 188: 7840–7852.
7 Barnes PJ, Shapiro SD, Pauwels RA. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *Eur Respir J* 2003; 22: 672–688.
8 Hippenstiel S, Opitz B, Schmeck B, Suttorp N. Lung epithelium as a sentinel and effector system in pneumonia molecular mechanisms of pathogen recognition and signal transduction. *Respir Res* 2006; 7: 97.
9 Mukaida N. Pathophysiological roles of interleukin-8/CXCL8 in pulmonary diseases. *Am J Physiol Lung Cell Mol Physiol* 2003; 284: L566–L577.
10 Richman-Eisenstat JB, Jorens PG, Hebert CA, Ueki I, Nadel JA. Interleukin-8: an important chemoattractant in sputum of patients with chronic inflammatory airway diseases. *Am J Physiol Lung Cell Mol Physiol* 1993; 264: L413–L418.
11 Di Stefano A, Capelli A, Donner CF. Role of interleukin-8 in the pathogenesis and treatment of COPD. *Chest* 2004; 126: 676–678.
12 Patel IS, Seemungal TAR, Wilks M, Lloyd-Owen SJ, Donaldson GC, Wedzicha JA. Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. *Thorax* 2002; 57: 759–764.
13 Schulz C, Wolf K, Harth M, Kratzel K, Kunz-Schughart L, Pfeifer M. Expression and release of interleukin-8 by human bronchial epithelial cells from patients with chronic obstructive pulmonary disease, smokers, and never-smokers. *Respiration* 2003; 70: 254–261.
14 Wickremasinghe MI, Thomas LH, Friedland JS. Pulmonary epithelial cells are a source of IL-8 in the response to *Mycobacterium tuberculosis*: essential role of IL-1 from infected monocytes in a NF-κB-dependent network. *J Immunol* 1999; 163: 3936–3947.
15 Griffin BD, Moynagh PN. *In vivo* binding of NF-κB to the IκB β promoter is insufficient for transcriptional activation. *Biochem J* 2006; 400: 115–125.
16 Li Y, Flanagan PM, Tschochner H, Kornberg RD. RNA polymerase II initiation factor interactions and transcription start site selection. *Science* 1994; 263: 805–807.
17 Slevogt H, Schmeck B, Jonatat C, *et al. Moraxella catarrhalis* induces inflammatory response of bronchial epithelial cells *via* MAPK and NF-κB activation and histone deacetylase activity reduction. *Am J Physiol Lung Cell Mol Physiol* 2006; 290: L818–L826.

18 Adcock IM, Chung KF, Caramori G, Ito K. Kinase inhibitors and airway inflammation. *Eur J Pharmacol* 2006; 533: 118–132.

19 Dempsey EC, Newton AC, Mochly-Rosen D, *et al.* Protein kinase C isozymes and the regulation of diverse cell responses. *Am J Physiol Lung Cell Mol Physiol* 2000; 279: L429–L438.

20 Jordan NJ, Watson ML, Yoshimura T, Westwick J. Differential effects of protein kinase C inhibitors on chemokine production in human synovial fibroblasts. *Br J Pharmacol* 1996; 117: 1245–1253.

21 Liu WS, Heckman CA. The sevenfold way of PKC regulation. *Cell Signal* 1998; 10: 529–542.

22 Clark AS, West KA, Blumberg PM, Dennis PA. Altered protein kinase C (PKC) isoforms in non-small cell lung cancer cells: PKCδ promotes cellular survival and chemotherapeutic resistance. *Cancer Res* 2003; 63: 780–786.

23 Webb BLJ, Lindsay MA, Seybold J, *et al.* Identification of the protein kinase C isoenzymes in human lung and airways smooth muscle at the protein and mRNA level. *Biochem Pharmacol* 1997; 54: 199–205.

24 Woo CH, Lim JH, Kim JH. VCAM-1 upregulation *via* PKCδ-p38 kinase-linked cascade mediates the TNF-α-induced leukocyte adhesion and emigration in the lung airway epithelium. *Am J Physiol Lung Cell Mol Physiol* 2005; 288: L307–L316.

25 Slevogt H, Tiwari KN, Schmeck B, *et al.* Adhesion of *Moraxella catarrhalis* to human bronchial epithelium characterized by a novel fluorescence-based assay. *Med Microbiol Immunol (Berl)* 2005; 195: 73–83.

26 Slevogt H, Seybold J, Tiwari KN, *et al. Moraxella catarrhalis* is internalized in respiratory epithelial cells by a trigger-like mechanism and initiates a TLR2- and partly NOD1-dependent inflammatory immune response. *Cell Microbiol* 2007; 9: 694–707.

27 Bergmann M, Hart L, Lindsay M, Barnes PJ, Newton R. IκBα degradation and nuclear factor-κB DNA binding are insufficient for interleukin-1β and tumor necrosis factor-α-induced κB-dependent transcription. Requirement for an additional activation pathway. *J Biol Chem* 1998; 273: 6607–6610.

28 N'Guessan PD, Etouem MO, Schmeck B, *et al. Legionella pneumophila*-induced PKCα-, MAPK-, and NF-κB-dependent COX-2 expression in human lung epithelium. *Am J Physiol Lung Cell Mol Physiol* 2007; 292: L267–L277.

29 Yang F, Haile DJ, Wang X, Dailey LA, Stonehuerner JG, Ghio AJ. Apical location of ferroportin 1 in airway epithelia and its role in iron detoxification in the lung. *Am J Physiol Lung Cell Mol Physiol* 2005; 289: L14–L23.

30 Li YH, Martin LD, Spizz G, Adler KB. MARCKS protein is a key molecule regulating mucin secretion by human airway epithelial cells *in vitro*. *J Biol Chem* 2001; 276: 40982–40990.

31 Aebi C, Lafontaine ER, Cope LD, *et al.* Phenotypic effect of isogenic uspA1 and uspA2 mutations on *Moraxella catarrhalis* 035E. *Infect Immun* 1998; 66: 3113–3119.

32 Opitz B, Vinzing M, van Laak V, *et al. Legionella pneumophila* induces IFN-β in lung epithelial cells *via* IPS-1 and IRF3, which also control bacterial replication. *J Biol Chem* 2006; 281: 36173–36179.

33 Juan-Vergara H, Peeples ME, Lockey RF, Mohapatra SS. Protein kinase C-α activity is required for respiratory syncytial virus fusion to human bronchial epithelial cells. *J Virol* 2004; 78: 13717–13726.

34 Hippenstiel S, Kratz T, Krull M, Seybold J, Eichel-Streiber C, Suttorp N. Rho protein inhibition blocks protein kinase C translocation and activation. *Biochem Biophys Res Commun* 1998; 245: 830–834.

35 Lallena MJ, Diaz-Meco MT, Bren G, Paya CV, Moscat J. Activation of IκB kinase β by protein kinase C isoforms. *Mol Cell Biol* 1999; 19: 2180–2188.

36 Griffin BD, Moynagh PN. *In vivo* binding of NF-κB to the IκB β promoter is insufficient for transcriptional activation. *Biochem J* 2006; 400: 115–125.

37 Chen D, Barniak V, VanDerMeid KR, McMichael JC. The levels and bactericidal capacity of antibodies directed against the UspA1 and UspA2 outer membrane proteins of *Moraxella (Branhamella) catarrhalis* in adults and children. *Infect Immun* 1999; 67: 1310–1316.

38 Attia AS, Lafontaine ER, Latimer JL, Aebi C, Syrogiannopoulos GA, Hansen EJ. The UspA2 protein of *Moraxella catarrhalis* is directly involved in the expression of serum resistance. *Infect Immun* 2005; 73: 2400–2410.

39 Bootsma HJ, van der Heide HGJ, van de Pas S, Schouls LM, Mooi FR. Analysis of *Moraxella catarrhalis* by DNA typing: evidence for a distinct subpopulation associated with virulence traits. *J Infect Dis* 2000; 181: 1376–1387.

40 Bergmann M, Barnes PJ, Newton R. Molecular regulation of granulocyte macrophage colony-stimulating factor in human lung epithelial cells by interleukin (IL)-1β, IL-4, and IL-13 involves both transcriptional and post-transcriptional mechanisms. *Am J Respir Cell Mol Biol* 2000; 22: 582–589.

41 Newton R, Hart LA, Stevens DA, *et al.* Effect of dexamethasone on interleukin-1β-(IL-1β)-induced nuclear factor-kappaB (NF-κB) and κB-dependent transcription in epithelial cells. *Eur J Biochem* 1998; 254: 81–89.

42 Wyatt TA, Heires AJ, Sanderson SD, Floreani AA. Protein kinase C activation is required for cigarette smoke-enhanced C5a-mediated release of interleukin-8 in human bronchial epithelial cells. *Am J Respir Cell Mol Biol* 1999; 21: 283–288.

43 Krunkosky TM, Fischer BM, Martin LD, Jones N, Akley NJ, Adler KB. Effects of TNF-α on expression of ICAM-1 in human airway epithelial cells *in vitro*. Signaling pathways controlling surface and gene expression. *Am J Respir Cell Mol Biol* 2000; 22: 685–692.

44 Reibman J, Talbot AT, Hsu Y, *et al.* Regulation of expression of granulocyte-macrophage colony-stimulating factor in human bronchial epithelial cells: roles of protein kinase C and mitogen-activated protein kinases. *J Immunol* 2000; 165: 1618–1625.

45 Hewson CA, Edbrooke MR, Johnston SL. PMA induces the MUC5AC respiratory mucin in human bronchial epithelial cells, *via* PKC, EGF/TGF-α, Ras/Raf, MEK, ERK and Sp1-dependent mechanisms. *J Mol Biol* 2004; 344: 683–695.

46 Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. *Nat Rev Cancer* 2007; 7: 281–294.

47 Deva R, Shankaranarayanan P, Ciccoli R, Nigam S. *Candida albicans* induces selectively transcriptional activation of cyclooxygenase-2 in HeLa Cells: pivotal roles of toll-like

receptors, p38 mitogen-activated protein kinase, and NF-κB. *J Immunol* 2003; 171: 3047–3055.

48 Chang YJ, Wu MS, Lin JT, *et al.* Induction of cyclooxygenase-2 overexpression in human gastric epithelial cells by *Helicobacter pylori* involves TLR2/TLR9 and c-Src-dependent nuclear factor-κB activation. *Mol Pharmacol* 2004; 66: 1465–1477.

49 Lin CH, Kuan IH, Lee HM, *et al.* Induction of cyclooxygenase-2 protein by lipoteichoic acid from *Staphylococcus aureus* in human pulmonary epithelial cells: involvement of a nuclear factor-κB-dependent pathway. *Br J Pharmacol* 2001; 134: 543–552.

50 Dempsey EC, Cool CD, Littler CM. Lung disease and PKCs. *Pharmacol Res* 2007; 55: 545–559.