AIRWAY BIOLOGY

Phosphodiesterase 4 inhibition decreases MUC5AC expression induced by epidermal growth factor in human airway epithelial cells

M Mata, B Sarriá, A Buenestado, J Cortijo, M Cerdá, E J Morcillo


Background: A common pathological feature of chronic inflammatory airway diseases such as asthma and chronic obstructive pulmonary disease (COPD) is mucus hypersecretion. MUC5AC is the predominant mucin gene expressed in healthy airways and is increased in asthmatic and COPD patients. Recent clinical trials indicate that phosphodiesterase type 4 (PDE4) inhibitors may have therapeutic value for COPD and asthma. However, their direct effects on mucin expression have been scarcely investigated.

Methods: MUC5AC mRNA and protein expression were examined in cultured human airway epithelial cells (A549) and in human isolated bronchial tissue stimulated with epidermal growth factor [EGF; 25 ng/ml]. MUC5AC mRNA was measured by real time RT-PCR and MUC5AC protein by EUSA (cell lysates and tissue homogenates). Western blotting (tissue homogenates) and immunohistochemistry.

Results: EGF increased MUC5AC mRNA and protein expression in A549 cells. PDE4 inhibitors produced a concentration dependent inhibition of the EGF induced MUC5AC mRNA and protein expression with potency values (−log IC50): rolipram (−7.5) > cilomilast (−6.5) > cilomilast (−5.5). Roflumilast also inhibited the EGF induced expression of phosphotyrosine proteins, EGF receptor, and phospho-p38- and p44/42-MAPK measured by Western blot analysis in A549 cells. In human isolated bronchus, EGF induced MUC5AC mRNA and protein expression was inhibited by roflumilast (1 μM) as well as the MUC5AC positive staining shown by immunohistochemistry.

Conclusion: Selective PDE4 inhibition is effective in decreasing EGF induced MUC5AC expression in human airway epithelial cells. This effect may contribute to the clinical efficacy of this new drug category in mucus hypersecretory diseases.

Mucus hypersecretion is an important feature of chronic inflammatory airway diseases such as chronic obstructive pulmonary disease (COPD) and asthma, and contributes to their morbidity and mortality.1 2 MUC5AC is the predominant mucin gene expressed in healthy human airway epithelial cells and its expression is increased in asthmatic and COPD patients,3 yet MUC5B upregulation is a significant component of airway mucus in asthma4 and COPD.5 Mucin MUC5AC expression in response to many different stimuli appears regulated by an epidermal growth factor receptor (EGFR) signalling cascade.6 Although sparse in healthy adult human airways, EGFR expression is upregulated by proinflammatory cytokines and in chronic airway diseases such as asthma, suggesting that it may have a role in the pathogenesis of mucus hypersecretion in these conditions.1 7

Cyclic AMP (cAMP) is an important second messenger determining many aspects of cellular function through the activation of protein kinase A (PKA). This cyclic nucleotide is inactivated by phosphodiesterases (PDEs). Many distinct forms of PDEs have been described, but PDE4 appears to be the major PDE isoenzyme involved in the regulation of cAMP mediated functions in airway inflammatory and structural cells.8 In vitro and in vivo studies have established that selective PDE4 inhibitors suppress the activity of many proinflammatory and immune cells, indicating that they may be effective in the treatment of airway inflammatory diseases. Indeed, oral PDE4 inhibitors are in phase II/III clinical trials for COPD and asthma.9 Recent work has shown that rolipram, the archetypal PDE4 inhibitor, markedly decreased goblet cell hyperplasia in animal models of secondary allergen challenge and chronic lipopolysaccharide exposure.5 10 This effect of rolipram was attributed to its known ability to reduce the release of inflammatory mediators which activate goblet cells. However, the direct effects of PDE4 inhibitors on mucin gene expression and production by airway epithelial cells have not so far been investigated to our knowledge.

Normal human airway epithelial cells as well as the human pulmonary epithelial A549 cells predominantly express PDE4 with lesser activity of other PDEs.11 12 Epithelial PDE4 activity may therefore be an important target for monoselective PDE4 inhibitors in the control of those inflammatory mediators produced by these cells. Furthermore, the functioning of the cAMP/PKA pathway appears to be linked to that of the extracellular signal regulated kinase (ERK)/mitogen activated protein kinase (MAPK) pathway, the downstream signalling of the EGFR.13

The aim of this study was to examine the effects of PDE4 inhibition on the MUC5AC mucin gene expression and production triggered by the activation of the EGFR with one of its endogenous ligands, the epidermal growth factor (EGF), in cultured human airway epithelial cells (A549 cells) and in human isolated bronchus.

METHODS

Preparations and chemicals

The human pulmonary epithelial cancer cell line (A549) was purchased from ATCC (American Type Culture Collection; Rockville, MD, USA). This cell line has previously been shown to be appropriate for studies of MUC5AC mRNA and protein expression.14 A549 cells were grown on 24-well cultured plates for MUC5AC mRNA experiments or T25 flasks for MUC5AC protein experiments (Corning, NY, USA).
in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% endotoxin-free fetal calf serum (FCS), 10 mM HEPES, l-glutamine (4 mM), and standard antimicrobials.

Human lung tissue was obtained from patients (five men, one woman) of mean age 59 years (range 48–69) who had undergone surgery for lung carcinoma as previously outlined. Experiments were approved by the local ethics committee and informed consent was obtained. At the time of operation all patients were active smokers but lung function was within normal limits by spirometry. None of the patients was being chronically treated with theophylline, and all patients were active smokers but lung function was within normal limits by spirometry. None of the patients was being chronically treated with theophylline.

To study the role of EGF in MUC5AC expression, the comparative Ct (ΔCt) method was used for obtaining quantitative data of relative gene expression, the comparative Ct (ΔΔCt) method, was as described by the manufacturer (PE-ABI PRISM 7700 Sequence Detection System; Perkin-Elmer Applied Biosystems, Perkin-Elmer Corporation, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as the endogenous control gene. Total RNA was extracted using TriPure isolation reagent (Roche, IN, USA). The PCR primers and probes for human MUC5AC and GAPDH were designed using the Primer Express (PE Biosystems, Morrisville, NC, USA) according to the published human MUC5AC and GAPDH cDNA sequences (table 1).

MUC5AC protein in A549 cells and human bronchial tissues was measured by enzyme linked immunosorbent assay (ELISA) as outlined previously. In brief, for cell lysates, A549 cells cultured in T25 flasks were trypsinised, washed in PBS, centrifuged (5 minutes, 300 g, 4 °C), and resuspended in five volumes of ice cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1% SDS, 50 mM NaCl, 2 mM EDTA, 1 mM MgCl2, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM diithiothreitol (DTT), 2 µg/ml leupeptin, 5 µg/ml aprotinin, 5 µg/ml pepstatin), vortexed for 20 seconds, sonicated, and centrifuged (30 minutes, 13 000 g, 4 °C). Human bronchial tissues were homogenised in five volumes of ice cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 2 mM MgCl2, 1 mM phenylmethylsulphonyl fluoride, 1 mM diithiothreitol, 2 µg/ml leupeptin, 5 µg/ml aprotinin, and 5 µg/ml pepstatin) and centrifuged (35 minutes, 13 000 g, 4 °C). The total protein in cell and tissue samples was estimated using the Bradford assay.

Experimental protocol

In preliminary experiments with A549 cells the MUC5AC expression in response to EGF stimulation was determined at 3, 12, 18 and 24 hours. Peak responses were observed at 18–24 hours for MUC5AC mRNA and at 24 hours for MUC5AC protein; an incubation time of 24 hours was therefore selected. Also, 25 ng/ml EGF was selected as a near maximal response from pilot experiments with EGF (5–50 ng/ml). The selected EGF concentration and time of observation are within the values reported by others in cultured airway epithelial cells. For human isolated bronchus, MUC5AC responses to EGF stimulation were studied at 0.5, 1, 3, 12 and 24 hours. In inhibition studies A549 cells and human bronchus were pretreated with drugs or their vehicles for 15 minutes before stimulation with EGF and remained until termination of experiments. When used, antagonists were added 15 minutes before the corresponding drug and remained for the rest of the experiment.

### Table 1

<table>
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<tr>
<th>Gene</th>
<th>Primers and probes</th>
<th>Sequence</th>
<th>Product size (bp)</th>
<th>GenBank accession no</th>
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<td>102</td>
<td>U60711</td>
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<tr>
<td></td>
<td>Reverse</td>
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<tr>
<td></td>
<td>TaqMan probe</td>
<td>5′-TGACCGTGTGCAATTAGCGGGA-3′</td>
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<tr>
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<td>TaqMan probe</td>
<td>5′-CAAGCTTTCTGCTCAGGCC-3′</td>
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</table>

bp, base pairs.

For MUC5AC, reverse transcription of RNA to generate cDNA was performed with Taqman RT reagents (ref. N808-0234; Applied Biosystems, NJ, USA) and the PCR was performed with TaqMan Universal PCR Master Mix (ref. 4304437; Applied Biosystems). The specificity of PCR primers was tested under normal PCR conditions and the products of the reaction were electrophoresed into a 2.5% NuSieve® GTG® agarose gel (BMA, Rockland, ME, USA). One single band with the expected molecular size was observed for MUC5AC and GAPDH. For the validation of the ΔΔCt method, the Ct values for target (MUC5AC) and reference (GAPDH) genes were measured at different input amounts of total RNA (2.34–300 ng); ΔCt values (target vs reference) were then plotted against log total RNA and the absolute value of the slope was found to be 0.008 (i.e. <0.1), indicating similar efficiency of the two systems.
MUC5AC and has no cross reactivity with other mucins). After 1 hour the plates were washed with PBS and then incubated with 100 μl horseradish peroxidase-goat anti-mouse IgG conjugated (1:10 000). The colour reaction was developed with TMB peroxidase solution (Sigma) and stopped with 1 M H2SO4. Absorbance was read at 450 nm.

In addition, Western blot analysis of MUC5AC was carried out in human bronchial homogenates as previously described.

![Figure 1](http://thorax.bmj.com/)

**Figure 1** Relative quantitation of MUC5AC mRNA and protein levels in A549 cells unstimulated (control) or stimulated with epidermal growth factor (EGF; 25 ng/ml, 24 hours incubation) in the absence or presence of selective inhibitors of EGF receptor tyrosine kinase activity ( tyrphostin A46 and AG1478; upper panels) or a selective phosphodiesterase 4 inhibitor (roflumilast; lower panels). Incubation with DMSO (0.1% v/v) was without significant effect on MUC5AC expression in the absence and presence of EGF (upper panel). The EGF induced increase in MUC5AC expression was abolished by pre-incubation with EGF receptor tyrosine kinase inhibitors (A46 100 μM or AG1478 3 μM) or roflumilast (1 μM). MUC5AC mRNA was determined using real time RT-PCR by the ΔΔCT method; columns show the fold increase in expression of MUC5AC relative to GAPDH values as mean (SE) of the 2-ΔΔCT values of three independent experiments. MUC5AC protein was determined by enzyme linked immunosorbent assay (ELISA); columns show the fold increase from control levels as mean (SE) values of three independent experiments. *p<0.05 v control; †p<0.05 v EGF.

![Figure 2](http://thorax.bmj.com/)

**Figure 2** Concentration-response curves for inhibition by the selective PDE4 inhibitors roflumilast, cilomilast and rolipram of the epidermal growth factor (25 ng/ml; 24 hours incubation) induced expression of MUC5AC mRNA (left panel) and protein (right panel) in A549 cells. MUC5AC mRNA and protein were determined as indicated in fig 1. Points are mean (SE) values of three to five independent experiments. The corresponding IC50 value for each PDE4 inhibitor is shown in the Results section.
reported. In brief, aliquots of supernatants from 13 000 g centrifugation of the tissue homogenate containing 25 μg total protein were suspended in SDS sample buffer and boiled for 5 minutes. Proteins were separated by SDS-PAGE electrophoresis in 8% acrylamide-bisacrylamide (80:1). The resulting gel was equilibrated in the transfer buffer: 25 mM Tris-HCl, 192 mM glycine, and 20% (v/v) methanol, pH 8.3. The proteins were then transferred electrophoretically to nitrocellulose membranes which were incubated with 5% fat-free skimmed milk in phosphate buffered saline (PBS) containing 0.5% BSA and 0.05% Tween 20 for 1 hour, and incubated with mAb to MUC5AC (clone 45M1, 1:500, NeoMarkers) for 2 hours at room temperature. Bound antibody was visualised according to standard protocols for the avidin-biotin-alkaline phosphatase complex method (ABC kit; Vector Laboratories, Burlingame, CA, USA).

For MUC5AC immunocytochemical analysis of human bronchus, specimens were fixed, cut into sections, stained with haematoxylin-eosin and periodic acid-Schiff (PAS) reagent (to visualise goblet cells), and incubated with mouse monoclonal antibody to MUC5AC (clone 45M1, 1:100; NeoMarkers, Fremont, CA) as previously reported.

### Western blotting of EGFR, phospho-p38 MAPK, phospho-p44/42 MAPK and phosphotyrosine

A549 cells were prepared for western blot analysis as indicated above, and preparations were incubated with either EGFR mouse mAb (Ab-12, cocktail R19/48, NeoMarkers, CA, USA), phospho-p38 MAPK (Thr180/Tyr182) mAb (28B10; Cell Signaling Technology, Beverly, MA, USA), phospho-p44/42 MAPK (Thr202/Tyr204) mAb (20G11; Cell Signaling Technology), or anti-phosphotyrosine mAb (clone PY20; ICN Biomedical Inc, Aurora, OH, USA) according to the manufacturer (RPN225; Amersham Life Sciences, UK).

Western blot analysis with PY20 anti-phosphotyrosine antibody, anti-EGFR antibody, and phospho-p38 and phospho-p44/42 MAPK antibodies in A549 cell lysates as indicated. Control levels are shown in lane A and the activation produced by EGF (25 ng/ml) is shown in lane B. Pretreatment with roflumilast (1 μM; lane C) reduced the EGF induced response. The duration of EGF exposure was 24 hours for anti-phosphotyrosine and EGFR experiments and 15 minutes for phospho-p38 and phospho-p44/42 MAPK experiments. Data presented are representative of three separate experiments.

### Measurement of cAMP accumulation

Formation of cAMP was measured as previously outlined. Cultured A549 cells were exposed to EGF or vehicle in the absence or presence of roflumilast for the indicated times, and the cAMP content was quantified using an enzyme immunoassay kit according to the assay protocol provided by the manufacturer (RPN225; Amersham Life Sciences, UK).

### Cytotoxicity assessment

To exclude the presence of non-selective detrimental effects of the compounds used, the percentage of lactate...
Statistical analysis
Data are expressed as mean (SE) of n experiments. In concentration-response experiments the \(-\log\) inhibitory concentration 50% (IC\(_{50}\)) was calculated by non-linear regression to express compound potency (GraphPad Software Inc, San Diego, USA). Statistical analysis was carried out by analysis of variance followed by appropriate post hoc tests including Bonferroni correction. Significance was accepted as \(p<0.05\).

RESULTS
Cytotoxicity studies and drug vehicle effects
None of the compounds at their maximal concentrations used showed any significant cytotoxicity (values for LDH release were below 5%).

DMSO (0.1% v/v) did not alter the MUC5AC mRNA and protein expression in the absence and presence of EGF 25 ng/ml (fig 1).

Effect of PDE4 inhibition on EGF induced MUC5AC expression and EGFR signalling cascade in A549 cells
EGF (25 ng/ml; 24 hours incubation) increased MUC5AC gene expression and protein production in A549 cells (fig 1). This finding was confirmed by immunocytochemical staining for MUC5AC (not shown). The dependency of this response on the tyrosine kinase activity of the EGFR was confirmed by inhibition of the EGF induced increase in MUC5AC mRNA.
PDE4 inhibition and MUC5AC expression in airway epithelial cells

and protein in the presence of two different selective inhibitors of EGFR tyrosine kinase (tyrophostin A46 and AG1478, fig 1). Rolflumilast (1 μM), a PDE4 inhibitor, did not change basal MUC5AC expression but prevented the increase in MUC5AC mRNA and protein production in response to EGF (fig 1). The relationship between the suppression of EGF induced MUC5AC expression and the PDE4 inhibition was further explored by examining the inhibitory effects of other structurally unrelated PDE4 inhibitors and by exploring their concentration dependency. The increase in MUC5AC mRNA and protein by EGF was inhibited in a concentration-related fashion by pretreatment of cells with the PDE4 inhibitors rolflumilast, cilomilast, and rolipram (fig 2). The rank order of potencies (−log IC₅₀ values) was rolflumilast (7.59 (0.27)) > rolipram (6.66 (0.26)) > cilomilast (5.58 (0.23)) for MUC5AC mRNA, and rolflumilast (7.37 (0.12)) > rolipram (6.17 (0.16)) > cilomilast (5.27 (0.10)) for MUC5AC protein. A fully active concentration of rolflumilast (1 μM) was selected for additional experiments.

Addition of EGF (25 ng/ml; 24 hours incubation) to A549 cells resulted in the phosphorylation of the tyrosine residues of different intracellular proteins and the augmented expression of the EGFR, as shown by Western blot analysis of cell lysates with the corresponding specific antibodies (fig 3). Expression of phospho-p38 MAPK and phospho-p44/42 MAPK reached peak values after 15 minutes of exposure to EGF (25 ng/ml). Treatment with rolflumilast (1 μM) abolished these EGF induced responses (fig 3). The functional requirement for p38 MAPK and for p44/42 MAPK in the EGF induced augmentation of MUC5AC mRNA was shown by using their respective selective inhibitors SB202190 and PD98059 (fig 4).

Relationship between inhibition of EGF induced MUC5AC expression by PDE4 inhibitors and the cAMP/PKA pathway in A549 cells

We then examined whether the inhibitory effect of rolflumilast on the overexpression of MUC5AC promoted by EGF was related to its ability to inhibit PDE4, thus increasing cAMP and subsequently activating PKA. EGF alone failed to alter the cellular content of cAMP significantly. Rolflumilast (1 μM) produced an early (peak at 5 minutes) and transient increase in the cAMP content of A549 cells (fig 5). The inhibitory effect of rolflumilast on the EGF induced MUC5AC response was reversed in the presence of H-89 (5 μM), an inhibitor of PKA, thus reinforcing the view of a mechanism of action for rolflumilast related to the cAMP/PKA pathway (fig 6).

To establish the ability of the cAMP/PKA pathway to interfere with the EGF induced overexpression of MUC5AC we showed that forskolin (10 μM), a direct activator of 149
adenylyl cyclase, 24 db-cAMP (100 μM), a membrane permeable analogue of cAMP, 25 and Sp-5,6-DCl-cBIMPS (100 μM), an activator of PKA 26—while not altering the control level of MUC5AC expression—were impeding the enhanced expression of MUC5AC elicited by EGF (fig 6).

Effect of PDE4 inhibition on EGF induced MUC5AC expression in human isolated bronchus

Since A549 cells are a cancer cell line, the results obtained with these cells may differ from responses of normal airway epithelium. Additional experiments were therefore performed using human isolated bronchial tissue. In this preparation EGF (25 ng/ml) augmented the MUC5AC mRNA and protein expression with peak values reached at 1 hour and 3 hours after EGF exposure, respectively (fig 7). These effects of EGF were suppressed in the presence of tyrphostin A46 (not shown). Roflumilast (1 μM) prevented the EGF induced overexpression of MUC5AC (fig 8).

Immunohistochemistry experiments showed that MUC5AC immunoreactivity was localised in goblet cells that were stained with PAS (fig 9). The MUC5AC positive staining in airway epithelium was increased in EGF exposed preparations, and this augmentation was reduced in roflumilast treated tissues.

DISCUSSION

In this study we found that PDE4 inhibition abolished the EGF induced augmentation of MUC5AC mRNA and protein expression in cultured human airway epithelial cells and in human bronchial tissue in vitro. To our knowledge, this is the first report of a direct inhibitory effect on mucin production of PDE4 inhibitors, a new class of drugs with potential therapeutic interest in the treatment of COPD and asthma—diseases in which mucus hypersecretion is considered pathologically relevant.

EGF activates EGFR signalling cascade and MUC5AC expression in A549 cells

The EGFR signalling cascade is important for regulating MUC5AC mucin gene expression and protein production by airway epithelial cells,6 and both the EGFR and the MUC5AC expression are upregulated in chronic airway diseases such as asthma and COPD.13 37 The EGFR signalling pathway translates into increased MUC5AC expression, the activation produced by many different stimuli including oxidative stress, neutrophil elastase, tobacco smoke, bacterial and viral products, and inflammatory cytokines.17 18 27 In this study we have selected EGF, an endogenous ligand of the EGFR, as a direct activator of this pathway based on previous studies in cultured human airway epithelial NCI-H292 cells.61 8

We confirmed that A549 cells have a constitutive expression of EGFR28 as shown by the faint band observed in Western blot analysis with anti-EGFR mAb in the control group (fig 3). The activation of the EGFR system results in an increase of about twofold in MUC5AC mRNA and protein expression as shown by ELISA data obtained after 24 hours of incubation with EGF. Immunocytochemistry of A549 cells confirmed this finding. The increase in MUC5AC mRNA and protein at 24 hours is within the time dependency shown in cultured human airway epithelial cells for MUC5AC.
production elicited with various stimuli activating EGFR including EGF.\textsuperscript{6, 17–18}

Consistent with the notion that the overexpression of MUC5AC is the consequence of the activation of the EGFR signalling cascade, we also found that preincubation with EGFR tyrosine kinase inhibitors prevented the EGFR induced augmentation of the MUC5AC mRNA expression and protein production (fig 1). EGF therefore increases the protein-tyrosine kinase activity of its receptor and thereby activates other kinase cascades such as MAPKs including p38 and p44/42 MAPKs.\textsuperscript{27} As expected, we found an early activation of p38 and p44/42-MAPKs as well as phosphorylation of tyrosine residues of different cell proteins and upregulation of the EGFR after exposure to EGF for 24 hours (fig 3). Furthermore, inhibition of p38 and p44/42-MAPKs with the selective inhibitors SB202190 and PD98059 abrogated the EGF induced MUC5AC mRNA expression.

**PDE4 inhibitors suppress the EGFR induced MUC5AC expression in A549 cells by activating the cAMP/PKA pathway**

There is evidence to indicate that the functioning of the cAMP/PKA pathway is linked with that of the ERK/MAPK pathway. Thus, agents that increase the intracellular cAMP concentration block growth factor stimulated ERK activation in a number of cell types by inhibiting the activation of Raf proteins.\textsuperscript{13, 15} In fact, PDE4 isoenzymes may provide a pivotal point for integrating cAMP and ERK signal transduction in cells.\textsuperscript{30} The known relevance of PDE4 isoenzyme activity in the regulation of cAMP levels in human airway epithelial cells, including A549 cells,\textsuperscript{12} prompted us to investigate the effects of non-selective PDE4 inhibitors on the EGFR induced MUC5AC expression and related events occurring in A549 cells.

We found that three different structurally unrelated PDE4 inhibitors—the archetypal PDE4 inhibitor rolipram and the second generation PDE4 inhibitors cilomilast and rolflumilast—produced concentration dependent inhibitions of the EGFR induced MUC5AC mRNA and protein expression. The potency order of their activities (expressed as –log IC\textsubscript{50} values) was rolflumilast (–7.5) > rolipram (–6.5) > cilomilast (–5.5). These differences in potencies are consistent with results obtained in other in vitro human cell systems, yet variation may depend on the stimulus and the cell type studied.\textsuperscript{38} Since rolumilast (1 μM) suppressed both MUC5AC mRNA and protein production in response to EGF, this concentration was selected for further studies.

The inhibitory action of rolumilast appears to be exerted at different levels of the EGFR signalling cascade. Thus, we showed that rolumilast (1 μM) markedly inhibited the early phospho-p38 MAPK expression as well as the phosphorylation of tyrosine residues of proteins and the overexpression of EGFR in response to EGF stimulation measured at 24 hours EGF exposure.

The inhibitory effects of rolumilast on the EGFR cascade events leading to enhanced MUC5AC expression are probably related to the activation of the cAMP/PKA pathway since this selective PDE4 inhibitor elicited a transient early increase in cAMP levels in A549 cells, and its inhibitory effects on MUC5AC expression were reversed by preincubation with H-89, an inhibitor of PKA activity.\textsuperscript{34} Furthermore, forskolin (a direct activator of adenyl cyclase),\textsuperscript{35} db-cAMP (a membrane permeant analogue of cAMP),\textsuperscript{36} and Sp-5,6-DCI-cBIMPS (a specific activator of PKA)\textsuperscript{36} prevented the enhanced expression of MUC5AC elicited by EGF (fig 6), thus supporting the notion that the activation of the cAMP/PKA pathway is effective in exerting an inhibitory influence on the EGFR cascade leading to MUC5AC expression in A549 cells.

**PDE4 inhibition attenuates EGF induced MUC5AC expression in human airways in vitro**

The inhibitory effects resulting from PDE4 inhibition with rolumilast in cultured A549 cells may not necessarily be representative of the responses of the epithelial cells in the human airways. MUC5AC expression was therefore also examined in human isolated bronchi, a preparation that has previously been shown to have a basal secretion of mucin MUC5AC produced principally by goblet cells.\textsuperscript{26} In the human airways in vitro, MUC5AC mRNA expression reached a peak at 1 hour after stimulation with EGF, while peak MUC5AC protein production in tissue and medium was observed at 3 hours (fig 7). This represents faster kinetics of MUC5AC expression than in cultured A549 cells, but we have not investigated the reason for this difference. Pretreatment with rolumilast (1 μM) markedly inhibited this augmented expression of MUC5AC induced by EGF activation, indicating that the direct inhibitory effects produced by this PDE4 inhibitor in cultured A549 cells are reproducible in intact airway epithelial cells. Immunohistochemical analysis of human bronchial tissues confirmed that EGF exposure resulted in an augmented expression of MUC5AC positive stained cells in airway epithelium and treatment with rolumilast effectively prevented this EGF induced overexpression of MUC5AC (fig 9).

In summary, the results of this study indicate that putative PDE4 inhibitors, in addition to their established inhibitory effects on the airway inflammatory cells,\textsuperscript{10} may also exert direct effects on human airway epithelial cells inhibiting the MUC5AC expression that follows the activation of the EGFR signalling cascade. These findings may be of added value to results from recent phase II/III clinical trials which suggest a therapeutic benefit for PDE4 inhibitors in mucus hypersecretory diseases such as COPD and asthma.\textsuperscript{8}

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Correlation

It has been brought to our attention that there is an error in figure 3 on page ii55 of the Pleural Disease Guideline available at www.brit-thoracic.org.uk/docs/PleuralDiseaseChestDrain.pdf. Below is a corrected diagram illustrating the “safe triangle” for a chest drain. The publishers apologise for this error.
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