Phosphodiesterase 4 Inhibitors Augment the Ability of Formoterol to Enhance Glucocorticoid-Dependent Gene Transcription in Human Airway Epithelial Cells: A Novel Mechanism for the Clinical Efficacy of Roflumilast in Severe Chronic Obstructive Pulmonary Disease

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ABSTRACT

Post-hoc analysis of two phase III clinical studies found that the phosphodiesterase 4 (PDE4) inhibitor, roflumilast, reduced exacerbation frequency in patients with severe chronic obstructive pulmonary disease (COPD) who were taking inhaled corticosteroids (ICS) concomitantly, whereas patients not taking ICS derived no such benefit. In contrast, in two different trials also performed in patients with severe COPD, roflumilast reduced exacerbation rates in the absence of ICS, indicating that PDE4 inhibition alone is sufficient for therapeutic activity to be realized. Given that roflumilast is recommended as an “add-on” medication to patients with severe disease who will inevitably be taking a long-acting β2-adrenoceptor agonist (LABA)/ICS combination therapy, we tested the hypothesis that roflumilast augments the ability of glucocorticoids to induce genes with anti-inflammatory activity. Using a glucocorticoid response element (GRE) luciferase reporter transfected into human airway epithelial cells [both bronchial epithelium + adenovirus 12 - SV40 hybrid (BEAS-2B) cells and primary cultures], roflumilast enhanced fluticasone propionate–induced GRE-dependent transcription. Roflumilast also produced a sinistral displacement of the concentration-response curves that described the augmentation of GRE–dependent transcription by the LABA formoterol. In BEAS-2B cells and primary airway epithelia, roflumilast interacted with formoterol in a positive cooperative manner to enhance the expression of several glucocorticoid-inducible genes that have anti-inflammatory potential. We suggest that the ability of roflumilast and formoterol to interact in this way supports the concept that these drugs together may impart clinical benefit beyond that achievable by an ICS alone, a PDE4 inhibitor alone, or an ICS/LABA combination therapy. Roflumilast may, therefore, be especially effective in patients with severe COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a general term that encompasses several debilitating pathologies that often coexist, and is characterized by a progressive and largely irreversible decline in lung function. Persistent chronic airflow limitation, usually associated with airway collapse, edema, mucus hypersecretion, and fibrosis, is present to a greater or lesser extent and accounts for the wide spectrum of disease. Typically, COPD afflicts middle-aged and elderly people and is caused, predominantly, by chronic cigarette smoking (Hogg, 2004; Hogg et al., 2004). COPD is characterized by neutrophilic inflammation of the small airways and lungs that, contrary to asthma, is relatively resistant to inhaled corticosteroids (ICS) (Barnes, 2008). Consequently, the management of COPD is hampered by a lack of disease-modifying, anti-inflammatory therapies. However, in April 2010, the European Medicines Agency recommended approval of the phosphodiesterase 4 (PDE4) inhibitor roflumilast for the “maintenance treatment of patients

ABBREVIATIONS: BEBM, bronchial epithelial cell basal medium; CD, cluster of differentiation; CMV, cytomegalovirus; COPD, chronic obstructive pulmonary disease; CRISPLD2, cysteine-rich secretory protein LCL (limulus clotting factor C, cochlín, Lgl1) domain-containing 2; E(A), concentration effect; FP, fluticasone propionate; GILZ, glucocorticoid-induced leucine zipper; GOLD, Global Initiative for Chronic Obstructive Lung Disease; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK 256066, (6-[[3-(dimethylamino)-carbonyl]phenyl][sulphonyl]-4-[3-methoxyphenyl]amino]-8-methyl-3-quinoline carboxamide); ICS, inhaled corticosteroid; HpAEC, human primary airway epithelial cell; LABA, long-acting β2-adrenoceptor agonist; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKI, cAMP-dependent protein kinase inhibitor; p57kip2, kinase inhibitor protein 2 of 57 kDa; qPCR, quantitative polymerase chain reaction; RGS, regulator of G-protein signaling; SFM, serum-free medium.
with severe COPD associated with chronic bronchitis who have a history of frequent exacerbations” (http://www.ema.europa.eu/docs/en_GB/document_library/Summary_of_opinion_-_Initial_authorisation/human/001179/WC500089626.pdf). Subsequently, in 2011, roflumilast was approved by Health Canada (www.hc-sc.gc.ca/dhp-mps/prodpharma/applique-demande/regist/reg_innov_dr-eng.php) and the U. S. Food and Drug Administration (www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm244989.htm). The approval of roflumilast for COPD is significant because it is believed to provide clinical benefit by suppressing inflammation (Giambryz and Field, 2010; Gross et al., 2010; Hatzellmann et al., 2010).

The Global Initiative for Chronic Obstructive Lung Disease (GOLD) recently updated its recommendations for the treatment of stable COPD to include roflumilast as a second choice medication in high-risk patients with severe, symptomatic disease (GOLD stages 3–4) (www.goldcopd.org/uploads/users/files/GOLD_Report_2011_Jan21.pdf). Thus, national and international guidelines, when revised, are likely to advocate that roflumilast be used as an “add-on” medication to patients already taking tiotropium bromide and a long-acting β2-adrenocceptor agonist (LABA)/ICS combination therapy.

Clinically, there may be a scientific rationale for using a PDE4 inhibitor in combination with an ICS. In a pooled post-hoc analysis of two phase III studies, roflumilast reduced exacerbation frequency in a subgroup of patients with severe COPD who were taking an ICS concomitantly, whereas patients not taking an ICS derived no such benefit (Rennard et al., 2011). Roflumilast also improved lung function in patients diagnosed with COPD associated with chronic bronchitis, with or without coexisting emphysema, that was greater if they had received concomitant ICS relative to placebo (Rennard et al., 2011). However, two additional phase III trials, also in patients with severe COPD, found that roflumilast reduced exacerbation rates in the absence of an ICS (Calverley et al., 2009), indicating that PDE4 inhibition alone may be sufficient for clinical benefit to be realized. Although the reason for this discrepancy remains unclear, the possibility that the efficacy of roflumilast and an ICS together may be superior to either drug alone is attractive from a therapeutics standpoint. Indeed, frequent exacerbations of COPD are associated with a high level of inflammation (Perera et al., 2007) that may be more responsive to these anti-inflammatory therapies when given in combination.

Glucocorticoids exert anti-inflammatory activity principally by repressing the expression of proinflammatory genes (Newton et al., 2010). Two general mechanisms have been described. The most widely accepted of these is transrepression, in which the agonist-bound glucocorticoid receptor (GR) hinders the ability of transcription factors, such as nuclear factor-κB and activator protein-1, to promote the transcription of proinflammatory genes. Recently, transrepression via a direct interaction of the agonist-bound GR to negative glucocorticoid response elements (GREs) was also defined (Surjit et al., 2011). However, in simple systems, glucocorticoids are often only partial inhibitors of proinflammatory gene transcription, implying that processes in addition to transrepression must be operative to explain the anti-inflammatory effects seen in bona fide models of inflammation (Clark, 2007; Newton et al., 2010). Indeed, there is now persuasive evidence that the induction (transactivation) of genes, many encoding proteins with anti-inflammatory potential, constitutes a major mechanism of glucocorticoid action (Clark, 2007; Newton et al., 2010; Clark and Belvisi, 2012).

Herein, we have used human bronchial epithelial cells as a model system to test the hypothesis that PDE4 inhibitors enhance GRE-dependent transcription in a manner similar to that described for LABA (Kaur et al., 2008). As roflumilast will be prescribed to patients with severe disease taking a LABA/ICS combination therapy, a primary objective of this study was to determine the effect of roflumilast alone and in combination with a LABA on glucocorticoid-induced gene expression. We submit that PDE4 inhibitors, by enhancing the transcription of glucocorticoid-inducible, anti-inflammatory genes above the maximum level that can be achieved by an ICS alone or an ICS/LABA combination therapy, could help reduce exacerbation frequency and improve lung function in patients with severe COPD.

Materials and Methods

Generation of a Stable 2×GRE BEAS-2B Cells. Stable transfection was used to generate a GRE reporter cell line. The construct, pGL3.neo.TATA.2GRE, contains two copies of a consensus simple GRE site (sense strand, 5'-TGT ACA GGA TGT TCT-3') positioned upstream of a modified minimal β-globin promoter driving a luciferase gene and a separate neomycin gene to confer resistance to geneticin (see Kaur et al., 2008). BEAS-2B cells at ~70% confluence in T162 flasks were transfected with 8 μg of plasmid DNA and 20 μl of lipofectamine 2000 (Invitrogen, Burlington, ON, Canada). After 24 hours, geneticin (200 μg/ml) was added until foci of stable transfectants appeared, which were harvested to create heterogeneous populations of cells in which the site of integration was randomized.

Culture of 2×GRE BEAS-2B Reporter Cells and Measurement of Luciferase. Cells were cultured for two days under a 5% CO2/air atmosphere at 37°C in 24-well plastic plates (Corning Life Sciences, Lowell, MA) containing Dulbecco’s modified Eagle’s medium/f10 (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), L-glutamine (2.5 mM), and sodium bicarbonate (0.15% v/v). The cells were then cultured for a further 24 hours in serum-free medium (SFM) and treated with fucitansone propionate (FP) or dexamethasone in the absence and presence of PDE4 inhibitors and/or formoterol as indicated. At 6 hours, cells were treated with 1× reporter lysis buffer (Promega, Madison, WI), and luciferase activity was measured using a Monolight Luminometer (BD Biosciences, San Diego, CA) according to the manufacturer’s instructions. Data are expressed as -fold induction of luciferase relative to unstimulated cells.

Culture of Human Primary Airway Epithelial Cells. Cells were obtained from both male and female donors by proteinase digestion of nontransplanted normal human lung (International Institute for the Advancement of Medicine, Edison, NJ) and seeded in 12-well plates (Corning Life Sciences) containing bronchial epithelial cell growth medium (Lonza, Walkersville, MD) supplemented with penicillin (50 μg/ml) and streptomycin (10 μg/ml). Initially, the cells were cultured under a 5% CO2/air atmosphere at 37°C until confluent (typically 14 days; medium changed every 3–4 days). They were then cultured for a further 24 hours in supplement-free, bronchial epithelial cell basal medium (BEBM; Lonza) and processed as described later. Ethics approval for the use of human tissues has been granted by the Conjoint Health Research Ethics Board of the University of Calgary.

Transient Transfection of Human Primary Airway Epithelial Cells with a 2×GRE-Reporter Construct. Plasmid DNA (500 ng) containing the pGL3.neo.TATA.2GRE construct was incubated for 30 minutes with 0.5 μl of Lipofectamine 2000 (Invitrogen) in 100 μl of BEBM (without supplements) at room temperature. Human primary airway epithelial cells (HpAECs) at ~85% confluence in 24-well plates
were washed and transfected by the addition of 250 μl of BEBM containing DNA/lipofectamine 2000 for 6 hours. The complex was removed and the cells were incubated with 500 μl of bronchial epithelial cell growth medium with supplements for 18 hours and then starved for an additional 24 hours in BEBM (without supplements) before beginning experiments. Cells were harvested at 6 hours for luciferase determination, as described earlier.

**Infection of 2×GRE BEAS-2B Reporter Cells with Ad5.Cytomegalovirus-AMP-Dependent Protein Kinase Inhibitor α.** Cells were infected (multiplicity of infection = 30) with an adenovirus vector [Ad5.CMV(cytomegalovirus)PKI] encoding the complete amino acid sequence of the α-isof orm of cAMP-dependent protein kinase (PKA) inhibitor downstream of the constitutively active CMV immediate early promoter. The expression of the PKIα transgene was confirmed by Western blotting. See Meja et al. (2004) for further details.

**Measurement of cAMP.** BEAS-2B cells at 37°C were grown to confluence in 12-well plates, growth arrested for 24 hours in SFM, and incubated for 1 hour with vehicle, roflu milast (2 nM), formoterol (100 pM), and roflumilast and formoterol in combination. The SFM was incubated for 1 hour with vehicle, roflumilast (2 nM), formoterol (100 pM), and roflumilast and formoterol in combination. The SFM was decanted and the cells lysed in HCl (0.1 M). cAMP was measured in pM, and roflumilast and formoterol in combination. The SFM was incubated for 1 hour with vehicle, roflumilast (2 nM), formoterol (100 pM), and roflumilast and formoterol in combination. The SFM was decanted and the cells lysed in HCl (0.1 M). cAMP was measured in cell lysates by enzyme-linked immunosorbent assay (Enzo Life Sciences, Inc., Foster City, CA), encoding glucocorticoid-induced leucine zipper factor C, cochlin, Lgl1) domain-containing 2 (CRISPLD2), regulator of megalovirus cAMP-Dependent Protein Kinase Inhibitor downstream of the constitutively active CMV immediate early sequence of the gene. Reverse transcribed using a qScript cDNA synthesis kit according to the manufacturer instructions (Quanta Biosciences, Gaithersburg, MD).

**RNA Isolation, Reverse Transcription, and Real-Time Polymerase Chain Reaction (PCR).** Total RNA was extracted from BEAS-2B cells and HPAECs that had been treated with glucocorticoid, PDE4 inhibitor, or LABA alone and in combination as indicated using RNeasy Mini Kits (Qiagen Inc., Mississauga, ON, Canada), and was reverse transcribed using a qScript cDNA synthesis kit according to the manufacturer's instructions, and is expressed in units of pmol/ml.

**Table 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide</th>
<th>Accession Number(s)</th>
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<tbody>
<tr>
<td>p57kip1 (CDKN1C)</td>
<td>5'-CGG CGA TCA AGA ACG TGT C-3'</td>
<td>NM_000767.2, NM_001122630.1, NM_001122631.1</td>
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<tr>
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<td>NM_001015881.1, NM_198057.2, NM_004089.3</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCC TAT AGA CAA GAT CCG-3'</td>
<td>NM_001122630.1, NM_001122631.1</td>
</tr>
<tr>
<td>GILZ (TSC22D3)</td>
<td>5'-AGG AGG CAT TGC TGA TGA TCT-3'</td>
<td>NM_031476.3</td>
</tr>
<tr>
<td>Forward</td>
<td>5'-AGC ACC TTA CTC ATT CAT G-3'</td>
<td>NM_002046.4, NM_001256799.1</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCC TGT GTC GAA GTC CAA ATC C-3'</td>
<td>NM_005944.5, NM_001004196.2</td>
</tr>
<tr>
<td>RGS2</td>
<td>5'-GGG CCA TCA AGA ACG TGT C-3'</td>
<td>NM_002046.4, NM_001256799.1</td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GCC TAT AGA CAA GAT CCG-3'</td>
<td>NM_001122630.1, NM_001122631.1</td>
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<tr>
<td>Reverse</td>
<td>5'-GGG CCA TCA AGA ACG TGT C-3'</td>
<td>NM_002046.4, NM_001256799.1</td>
</tr>
<tr>
<td>CD200</td>
<td>5'-AGG ACC ATG GAG AAC C-3'</td>
<td>NM_002046.4, NM_001256799.1</td>
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<tr>
<td>Forward</td>
<td>5'-GCC TGT GTC GAA GTC CAA ATC C-3'</td>
<td>NM_005944.5, NM_001004196.2</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AGG ACC ATG GAG AAC C-3'</td>
<td>NM_002046.4, NM_001256799.1</td>
</tr>
</tbody>
</table>

GAPDH, glycerinaldehyde 3-phosphate dehydrogenase.

**Curve Fitting.** Monophasic, agonist concentration-effect (E/[A]) curves were fitted by least-squares, nonlinear iterative regression to the following form of the Hill equation using Prism 4 (GraphPad Software Inc., San Diego, CA):

$$E = E_{\text{min}} + \left( E_{\text{max}} - E_{\text{min}} \right) \left( 1 + \frac{[A]^p}{\text{IC}_{50}^p} \right)$$

where $E$ is the effect, $E_{\text{min}}$ and $E_{\text{max}}$ are the lower and upper asymptote (i.e., the basal response and maximum agonist-induced response, respectively), $p[A]$ is the log molar concentration of agonist, $E_{\text{IC}_{50}}$ is a location parameter equal to the log molar concentration of agonist producing $(E_{\text{max}} - E_{\text{min}})/2$, and $n$ is the gradient of the E/[A] curve at the $E_{\text{IC}_{50}}$ level.

**Drugs and Analytical Reagents.** Roflumilast [3-(cyclopropylmethyl)-N-(3,5-dichloropyridin-4-yl)-4-(difluoromethoxy)benzamide], roflu milast N-oxide (3-(cyclopropylmethyl)-N-(3,5-dichloro-1-oxo-pyrrolo-4-yl)-4-(difluoromethoxy) benzamide), cilomilast (4-cyano-4-[3-(cyclopentyl)-4-methoxyphenyl] cyclohexene carboxylic acid), tetomilast (6-[2-(3,4-dihydro-4-yl)-1,3-thiazol-4-yl]pyridine-2-carboxylic acid), apremilast (N-[2-[1-LS]-1-3-ethoxy-4-methoxyphenyl]-2-methylsulphonyl-ethyl)-1,3-dioxosindol-4-ylacetamide), and AWD 12-281 (N-(3,5-dichloropyrid-4-yl)-[1-(4-fluorobenzyl)-5-hydroxy-indole-3-yl]-glyoxylic acid amide) were synthesized by Nycomed (Konstanz, Germany). GSK 258066 and dexamethasone were from Gilead Sciences (Seattle, WA) and Steraloids (Newport, RI), respectively. Formoterol, FP, and all other drugs, chemicals, and reagents were from Sigma-Aldrich (Oakville, ON, Canada). PDE4 inhibitors, formoterol, and glucocorticoids were dissolved in dimethylsulfoxide and diluted to the desired working concentrations in culture medium. The final concentrations of dimethylsulfoxide did not exceed 0.5% (v/v).

**Definitions and Statistics.** In the text, the term “additivity” refers to an effect produced by two or more drugs that, when combined, is the sum of their individual components. Conversely, the term “positive cooperativity” describes a response produced by two or more drugs in combination that is greater than the sum of their individual effects.

Data points and values in the text and figure legends are presented as the mean ± S.E. mean of $N$ independent determinations. Data were analyzed using a one-way or two-way analysis of variance as indicated, followed, when appropriate, by Tukey's multiple comparison.
Results

Effect of PDE4 Inhibitors on GRE-Dependent Transcription in BEAS-2B Cells: A Comparison with the LABA, Formoterol. Treatment of 2 × GRE BEAS-2B reporter cells with FP (0.1 nM to 1 μM) for 6 hours induced GRE-dependent transcription in a concentration-dependent manner, with an [A50] and E_max of 3.1 nM and 18-fold, respectively (Fig. 1, A and B; Table 2). In contrast, neither roflumilast (1 μM) nor cilomilast (10 μM) alone induced the expression of luciferase (unpublished data). However, when added to 2 × GRE BEAS-2B reporter cells concurrently with FP, both PDE4 inhibitors enhanced (1.6- to 1.9-fold), in a positive cooperative fashion, FP-induced transcription above that produced by the glucocorticoid alone (Fig. 1, A and B). Analysis of the E[A] relationship that describes FP-induced, GRE-dependent transcription in BEAS-2B cells harboring the same 2 × GRE construct (Fig. 1, C and D; Tables 2 and 3). However, the maximum fold induction produced by formoterol was significantly greater than that produced by either cilomilast or roflumilast (Fig. 1, A–D; Tables 2 and 3) in the absence of any change in the potency of FP (Fig. 1, A and B; Table 2).

The interaction between FP and roflumilast and between FP and cilomilast was not peculiar to these drug combinations. Indeed, six other PDE4 inhibitors of varied structural classes (AWD 12-281, apremilast, GSK 256066 [(6-({3-[(dime-...

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>p[A50] of FP</th>
<th>E_max of FP</th>
<th>Enhancement of Maximum FP-Induced Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>FP</td>
<td></td>
<td>8.51 ± 0.09</td>
<td>18.0 ± 0.9</td>
<td>1</td>
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<tr>
<td>+ Cilomilast (10 μM)</td>
<td>9</td>
<td>8.35 ± 0.07</td>
<td>34.9 ± 1.9*</td>
<td>1.9</td>
</tr>
<tr>
<td>+ Roflumilast (1 μM)</td>
<td>12</td>
<td>8.29 ± 0.10</td>
<td>28.6 ± 1.1*</td>
<td>1.6</td>
</tr>
<tr>
<td>+ Formoterol (1 nM)</td>
<td>12</td>
<td>8.37 ± 0.03</td>
<td>41.4 ± 1.2*</td>
<td>2.3</td>
</tr>
</tbody>
</table>

*P < 0.05, E_max significantly different from that produced by FP alone.

maximal effects being 2- to 2.5-fold greater than that produced by FP alone (Fig. 1, C and D; Table 3).

Consistent with a previous report (Kaur et al., 2008), formoterol also enhanced FP (100 nM)-induced, GRE-dependent transcription in BEAS-2B cells harboring the same 2 × GRE construct (Fig. 1, C and D; Table 3). However, the maximum fold induction produced by formoterol was significantly greater than that produced by either cilomilast or roflumilast (Fig. 1, A–D; Tables 2 and 3) in the absence of any change in the potency of FP (Fig. 1, A and B; Table 2).

Effect of Cilomilast and Roflumilast on the Kinetics of GRE-Dependent Transcription in BEAS-2B Cells. Treatment of 2 × GRE BEAS-2B reporter cells with FP (100 nM) induced the luciferase gene in a time-dependent manner (Fig. 3, A and B). Transcription reached a maximum at the 6-hour time point, and this level of luciferase activity then gradually declined over the next 18 hours. In the presence of roflumilast (1 μM) or cilomilast (10 μM), which were inactive alone on the reporter, the ability of FP to promote GRE-dependent transcription was significantly augmented (1.8- to 2.1-fold; Fig. 2).

Effect of Cilomilast and Roflumilast on the Kinetics of GRE-Dependent Transcription in BEAS-2B Cells. Treatment of 2 × GRE BEAS-2B reporter cells with FP (100 nM) induced the luciferase gene in a time-dependent manner (Fig. 3, A and B). Transcription reached a maximum at the 6-hour time point, and this level of luciferase activity then gradually declined over the next 18 hours. In the presence of roflumilast (1 μM) or cilomilast (10 μM), which were inactive alone on the reporter, the ability of FP to promote GRE-dependent transcription was significantly augmented (1.8- to 2.1-fold; Fig. 2).

Effect of a fixed concentration of cilomilast, roflumilast, or formoterol on the E[A] relationship that describes FP-induced, GRE-dependent transcription in 2 × GRE BEAS-2B reporter cells Data are derived from the graphs in Fig. 1, A and B, and were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test.

Table 3 Concentration dependence of the enhancement of FP-induced, GRE-dependent transcription in 2 × GRE BEAS-2B reporter cells by cilomilast, roflumilast, and formoterol Data are derived from the graphs in Fig. 1, C and D, and were analyzed by one-way analysis of variance followed by Tukey’s multiple comparison test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>p[A50] of FP</th>
<th>Induction</th>
<th>Enhancement of FP-Induced Response</th>
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<tr>
<td>FP (100 nM)</td>
<td></td>
<td>11.8 ± 0.7</td>
<td>1.0</td>
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<tr>
<td>+ Cilomilast (10 μM)</td>
<td>8</td>
<td>5.48 ± 0.98</td>
<td>23.7 ± 3.3*</td>
<td>2.0</td>
</tr>
<tr>
<td>+ Formoterol (1 nM)</td>
<td>7</td>
<td>10.41 ± 0.08</td>
<td>32.2 ± 1.3*</td>
<td>2.7</td>
</tr>
<tr>
<td>+ Roflumilast (1 nM)</td>
<td>7</td>
<td>10.41 ± 0.08</td>
<td>32.2 ± 1.3*</td>
<td>2.7</td>
</tr>
</tbody>
</table>

*P < 0.05, E_max significantly different from that produced by FP alone.
transcription was augmented at all time points examined (Fig. 3, A and B). The greatest effects were seen at 4–6 hours, and this additional level of luciferase induction was largely preserved over the time course of the experiment, although the absolute luciferase activity waned (Fig. 3, A and B).

**Effect of Selective Inhibitors of Other cAMP PDEs on GRE-Dependent Transcription in BEAS-2B Cells.** BEAS-2B cells and HpAECS express multiple cAMP PDEs, including PDE2, PDE3, PDE4, and PDE7 (Dent et al., 1998; Fuhrmann et al., 1999; Smith et al., 2003). Figure 4 shows the effect on GRE-dependent transcription of inhibitors of these PDEs at the highest concentrations where isoenzyme selectivity is preserved. None of the PDE inhibitors alone activated the 2×GRE BEAS-2B reporter, whereas dexamethasone (1 μM) increased luciferase activity by 19.4-fold. Selective inhibitors of PDE2 (Bay 60-7550; 500 nM) and PDE3 (siguazodan; 10 μM), each added concurrently with dexamethasone, had no further effect on GRE-dependent transcription (Fig. 4A). Indeed, siguazodan (10 μM) did not significantly modify the dexamethasone E/A relationship in terms of either its p[A]50 or Emax (Fig. 4B). In contrast, BRL 50481 (3-(N,N-dimethylsulphonamido)-4-methyl-nitrobenzene) (100 μM), an inhibitor of PDE7 (Smith et al., 2004), and rolipram (10 μM), an archetypical PDE4 inhibitor, significantly enhanced dexamethasone-induced luciferase activity from 19.4-fold to 25- and 45-fold, respectively (Fig. 4A). Moreover, when added concurrently with dexamethasone to 2×GRE BEAS-2B reporter cells, rolipram and BRL 50481 increased transcription at 6 hours to 53.3-fold. All other cAMP PDE inhibitor combinations that were examined failed to enhance dexamethasone-induced GRE-dependent transcription to a greater extent than rolipram alone (Fig. 4A).

**Effect of PKIα on the Enhancement by Roflumilast, Roflumilast N-Oxide, and Cilomilast of GRE-Dependent Transcription in BEAS-2B Cells.** To examine a possible role for PKA in the enhancement of GRE-dependent transcription by PDE4 inhibitors, a highly selective, endogenous inhibitor, PKIα, was used that was delivered to BEAS-2B cells by a adenovirus vector, Ad5.CMV.PKIα (see Mejia et al. (2004) and references therein). In uninfected cells, PKIα was not detected by Western blotting in any experiment. However, 48 hours after infection of BEAS-2B cells with Ad5.CMV.PKIα (multiplicity of infection = 30), a single protein was labeled by the anti-PKIα antibody that migrated as a 12-kDa band on SDS polyacrylamide gels (Fig. 5A). As shown in Fig. 5B, the concentration-dependent enhancement by cilomilast of FP (100 nM)-induced GRE-dependent transcription was abolished in cells expressing the PKIα transgene. In contrast, cells infected with a null virus, Ad5.CMV.Null, responded to cilomilast in a manner that was not significantly different from uninfected cells (Fig. 5B). Identical results were obtained with roflumilast (1 μM) and its active metabolite, roflumilast N-oxide (1 μM; Fig. 5C).

**Effect of Roflumilast on the Ability of Formoterol to Augment GRE-Dependent Transcription in BEAS-2B Cells.** We have previously reported that the LABA, formoterol, augments GRE-dependent transcription in 2×GRE BEAS-2B reporter cells (Giembycz et al., 2008; Kaur et al., 2008). Figure 6 shows the results of studies designed to assess whether this effect of formoterol could be potentiated by the PDE4 inhibitor, roflumilast. FP (100 nM) alone increased luciferase activity by ∼17-fold (Fig. 6A), and this effect was augmented in a concentration-dependent manner by formoterol (p[A]50 = −10.4 ± 0.02; Emax = 46-fold; Fig. 6A). Roflumilast, which, by itself, enhanced GRE-dependent transcription, significantly potentiated the effect of formoterol in a concentration-dependent manner (p[A]50 = −10.69 ± 0.03, −11.03 ± 0.02, and −11.2 ± 0.02 in the presence of 1, 10, and 100 nM roflumilast, respectively), although the maximum luciferase activity was unchanged (Fig. 6A). Replotting these data (Fig. 6B) showed that roflumilast at concentrations of 10 nM and higher significantly potentiated the effect of formoterol (10 pM). On cAMP, a similar effect was observed. Thus, roflumilast (2 nM), which was inactive by itself, significantly augmented formoterol-induced cAMP accumulation (from 75.2 ± 11.3 to 118.6 ± 5.3 pmol/ml; Fig. 6C) when measured at a time (1 hour) that preceded the peak in GRE-dependent transcription (Fig. 3).

**Effect of Roflumilast and Cilomilast on FP-Induced GRE-Dependent Transcription in HpAECS.** Additional studies were performed to confirm that PDE4 inhibitors and a LABA can augment GRE-dependent transcription in HpAECS transiently transfected with the same 2×GRE reporter construct. As shown in Fig. 7, FP (100 nM) doubled luciferase expression when compared with unstimulated cells, and this effect was enhanced if formoterol (1 nM) was added concurrently with the glucocorticoid. Significantly, formoterol (100 pM) and low concentrations of roflumilast (2 nM) or cilomilast (20 nM) that, by themselves, did not significantly enhance FP-induced transcription interacted in combination to produce an effect that was equal to that evoked by a maximally effective concentration of formoterol (1 nM).

**Effect of Roflumilast and Formoterol Alone and in Combination on the Expression of Glucocorticoid-Inducible Genes.** The data presented in the preceding sections demonstrate that a PDE4 inhibitor and/or formoterol, alone and in combination, enhanced glucocorticoid-induced transcription from a conventional, simple 2×GRE reporter. To determine whether these findings translate to the enhancement of real genes, we used data derived from two microarrays in

![Fig. 2. Effect of structurally dissimilar PDE4 inhibitors on luciferase activity in 2×GRE BEAS-2B reporter cells.](image-url)
which dexamethasone- and indacaterol-inducible mRNA transcripts were identified in pulmonary type II A549 cells and BEAS-2B cells, respectively. As briefly outlined in Table 4, there is a literature precedent that the expression of several of these genes, including *GILZ*, *RGS2*, *p57kip2*, *CRISPLD2*, and *CD200*, could suppress indices of inflammation and therefore reduce exacerbations, protect against bronchoconstriction, and limit airway remodeling (Samuelsson et al., 1999; Eddleston et al., 2007; Snelgrove et al., 2008; Wang et al., 2009; Holden et al., 2011). Accordingly, these genes were selected to determine if they are regulated in HpAECs and BEAS-2B cells in a manner similar to the 2×C2 GRE reporter.

**HpAECs.** Initially, a small proof-of-concept experiment was performed to assess the ability of a maximally effective concentration of roflumilast (1 μM; determined from the data presented in Fig. 1D) to augment FP-induced *GILZ* expression in HpAECs. To ensure the detection of any additive or positive cooperative interactions between these stimuli, qPCR was performed using cDNA prepared from cells treated over an 18-hour time frame. As shown in Fig. 8, FP (100 nM) increased *GILZ* expression in a time-dependent manner by a mechanism that was enhanced by roflumilast (from 1.1- to 3.3-fold, 2.9- to 8.9-fold, 2.5- to 13.5-fold, and 16.9- to 43.6-fold at 1, 2, 6, and 18 hours, respectively). Roflumilast (1 μM), alone, was inactive. Based on these results, all further gene expression analyses in HpAECs were performed at 2 and 6 hours, and were expanded to include *RGS2*, *p57kip2*, *CD200*, and *CRISPLD2*. In BEAS-2B cells, mRNA transcripts were measured 6 hours after treatments as we have reported previously (Kaur et al., 2008; Wilson et al., 2009). Finally, in both cell types, the concentration of roflumilast was reduced to 2 nM. This equates to the amount of free (unbound) roflumilast N-oxide (the primary, active metabolite) in plasma at steady state after administration of the recommended dose (500 μg once-a-day) (Bethke et al., 2007), which inhibits PDE4 subtypes with potencies similar to the parent compound (Hatzelmann et al., 2010).

Treatment of HpAECs with FP (100 nM) induced *RGS2* at 2 and 6 hours by 9.2- and 4.6-fold, respectively, relative to

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**Fig. 3.** Kinetics of GRE-dependent transcription in 2×GRE BEAS-2B reporter cells. Confluent cells were treated with FP (100 nM), cilomilast (10 μM), roflumilast (1 μM), or a combination of FP with either PDE4 inhibitor. Cells were then incubated for 1–24 hours and harvested for luciferase activity. Panels (A) and (B) refer to the FP/cilomilast and FP/roflumilast combinations. Data points represent the mean ± S.E. mean of 3 independent determinations. *P < 0.05, significant enhancement of transcription relative to time-matched, unstimulated cells; +P < 0.05, significant enhancement of transcription relative to time-matched, FP-treated cells. Data were analyzed by two-way ANOVA (analysis of variance) followed by Tukey’s multiple comparison test.

**Fig. 4.** Effect of isoenzyme-selective PDE inhibitors on luciferase activity in 2×GRE BEAS-2B reporter cells. In panel (A), cells were treated with dexamethasone (Dex; 1 μM) in the absence and presence of Bay 60-7550 (Bay; 500 nM), siguazodan (Sig; 10 μM), rolipram (Roli; 10 μM), BRL 50481 (BRL; 100 μM), or the following combinations: Bay 60-7550 and siguazodan, Bay 60-7550 and rolipram, Bay 60-7550 and BRL 50481, siguazodan and rolipram, siguazodan and BRL 50481, and rolipram and BRL 50481. In panel (B), cells were treated with dexamethasone (100 pM to 1 μM) in the absence and presence of the PDE3 inhibitor siguazodan (10 μM). At 6 hours, cells were harvested for the determination of luciferase activity. Bars and data points represent the mean ± S.E. mean of 3 independent determinations shown in parentheses. The dashed line in panel (A) defines the effect of dexamethasone alone. The numerals at the base of each bar in panel (A) refer to the PDE family targeted by the inhibitor(s). *P < 0.05, significant enhancement in luciferase activity relative to dexamethasone-treated cells. Data were analyzed by one-way ANOVA (analysis of variance) with Tukey’s multiple comparison test. NS, unstimulated cells.
time-matched, untreated cells (Fig. 9, A and B). Neither formoterol nor roflumilast induced RGS2 at either time point. However, at 2 hours, formoterol significantly enhanced the effect of FP (to 14.2-fold), although no further increase was seen if roflumilast was combined with formoterol. At 6 hours, formoterol failed to enhance FP-induced RGS2 expression, whereas a significant augmentation was produced when FP, formoterol, and roflumilast were used in combination (to 8.7-fold; Fig. 9B).

At 2 and 6 hours, FP, formoterol, and roflumilast failed to induce p57kip2 (Fig. 9, C and D). However, in combination, FP and formoterol increased p57kip2 expression by 3.1- and 6.2-fold at 2 and 6 hours, respectively. A combination of formoterol and roflumilast enhanced FP-induced p57kip2 expression at 2 hours to a significantly greater degree (to 4.4-fold) than did formoterol alone, although this interaction was lost at 6 hours.

Formoterol and roflumilast alone significantly enhanced the ability of FP to induce GILZ at 2 hours (from 6.5-fold to 19.5-fold and 12.3-fold, respectively), although no further increase was seen if formoterol was combined with roflumilast (Fig. 9E). At 6 hours, a similar profile of activity was found with the exception that roflumilast alone did not augment FP-induced GILZ expression (Fig. 9F).

None of the interventions significantly induced CD200 in HpAECs at either time point (Fig. 9, G and H) or CRISPLD2 at 2 hours (Fig. 9I). However, at 6 hours, FP and roflumilast in combination increased the expression of CRISPLD2 to an extent that was significantly greater than in time-matched, unstimulated cells (Fig. 9J).

**BEAS-2B Cells.** Treatment of BEAS-2B cells with FP (100 nM) or formoterol (100 pM) produced a 3.1- and 2.6-fold induction of RGS2, respectively, at 6 hours, whereas roflumilast alone significantly enhanced the transcription of GRE-dependent activity in cells treated with formoterol and FP, in combination at the concentrations indicated. At 6 hours, cells were harvested for the determination of luciferase activity. In panel (C), cells were exposed for 1 hour to roflumilast (Rof; 2 nM), formoterol (Form; 100 pM), a combination of roflumilast and formoterol (Rof + Form), or were left untreated, and cAMP was measured by enzyme-linked immunosorbent assay. In each panel, bars and data points represent the mean ± S.E. mean of N independent determinations. *P < 0.05, significant enhancement of transcription by roflumilast over the effect of FP (100 nM) and formoterol (10 pM) in combination. Data were analyzed by two-way ANOVA (analysis of variance) followed by Tukey’s multiple comparison test. ** and ***P < 0.05, significant increase in cAMP content relative to unstimulated cells (NS) and formoterol-stimulated cells, respectively. Data were analyzed by one-way ANOVA with Tukey’s multiple comparison test.
the sum of the effects produced by FP, formoterol, and roflumilast alone, and the combination of FP and formoterol (Fig. 10C).

FP (100 nM) or formoterol (100 pM) produced a 6.5- and 8.1-fold induction of CRISPLD2, respectively, whereas roflumilast (2 nM) was inactive (1.3-fold; Fig. 10D). Formoterol, but not roflumilast, interacted with FP in a positive cooperative manner, augmenting CRISPLD2 mRNA levels by 25.5-fold relative to time-matched, untreated cells. Exposure of cells concurrently to FP, formoterol, and roflumilast further enhanced CRISPLD2 expression (by 35-fold) to a level that was higher than the sum of the effects produced by FP, formoterol, and roflumilast alone and the combination of FP and formoterol (Fig. 10D).

Treatment of BEAS-2B cells with FP (100 nM) produced a 49-fold induction of GILZ at 6 hours, whereas formoterol (100 pM) and roflumilast (2 nM), individually and in combination, were inactive (Fig. 10E). Formoterol, but not roflumilast, augmented FP-induced GILZ expression to a similar degree (63.5-fold) as formoterol, but, again, this effect was not significantly greater than that produced by FP alone or the combination of FP and formoterol (Fig. 10E).

Discussion

The major finding of this study was that a panel of structurally dissimilar PDE4 inhibitors, including roflumilast and its primary metabolite, roflumilast N-oxide, augmented GRE-dependent transcription in BEAS-2B cells and HpaECs in a manner similar to LABAs (Giembycz et al., 2008; Kaur et al., 2008). Moreover, positive cooperative effects were often observed when a PDE4 inhibitor and a LABA were combined. These interactions were produced in a simple, 2 × GRE luciferase reporter and, more importantly, in epithelial cells in which the expression of a selection of glucocorticoid-inducible genes was determined. Indeed, formoterol and roflumilast in combination enhanced FP-induced gene expression to a significantly greater degree than either the LABA or PDE4 inhibitor alone. As outlined in the Introduction, transactivation is now believed to constitute a major mechanism of glucocorticoid action, which likely operates in tandem with the more established process of transrepression. Indeed, the expression of the anti-inflammatory gene GILZ is significantly upregulated in bronchial biopsies harvested from human asthmatic subjects given inhaled budesonide (Kelly et al., 2012). On this basis, we propose that roflumilast in combination with an ICS may be more effective in reducing exacerbation frequency and improving lung function in patients with severe COPD relative to either drug alone due to their ability, at least in part, to interact in a positive cooperative manner on the expression of a variety of glucocorticoid-inducible, anti-inflammatory genes. Moreover, in the context of COPD treatment guidelines, enhanced gene transactivation could provide a mechanistic rationale for “adding on” a PDE4 inhibitor to patients with severe COPD taking an ICS/LABA combination therapy who still suffer frequent exacerbations. In this respect, in 2011, the REACT (Roflumilast in the Prevention of COPD Exacerbations while Taking Appropriate Combination Treatment) study was initiated to compare the effects of roflumilast and placebo on exacerbation rates in patients who are treated
simultaneously with a fixed combination of a LABA and an ICS, with or without a long-acting muscarinic receptor antagonist (Calverley et al., 2012). The results of this trial are awaited with interest.

### Effect of PDE Inhibitors on GRE-Dependent Transcription.

In 2×GRE BEAS-2B reporter cells, PDE4 inhibitors augmented dexamethasone- and FP-induced luciferase activity in a time- and concentration-dependent manner. Inspection of the

### Table 4

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Putative Function(s)</th>
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<tr>
<td>GILZ</td>
<td>GILZ is a glucocorticoid-inducible gene that inhibits the transcriptional activity of both NF-κB and AP-1. Glucocorticoid-induced GILZ expression may also be modestly enhanced by LABAs. It is reported that induction of this gene suppresses various indices of inflammation (Mittelstadt and Ashwell, 2001).</td>
</tr>
<tr>
<td>RGS2</td>
<td>RGS2 is a GTPase-activating protein that switches off signaling from Gq-linked GPCRs. In airway smooth muscle, RGS2 is upregulated in a positive cooperative manner by glucocorticoid and LABA, and exerts a bronchoprotective effect; in epithelial and other cells. RGS2 expression may attenuate proinflammatory mediator release (Holden et al., 2011).</td>
</tr>
<tr>
<td>CD200</td>
<td>Pulmonary alveolar macrophages have high constitutive expression of CD200R. Signaling through this receptor involves the interaction of CD200R-bearing cells with other cells (e.g., airway epithelia) that express the glucocorticoid-inducible, cognate agonist CD200, which can be enhanced by formoterol and roflumilast alone and in combination. Studies in mice have shown that the CD200R/CD200 interaction blunts macrophage activation measured as proinflammatory cytokine generation. Acute exacerbations of COPD are triggered, primarily, by prolonged bouts of excessive inflammation in response to bacterial and viral infections. Pharmacological upregulation of CD200 on epithelial and other airway cells in COPD could attenuate inflammation and reduce exacerbation frequency (Sneldgrove et al., 2008).</td>
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</tr>
</tbody>
</table>
| CRISPLD2     | Previously known to mediate several other functions, CRISPLD2 was recently found to encode as a novel, secreted, mammalian LPS-binding protein in both humans and mice. Enhancement of FP-induced CRISPLD2 expression could contribute to the reduction in exacerbations in COPD produced by infections with gram-negative bacteria by downregulating TLR4-mediated proinflammatory responses (Wang et al., 2009).

### p57kip2

p57kip2 encodes a cell cycle kinase inhibitor that is induced by glucocorticoids in airway epithelial and other structural cells by a mechanism that is enhanced by formoterol and roflumilast, alone and in combination. Expression of this gene in relevant cells could arrest mitogenesis and suppress airway remodeling, which is a characteristic feature of COPD. p57kip2 may also block proinflammatory responses through its ability to inhibit one of the core mitogen-activated protein kinases, c-Jun-N-terminal kinase (Samuelsson et al., 1999; Chang et al., 2003).


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**Fig. 8.** Roflumilast augments the induction of GILZ in HpAECs by FP. Cells were treated with FP (100 nM) or roflumilast (Rof; 1 μM) alone and in combinations or left untreated (NS) for 1, 2, 6, and 18 hours (A–D). cDNA was prepared and real-time reverse-transcription polymerase chain reaction was performed for GILZ and normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Data are expressed as fold stimulation relative to GAPDH, and are plotted as means ± S.E. mean of N independent observations. The dashed lines define the effect of FP alone. *P < 0.05, significant enhancement of GILZ expression relative to FP-treated cells. Data were analyzed by one-way ANOVA (analysis of variance) followed by Tukey’s multiple comparison test.
E/A curves showed that PDE4 inhibitors increased the ability of FP to transactivate the reporter without affecting its potency. This was a positive cooperative interaction because the PDE4 inhibitors alone were inactive. Of potential clinical significance was the additional observation that roflumilast potentiated the ability of formoterol to promote GRE-dependent transcription, which was described by a sinistral displacement of formoterol E/A curves. Consistent effects were obtained with HpAECs transiently transfected with the same GRE reporter, indicating that these effects were not peculiar to BEAS-2B cells.

Fig. 9. Effect of roflumilast and formoterol, alone and in combination, on the expression of FP-inducible genes in HpAECs. Cells were treated with FP (100 nM), formoterol (Form; 100 pM), or roflumilast (Rof; 2 nM) alone and in combination or left untreated (NS) as indicated. After 2 and 6 hours, cDNA was prepared and real-time reverse-transcription polymerase chain reaction was performed for RGS2 (A and B), p57kip2 (C and D), GILZ (E and F), CD200 (G and H), and CRISPLD2 (I and J). Data are expressed as fold stimulation relative to GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and are plotted as means ± S.E. mean of N independent observations. The dashed lines define the effect of FP alone. *P < 0.05, significant enhancement of transcription relative to FP alone. †P < 0.05, significant enhancement of transcription relative to FP + formoterol. Data were analyzed by one-way ANOVA (analysis of variance) followed by Tukey's multiple comparison test.
Augmentation of GRE-dependent transcription was apparently restricted to inhibitors of PDE4. However, a high concentration (100 μM) of the PDE7 inhibitor BRL 50481 (Smith et al., 2003) also increased reporter activity and interacted additively with rolipram. Whether these were genuine additive effects due to inhibition of both PDE4 and PDE7 family members or an action of BRL 50481 on residual PDE4 activity that was not inhibited by 10 μM rolipram is unclear.

**Effect of PDE4 Inhibitors on Gene Expression.** The results of the reporter experiments prompted us to determine if “real” glucocorticoid-inducible genes are regulated similarly. Microarray profiling of A549 and BEAS-2B cells identified several candidate genes that were induced by dexamethasone and/or the LABA indacaterol, including RGS2, GILZ, p57kip2, CD200, and CRISPLD2. Although upregulation of these genes may, collectively, have clinical benefit in COPD (Table 4), they were selected, primarily, to provide proof of concept.

In HpAECs, a high concentration of roflumilast (1 μM), sufficient to abolish PDE4 activity in intact cells (Hatzelmann et al., 2010), augmented FP-induced GILZ expression in a time-dependent manner. In contrast, a lower concentration (2 nM) of roflumilast, comparable to the amount of free, unbound roflumilast N-oxide (which is equipotent) in plasma at steady state after administration of the recommended dose (Bethke et al., 2007), was inactive on the six genes studied. However, roflumilast and formoterol in combination enhanced the expression of several FP-inducible genes to levels that were significantly greater than those produced by formoterol alone. Although differences were apparent between BEAS-2B cells and HpAECs with regard to the genes affected, the magnitude of gene induction, and their sensitivity to formoterol and roflumilast, the data gathered from the two cell types were conceptually consistent. The reason(s) why these two cell types do not respond equivalently to glucocorticoids and/or cAMP-elevating drugs is unclear, but our experience is that the composition of culture media, passage number, and reagents used for the purification of primary epithelial cells are contributing factors.

The inactivity of a low concentration of roflumilast may suggest that, in vitro, a degree of tonic, cAMP "drive" is required for an impact on FP-induced gene expression to occur. This may be particularly relevant in cells such as airway epithelia that do not make appreciable quantities of cAMP-elevating autacoids such as prostaglandin E2 (cf., airway myocytes; Lazzeri et al., 2001). However, in vivo, cAMP-generating ligands will be released from a variety of cell types as well as through the therapeutic consumption of β2-adrenoceptor agonists.

**Mechanism of Action of PDE4 Inhibitors.** cAMP measurements and studies with the PKIα expression vector indicated that roflumilast, roflumilast N-oxide, and cilomilast augmented GRE-dependent transcription by activating the canonical cAMP/PKA signaling cascade. Although the downstream molecular targets of PKA are unknown, at least two possible mechanisms could explain this phenomenon. First, PDE4 inhibitors may augment the translocation of GRs from...
the cytosol to the nucleus. Although evidence is available to support this proposal (Miller et al., 2002), it necessarily implies that the transcription of all glucocorticoid-inducible genes would be enhanced by a cAMP-elevating drug, which does not seem to be the case (Giembycz et al., 2008; Kaur et al., 2008; Newton et al., 2010). Thus, to account for the enhancement of transcription of only a specific subset of glucocorticoid-inducible genes, it is necessary to consider alternative explanations. One possibility is PKA-mediated phosphorylation of components of the transcriptional apparatus. For example, the human GR features multiple phosphorylation sites, of which S211 is phosphorylated by PKA (Miller et al., 2007; Gallilher-Beckley and Cidlowski, 2009). It is possible that the phospho-GR would be better able to drive the transcription of some, but not all, glucocorticoid-inducible genes. Alternatively, PKA may target substrates downstream of the GR that regulate the activity and/or recruitment of specific cofactors (Moyer et al., 1993). Although such regulation of glucocorticoid-inducible genes remains undefined, phosphorylation could confer promoter specificity and explain why the expression of some glucocorticoid-inducible genes is augmented in a positive cooperative fashion by cAMP-elevating agents, while others are not.

**Clinical Relevance.** The concentration of free, unbound roflumilast N-oxide in the plasma of COPD patients at steady state (~2 nM; Bethke et al., 2007) after administration of the recommended dose of the parent compound, roflumilast, was at the threshold for enhancing GRE-dependent transcription in epithelial cells. One interpretation of these data is that this effect of roflumilast has limited clinical significance. However, many cells in the lung generate a variety of cAMP-elevating autacoids (e.g., PGE₂, adenosine) with which a low dose of a PDE4 inhibitor could interact in a positive cooperative fashion in a way similar to that shown for the induction of RGS2 and p57kip2. Credibility for this idea derives from the fact that the concentrations of PGE₂ and ATP (which is readily dephosphorylated into adenosine) are elevated in the exhaled breath condensate and in bronchoalveolar lavage fluid, respectively, of patients with COPD when compared with control subjects (Montuschi et al., 2003; Lommatzsch et al., 2010). Moreover, roflumilast is recommended for patients with severe disease (GOLD stages 3–4) who will already be taking a LABA/ICS combination therapy and a SABA (short-acting β2 adrenoceptor agonist) as a rescue medication.

**Conclusions**

The beneficial effects of roflumilast in COPD are suggested to be due to the suppression of inflammation (Hatzellmann et al., 2010). The results presented herein provide a novel mechanism to account for this anti-inflammatory effect, especially in patients with severe disease taking ICS/LABA combination therapy. We submit that an ICS, a LABA, and a PDE4 inhibitor in their various combinations work in several distinct, but mutually cooperative, ways (Fig. 11). Foremost, PDE4 inhibitors, particularly in the presence of a LABA, enhance the expression of some glucocorticoid-induced genes to levels that cannot be achieved by a glucocorticoid alone. A notable example is p57kip2, which was markedly induced by FP, formoterol, and roflumilast in combination, but largely unaffected by these drugs individually. Some of these same ICS-inducible genes may also be upregulated by a LABA (e.g., CD200). In addition (although not examined here), a LABA and/or PDE4 inhibitor may upregulate cAMP-inducible, anti-inflammatory genes that are insensitive to glucocorticoids, which could contribute to the therapeutic activity of roflumilast reported in COPD patients not taking ICS (Calverley et al., 2009). In concluding, it is appropriate to acknowledge that, although LABAs and PDE4 inhibitors alone can induce a variety of proinflammatory genes, this unwanted effect could be mitigated by an ICS and may not be overly problematic (Hertz et al., 2009; Holden et al., 2010). Thus, in severe COPD, the clinical efficacy of an ICS, a LABA, and a PDE4 inhibitor in combination may be attributable, in part, to their collective actions on gene transcription (Giembycz and Newton, 2011).

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**Authorship Contributions**

- **Participated in research design:** Giembycz, Newton.
- **Conducted experiments:** Joshi, Moodley, Rider, Sharma, Wilson, Yan.
- **Performed data analysis:** Giembycz, Joshi, Moodley, Rider, Sharma, Wilson, Yan.
- **Wrote or contributed to the writing of the manuscript:** Giembycz, Newton.
References


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