Superiority of Combined Phosphodiesterase PDE3/PDE4 Inhibition over PDE4 Inhibition Alone on Glucocorticoid- and Long-Acting β2-Adrenoceptor Agonist–Induced Gene Expression in Human Airway Epithelial Cells

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ABSTRACT

Glucocorticoids, also known as corticosteroids, induce effector gene transcription as a part of their anti-inflammatory mechanisms of action. Such genomic effects can be significantly enhanced by long-acting β2-adrenoceptor agonists (LABAs) and may contribute to the clinical superiority of inhaled corticosteroid (ICS)/LABA combinations in asthma and chronic obstructive pulmonary disease (COPD) over ICSs alone. Using models of cAMP- and glucocorticoid-induced transcription in human bronchial epithelial BEAS-2B cells, we show that combining inhibitors of phosphodiesterase (PDE) 3 and PDE4 provides greater benefits compared with inhibiting either PDE alone. In respect to cAMP-dependent transcription, inhibitors of PDE3 (siguazodan, cilostazol) and PDE4 (rolipram, GSK256066, roflumilast N-oxide) each sensitized to the LABA, formoterol. This effect was magnified by dual PDE3 and PDE4 inhibition. Siguazodan plus rolipram was also more effective at inducing cAMP-dependent transcription than either inhibitor alone. Conversely, the concentration-response curve describing the enhancement of dexamethasone-induced, glucocorticoid response element–dependent transcription by formoterol was displaced to the left by PDE4, but not PDE3, inhibition. Overall, similar effects were described for bona fide genes, including RGS2, CD200, and CRISPLD2. Importantly, the combination of siguazodan plus rolipram prolonged the duration of gene expression induced by formoterol, dexamethasone, or dexamethasone plus formoterol. This was most apparent for RGS2, a bronchoprotective gene that may also reduce the proinflammatory effects of constrictor mediators. Collectively, these data provide a rationale for the use of PDE3 and PDE4 inhibitors in the treatment of COPD and asthma where they may enhance, sensitize, and prolong the effects of LABA/ICS combination therapies.

Introduction

Glucocorticoids taken in an inhaled form, and referred to as inhaled corticosteroids (ICSs), are the mainstay anti-inflammatory treatment of mild/moderate asthma and work by suppressing airway inflammation (Barnes, 2006). However, in severe asthma, ICSs often do not provide full control. In these cases, international practice guidelines recommend combining an ICS with a long-acting β2-adrenoceptor agonist (LABA) for which superior disease control, including reduced exacerbation rates, is documented (Shrewsbury et al., 2000). Likewise, in chronic obstructive pulmonary disease (COPD), ICS/LABA therapy provides improved control relative to either monotherapy alone (Rabe et al., 2007). Equally, in acute bronchiolitis, nebulized adrenaline plus oral dexamethasone reduces hospitalization rates relative to either intervention alone (Plint et al., 2009). Thus, multiple strands of clinical evidence indicate cooperative interactions between β2-adrenoceptor (gene symbol: ADRB2) and glucocorticoid receptor (GR) (NR3C1) agonists (Giembycz et al., 2008; Newton et al., 2010).

Mechanisms accounting for the positive interaction between ICSs and LABAs are likely to be multiple (Newton et al., 2010). Glucocorticoids promote β2-adrenoceptor expression, and

ABBREVIATIONS: 8Br-cGMP, 8-bromo-cGMP; ASM, airway smooth muscle; CD200, cluster of differentiation 200; COPD, chronic obstructive pulmonary disease; CRE, cAMP response element; CREB, cAMP response element-binding protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK256066, 6-(6-(3-dimethylamino)carbonyl)phenyl[sulphonyl]-8-methyl-4-[3-methoxy]phenyl]-3-quinolincarboxamide; ICS, inhaled corticosteroid; KT5823, (9-dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK256066,(6-((3-(dimethylamino)carbonyl)phenyl)sulphonyl)-8-methyl-4-[3-methoxy]phenyl)-3-quinolincarboxamide; ICS, inhaled corticosteroid; KT5823, (9-dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK256066,(6-((3-(dimethylamino)carbonyl)phenyl)sulphonyl)-8-methyl-4-[3-methoxy]phenyl)-3-quinolincarboxamide; ICS, inhaled corticosteroid; KT5823, (9-dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK256066,(6-((3-(dimethylamino)carbonyl)phenyl)sulphonyl)-8-methyl-4-[3-methoxy]phenyl)-3-quinolincarboxamide; ICS, inhaled corticosteroid; KT5823, (9-dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK256066,(6-((3-(dimethylamino)carbonyl)phenyl)sulphonyl)-8-methyl-4-[3-methoxy]phenyl)-3-quinolincarboxamide; ICS, inhaled corticosteroid; KT5823, (9-dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK256066,(6-((3-(dimethylamino)carbonyl)phenyl)sulphonyl)-8-methyl-4-[3-methoxy]phenyl)-3-quinolincarboxamide; ICS, inhaled corticosteroid; KT5823, (9-dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK256066,(6-((3-(dimethylamino)carbonyl)phenyl)sulphonyl)-8-methyl-4-[3-methoxy]phenyl)-3-quinolincarboxamide; ICS, inhaled corticosteroid; KT5823, (9-dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK256066,(6-((3-(dimethylamino)carbonyl)phenyl)sulphonyl)-8-methyl-4-[3-methoxy]phenyl)-3-quinolincarboxamide; ICS, inhaled corticosteroid; KT5823, (9-dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; GSK256066,(6-((3-(dimethylamino)carbonyl)phenyl)sulphonyl)-8-methyl-4-[3-methoxy]phenyl)-3-quinolincarboxamide; ICS, inhaled corticosteroid; KT5823, (9-dehydro-
coupling to Gαs, and reduce receptor desensitization (Mak et al., 1995a,b; Chong et al., 1997; Kalavantvanch and Schramm, 2000). Conversely, β2-adrenoceptor agonists elicit various proinflammatory responses, such as the enhancement of interleukin-8 release (Holden et al., 2010), which are inhibited by glucocorticoids (Giambiczy and Newton, 2006; Newton et al., 2010). Although glucocorticoid/LABA combinations may show enhanced repression of inflammatory gene expression (Edwards et al., 2006), evidence for functional synergy on inflammatory outputs is less common (Pang and Knox, 2000), with some studies showing additivity (Pang and Knox, 2001; Newton et al., 2010). However, in terms of anti-inflammatory effects, it is clear that the transactivation of glucocorticoid-sensitive genes is important (Newton, 2000; Newton and Holden, 2007; Clark and Belvisi, 2012). Thus, glucocorticoid-dependent repression of multiple inflammatory genes appears to require GR-mediated gene expression (King et al., 2013). Indeed, the dual-specificity mitogen-activated protein kinase (MAPK) phosphatase, dual-specificity phosphatase 1 (DUSP1), is induced by glucocorticoids to switch off MAPK signaling and thereby reduce inflammatory gene expression (Clark et al., 2008). Since the effect of LABAs and glucocorticoids on DUSP1 expression appears additive (Kaur et al., 2008; Manetsch et al., 2012), this offers some explanation for the effects of combination therapy.

Notwithstanding effects on DUSP1 expression, synergistic enhancement of glucocorticoid-dependent transcription by the cAMP/protein kinase A (PKA) pathway is well established (Manetsch et al., 2012), this offers some explanation for the effects of combination therapy. Moreover, the expression of a variety of other genes with anti-inflammatory activity (Franciosi et al., 2013), evidence for functional synergy on inflammatory outputs is less common (Pang and Knox, 2000), with some studies showing additivity (Pang and Knox, 2001; Newton et al., 2010). However, in terms of anti-inflammatory effects, it is clear that the transactivation of glucocorticoid-sensitive genes is important (Newton, 2000; Newton and Holden, 2007; Clark and Belvisi, 2012). Thus, glucocorticoid-dependent repression of multiple inflammatory genes appears to require GR-mediated gene expression (King et al., 2013). Indeed, the dual-specificity mitogen-activated protein kinase (MAPK) phosphatase, dual-specificity phosphatase 1 (DUSP1), is induced by glucocorticoids to switch off MAPK signaling and thereby reduce inflammatory gene expression (Clark et al., 2008). Since the effect of LABAs and glucocorticoids on DUSP1 expression appears additive (Kaur et al., 2008; Manetsch et al., 2012), this offers some explanation for the effects of combination therapy.

Notwithstanding effects on DUSP1 expression, synergistic enhancement of glucocorticoid-dependent transcription by the cAMP/protein kinase A (PKA) pathway is well established (Rangarajan et al., 1992; Nordeen et al., 1993). Thus, although LABAs do not activate a simple glucocorticoid response element (GRE)-dependent reporter, they can increase the maximal response of a glucocorticoid (Kaur et al., 2008). Because this also occurs with glucocorticoid-induced genes, in vivo correlates may account for the benefits of ICS/LABA therapy (Giambiczy et al., 2008; Kaur et al., 2008). For example, regulator of G-protein signaling 2 (RGS2), which terminates signaling from the heterotrimeric G protein Gαs (Heximer, 2004; Kimple et al., 2009), is bronchoprotective in vivo and may also attenuate cytokine release (Holden et al., 2011, 2014; Xie et al., 2012). Importantly, in human airway smooth muscle (ASM) and bronchial epithelial cells, RGS2 expression is induced by LABAs and this is synergistically enhanced and prolonged by glucocorticoids (Holden et al., 2011, 2014). Likewise, the expression of a variety of other genes with anti-inflammatory potential are induced by glucocorticoids and/or LABAs and could contribute to the efficacy of ICS/LABA combination therapy (Giambiczy and Newton, 2014).

In terms of new therapeutics, the phosphodiesterase (PDE) 4 inhibitor roflumilast was approved in 2012 for patients with severe COPD with chronic bronchitis and frequent exacerbations (Giambiczy and Field, 2010). Although improvements in lung function may occur in the absence of ICSs (Calverley et al., 2009), the additional benefit of adding roflumilast for patients already taking ICSs fits with the fact that PDE4 inhibitors, like LABAs, also enhance glucocorticoid-dependent transcription (Miller et al., 2002; Kaur et al., 2008; Rennard et al., 2011; Moodley et al., 2013; Giambiczy and Newton, 2014). Since PDE3 inhibitors are bronchodilators in humans and may also have anti-inflammatory activity (Franciosi et al., 2013), combined PDE3/PDE4 inhibition may provide a superior treatment option compared with a PDE4 inhibitor alone (Giambiczy and Newton, 2011; Abbott-Banner and Page, 2014). In this study, we used human bronchial epithelial cells, which express PDE3 and PDE4 (Rabe et al., 1993; Dent et al., 1998; Wright et al., 1998) and respond to LABAs and glucocorticoids (Kaur et al., 2010), as a model system to test this hypothesis using anti-inflammatory gene expression that is induced by LABAs and glucocorticoids as a functionally relevant output.

Materials and Methods

Cell Culture, Drugs, and Stimuli. Human bronchial epithelial BEAS-2B cells were grown to confluence in Dulbecco’s modified Eagle’s medium/Ham’s F12 medium (Invitrogen, Burlington, ON, Canada), supplemented with 10% fetal calf serum, l-glutamine (2.5 mM), and NaHCO3 (0.15% w/v) (Invitrogen), prior to overnight incubation in serum-free medium. The cells were then changed to fresh serum-free medium prior to experiments.

Formoterol fumarate [(rac-1R,2R)-N-[2-hydroxy-5-[1-(4-methoxyphenyl)prop-2-ynamino][ethyl]phenyllumformamide] (formoterol; AstraZeneca, Mölndal, Sweden), forskolin ([3R-3a,4a,6a,10a,10b,10a)-5-acetoxyloxy]-3-ethenylodecachydro-6,10,16-trihydroxy-3,4a,7,7a,10a-pentamethyl-1H-naphtho[2,1-b]pyran-1-one), cilostazol (6-[4-(1-cytophyl)-1H-tetrazol-5-yl]butylox-3,4-dihydro-2H-quinolimine), dexamethasone [(8SH,10R,10S,11S,13S,14S,16R,17R)-17-fluroo-11,17-dihydroxy-17-(2-hydroxyacetoyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecachydro-3H-cyclopenta[af]phenanthen-3-one], sodium nitroprusside (SNP) (Sigma-Aldrich, Oakville, ON, Canada), 8-bromo-cGMP (8Br-cGMP), KT5823 (6S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diazolo[123-cy;3,2-1]pyrrolo[3,4-1]benzodiazicione-10-carboxylic acid, methyl ester), ODQ (1H-1,2,4-oxadiazole[4,3-a:3,4-quinolinaxin-1-one], rolipram [(R)-3-(3-cyclopentoloyl-4-methoxyphenyl)pyryridiol-2-one], siguazodon (N-cyano-N-methyl-“4-[4,1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyrazimidyl] phenyl]guanidine), zardaverine (6-[4-(difluoromethoxy)-3-methoxy-phenyl]-3H-(2H)-pyridazinone) (Tocris, Bristol, UK), GSK256066 (6-[[(3-[(dimethylamino)carbonyl]pheny])-8-methyl-4-[[(3-[(dimethylamino)phenyl]amino]]-3-quinolinaoxabite] (Gilead Sciences, Seattle, WA), and roflumilast N-oxide(3-cyclopolympethoxyl)-N-(3,5-dichloro-1-oxo-4-pyridinyl)-4-(difluoromethoxy) benzamide and N-(3,5-dichloro-1-oxo-4-pyridinyl)-4-(difluoromethoxy)3-cyclopropyl-methoxybenzamide) (Nycemed, Konstanz, Germany) were dissolved in dimethylsulfoxide. Final concentrations of dimethylsulfoxide on cells were always <0.1%.

Luciferase Assay. BEAS-2B cells stably transfected with a 6x CRE reporter (pADnese2-C6-BGL) or a 2x CRE reporter (pGL3neo. TATA 2GRE) were grown to confluence in 24-well plates (Chivers et al., 2004; Meja et al., 2004). Confluent cells were incubated in serum-free medium overnight before treatment with drugs. Cells were harvested after 6 hours, unless otherwise indicated, for luciferase activity assay using a commercial kit (Firefly Luciferase Assay Kit; Biotium, Hayward, CA) and a TD20/20 Luminometer (Promega, Madison, WI).

Western Blotting and Adenoviral Infection. Western blotting and adenoviral infections were carried out as previously described (King et al., 2009; Holden et al., 2011). Primary antibodies were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [4699-9555(ST)] (AbD Serotec, Raleigh, NC), cAMP response element-binding protein (CREB) (9197), and phospho-CREB (9191) (Cell Signaling Technology, Danvers, MA). Immunodetection was by enhanced chemiluminescence (GE Healthcare Bio-Sciences Inc., Pittsburgh, PA) and visualization was by autoradiography. For adenoviral infections, 6x CRE reporter BEAS-2B cells at approximately 70% confluence were incubated for 24 hours in Dulbecco’s modified Eagle’s medium/F12 plus 10% fetal calf serum with adenoviral (Ad5) expression vectors at the indicated multiplicity of infection (MOI). Ad5-cAMP response element (CREB-dependent, catalytic) inhibitor a (PKLa/PKIA) and the empty Ad5 vector (null) were as described (Meja et al., 2004).

RNA Isolation, cDNA Synthesis, and SYBR Green Real-Time Polymerase Chain Reaction. Total RNA (0.5 μg), extracted by the RNeasy mini kit method (Qiagen, Valencia, CA), was reverse transcribed
using QScript reverse transcriptase (Quanta Biotics, Gaithersburg, MD). SYBR GreenER (Invitrogen) real-time polymerase chain reaction (PCR) was performed using a StepOne Plus real-time PCR machine (Applied Biosystems Inc, Foster City, CA) as described (King et al., 2009). The relative cDNA concentration of target genes was derived from parallel analysis of a standard curve of serially diluted cDNA. Amplification primers for RGS2, cluster of differentiation 200 (CD200), cysteine-rich secretory protein LCCL domain containing 2 (CRISPLD2), DUSP1, and GAPDH were as described and the specificity of the primers was determined by dissociation (melt) curve analysis (King et al., 2009; Holden et al., 2011; Moodley et al., 2013).

Data Presentation and Statistical Analysis. All data are presented as means ± S.E. of n determinations as indicated in each figure. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA). Comparisons between groups were performed using one-way analysis of variance (type II error). In these situations, a Newman–Keuls multiple comparison test was used.

### Results

**PDE Family mRNA Expression Profile in BEAS-2B Cells.** Microarray analysis of BEAS-2B cells supports the presence of mRNAs for PDE1C, PDE3A, PDE4A, PDE4B, PDE4D, PDE5A, PDE6A, PDE6D, PDE7A, PDE8A, and PDE10A (Supplemental Table 1). Positive detection of probe sets corresponding to PDE1A, PDE2B, PDE4C, PDE7B, PDE9A, and PDE11A was more variable between arrays, whereas PDE1B, PDE2A, PDE6B, PDE6C, PDE6D, PDE6G, PDE6H, and PDE8B were not detected (Supplemental Table 1).

**Effect of PDE3 and PDE4 Inhibitors on CRE-Dependent Transcription.** PDE3 and PDE4 were previously identified in human airway epithelial cells and their ability to mediate anti-inflammatory activity is well studied (Dent et al., 1998; Wright et al., 1998; Banner and Press, 2009). PDE3 and PDE4 inhibitors were therefore tested for their ability to induce cAMP-dependent transcription using BEAS-2B cells harboring a luciferase reporter driven by six CRE sites (Meja et al., 2004). The PDE3 inhibitor siguazodan was inactive on the 6× CRE reporter except for a small enhancement produced at the highest concentrations tested (10 and 30 μM). By contrast, rolipram, a PDE4 inhibitor, increased CRE-dependent transcription in a concentration-dependent manner (EC_{50} = approximately 960 nM). However, the maximal induction was modest (~2-fold) compared with forskolin, which increased luciferase activity by almost 20-fold (Fig. 1A). Siguazodan, in combination with rolipram, both at 10 μM, produced an approximately 3-fold activation of the reporter (Fig. 1B). This effect was considerably greater than the sum of the responses produced by siguazodan and rolipram alone and is indicative of a positive cooperative interaction between the actions of these compounds.

**Effect of PDE3 and PDE4 Inhibitors on the Enhancement of CRE-Dependent Transcription by Formoterol.** Formoterol robustly and substantially activated CRE-dependent transcription with an EC_{50} value of 50–100 pM (Fig. 2; Table 1). In the presence of either siguazodan (10 μM) or rolipram (10 μM), the formoterol concentration-response curves were displaced almost a half-log to the left in the absence of changes to the maximum response (Fig. 2A). Thus, both PDE inhibitors increased the sensitivity of CRE-dependent transcription to the LABA (Fig. 2A; Table 1). Furthermore, with a shift in the EC_{50} for formoterol from 107 pM to 18 pM, this sensitization was considerably more pronounced when siguazodan plus rolipram were used in combination (Fig. 2A, right panel; Table 1). As before, there was no change in the maximal reporter activity produced by formoterol. To confirm this effect, the ability of rolipram to sensitize formoterol-induced responsiveness was assessed in the presence of siguazodan (Fig. 2B). Thus, the further addition of rolipram to formoterol plus siguazodan produced a significant leftward shift in the concentration-response relationship for formoterol (Fig. 2B; Table 1). Conversely, the ability of siguazodan to sensitize the effect of formoterol was assessed in presence of rolipram and again this resulted in a significant leftward shift in the formoterol concentration-response curve (Fig. 2B; Table 1). These data confirm that combining siguazodan with rolipram produces a positive combinatorial effect on the ability of formoterol to promote CRE-dependent transcription.

To assess possible off-target effects, structurally dissimilar PDE3 and PDE4 inhibitors were evaluated (Supplemental Fig. 1). The PDE3 inhibitor (cilostazol) and the two PDE4 inhibitors (GSK256066 and roflumilast N-oxide) showed little effect on CRE reporter activation (Supplemental Fig. 1A). However, each PDE inhibitor modestly, but significantly, sensitized the reporter to the effects of formoterol (Supplemental Fig. 1B; Table 1). This was consistent with the prior data obtained for siguazodan and rolipram (Fig. 2; Table 1). Equally, the dual PDE3/PDE4 inhibitor (zardaverine) modestly increased CRE reporter activity and significantly and
substantially sensitized the response to formoterol (Supplementary Fig. 1C; Table 1). This was similar to the effect of siguazodan plus rolipram in combination and indicates that inhibition of both PDE3 and PDE4 can interact to sensitize formoterol-induced CRE-dependent transcription.

Effect of Siguazodan and Rolipram on the Kinetics of CRE-Dependent Transcription. Whereas siguazodan (10 μM) failed to promote the CRE-dependent transcription, rolipram (10 μM) produced a modest, but transient, activation of the reporter that peaked between 4 and 8 hours (Fig. 2C, left panel). In the presence of siguazodan (10 μM), the kinetics of rolipram-induced, CRE-dependent transcription were enhanced from below 2-fold to a peak of around 3-fold and were also considerably extended. Indeed, at 12 and 18 hours, neither siguazodan nor rolipram had any marked effect on reporter activation (Fig. 2C, left panel), yet the combination of the two inhibitors produced a significantly greater response than either one alone. A maximally effective concentration of formoterol (1 nM) strongly activated the CRE reporter, with the peak in luciferase activity occurring from 4 to 8 hours (Fig. 2C, right panel). These kinetics were not materially affected by rolipram (10 μM) or siguazodan (10 μM) alone or in combination (Fig. 2C, right panel).

Effects of PKI Overexpression and Modulation of the cGMP/Protein Kinase G Pathway on CRE-Dependent Transcription. cAMP-dependent activation of PKA leads to the phosphorylation of CREB and the subsequent activation of CRE-dependent transcription (Giembycz and Newton, 2006). Infection of BEAS-2B 6× CRE reporter cells with an adenovirus expressing PKIα (MOI of 30), a potent and highly selective inhibitor of PKA (Meja et al., 2004), markedly (>80%) prevented the CRE-dependent transcription induced by both formoterol and formoterol in the presence of siguazodan plus rolipram (Fig. 3). Because null virus at this MOI was inactive, the sensitization of formoterol-induced CRE-dependent transcription by PDE3 plus PDE4 inhibitors appears to involve the classic PKA pathway.
the hydrolysis of cGMP generated constitutively by soluble guanylyl cyclase. For completeness, the role of cGMP-dependent protein kinase G (PKG) was evaluated using the inhibitor KT5823, which is approximately 50-fold selective for PKG over PKA (\(K_i = 0.2\) and \(10\ \mu M\) respectively). As shown in Supplemental Fig. 2C, KT5823 had no effect on activation of the CRE reporter by siguazodan, rolipram (alone and in combination), or formoterol (Supplemental Fig. 2C).

Effect of PDE3 and PDE4 Inhibitors on CREB Phosphorylation. Treatment of BEAS-2B cells with formoterol promoted a time-dependent phosphorylation of CREB at serine 133 that was readily detectible at 10 minutes and was maintained for at least 1 hour after treatment (Fig. 4A). Two hours after formoterol treatment, CREB phosphorylation had begun to wane and this had approached basal levels by 6 hours. It is important to note that because of apparent blocking of signal produced by the total CREB antibody after prior immunodetection of phospho-CREB, even after robust stripping of the membrane, normalization was necessarily performed relative to GAPDH (Fig. 4A). The validity of this approach was confirmed by examining total CREB expression relative to GAPDH, which did not change over the duration of these experiments (Fig. 4A; Supplemental Fig. 3A). Therefore, all subsequent normalizations were performed using GAPDH as the loading control. Although the phosphorylation of activating transcription factor (ATF1) was also detected and increased in parallel with CREB phosphorylation (Fig. 4), total ATF1 expression was not assessed and therefore changes in ATF1 expression cannot be excluded.

There was little or no effect of either siguazodan (10 \(\mu M\)) or rolipram (10 \(\mu M\)) alone or in combination on CREB phosphorylation (Fig. 4B). With a low concentration of formoterol (10 pM), there was an apparently modest increase in CREB phosphorylation produced by siguazodan. In the presence of rolipram, formoterol-induced CREB phosphorylation was significantly augmented at both 10 and 30 minutes after stimulation. The further addition of siguazodan produced a small increase in CREB phosphorylation that was not statistically significant. Conversely, the higher (100 pM) concentration of formoterol produced a robust increase in CREB phosphorylation that was not increased by a combination of siguazodan plus rolipram (both at 10 \(\mu M\)) (Fig. 4B). To examine possible effects on total CREB expression, the samples from these experiments were separately subjected to Western blot analysis for total CREB and GAPDH. As in Fig. 4A and Supplemental Fig. 3A, there was no change in total CREB expression relative to GAPDH for any treatment (Supplemental Fig. 3B). Taken together, these data mimic the effects produced on the CRE reporter, albeit with a lower level of sensitivity.

![Fig. 3. Effect of PKI\(_\alpha\) overexpression on CRE-dependent transcription. 6×CRE BEAS-2B cells were either not treated or infected at a MOI of 30 with either Ad5.CMV.PKI\(_\alpha\) or Ad5.CMV.Null for 48 hours. Thereafter, cells were left not stimulated (NS) or treated for 30 minutes with siguazodan (10 \(\mu M\)) plus rolipram (10 \(\mu M\)) (S + R), before being exposed to various concentrations of formoterol (Form). After 6 hours, cells were harvested for luciferase assay. Data are expressed as fold induction relative to NS and are plotted as means ± S.E.](image-url)

<table>
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<tr>
<th>Treatment</th>
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<th>Determinations</th>
<th>Paired t Test</th>
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<tr>
<td>Naive</td>
<td>10.6 ± 0.0</td>
<td>7</td>
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<td>***</td>
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</tr>
<tr>
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<td>10.75 ± 0.09</td>
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<td>10.54 ± 0.04</td>
<td>5</td>
<td>***</td>
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<td>+ zaraverine</td>
<td>11.2 ± 0.11</td>
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\(*P < 0.05; **P < 0.001.\)
Effect of PDE3 Plus PDE4 Inhibitors on Formoterol-Induced Gene Expression. Treatment of BEAS-2B cells with siguazodan plus rolipram produced modest, nonsignificant increases in RGS2, CD200, CRISPLD2, and DUSP1 mRNA expression (Fig. 5; Supplemental Fig. 4A). By contrast, formoterol (1 nM) significantly, but transiently, increased the mRNA expression of all four genes. RGS2 and DUSP1 expression peaked at 1 hour, whereas CD200 and CRISPLD2 were maximal at 2 hours and, in each case, these responses were unaffected by siguazodan plus rolipram. However, in the presence of siguazodan plus rolipram, there was a significantly reduced decline in RGS2 expression observed by 6 hours and at all times thereafter (Fig. 5). Similar, but considerably more modest, effects were apparent for CD200 and DUSP1 mRNA at 12 and 18 hours (Fig. 5; Supplemental Fig. 4A).

To examine the contribution of each inhibitor to this effect, BEAS-2B cells were treated with siguazodan plus rolipram in the absence or presence of formoterol at 2 and 12 hours (Fig. 6). However, due to the low fold enhancement by formoterol, or formoterol plus siguazodan plus rolipram, DUSP1 expression was not examined (Supplemental Fig. 4A). As described for the CRE reporter (Fig. 1), there was little effect of siguazodan on RGS2, CD200, and CRISPLD2 mRNA expression at 2 hours. Rolipram produced modest increases in RGS2, CD200, and CRISPLD2 mRNA at 2 hours (Fig. 6A) and the combination of both PDE inhibitors significantly increased the expression of all three genes to a level that was greater than the sum of their individual effects (Fig. 6A). At 2 hours, the concentration-response curves that described formoterol-induced RGS2, CD200, and CRISPLD2 expression were displaced to the left by approximately 1 log\textsubscript{10} unit by the combination of siguazodan and rolipram without changing the maximal responses (Fig. 6B; Table 2). With a low concentration of formoterol (10 pM), the enhancement of formoterol-induced gene expression by the combined PDE inhibitors was primarily due to rolipram with modest increases, significant for CD200, produced by the further addition of siguazodan (Fig. 6C).
that at early time points (2 hours) the formoterol concentration-response curve was displaced to the left by approximately 1 log10 unit, in the absence of any change in the maximal response (Fig. 7A; Table 2). In the presence of a low concentration of formoterol (1 nM) (Form), the formoterol concentration-response relationship for each gene relative to dexamethasone alone. This effect was maximal from 1 to 2 hours and declined thereafter. Concurrent addition of the PDE3 with PDE4 inhibitor had no effect on gene expression produced by formoterol plus dexamethasone at 1 and 2 hours (Fig. 8A). However, at 6, 12, and 18 hours, when dexamethasone plus formoterol-induced expression of RGS2 mRNA had declined from the peak value, the combination of siguazodan and rolipram significantly augmented mRNA expression above that achieved by the LABA plus glucocorticoid. Thus, the half-life for RGS2 expression taken from the 1-hour maximum was extended from approximately 5 to 8 hours in the presence of the PDE3 plus PDE4 inhibitors (Fig. 8B). Likewise, the time required for RGS2 expression to decline to 25% of the 1-hour maximum value was extended from approximately 9.3 to almost 16 hours. Qualitatively similar data were obtained for CD200 and CRISPLD2, but not for DUSP1, which was not further studied (Fig. 8A; Supplemental Fig. 4B).

To explore the contribution of PDE3 and PDE4 inhibition to these effects, BEAS-2B cells were treated with dexamethasone (1 μM) in the absence and presence of each inhibitor. At 2 hours, dexamethasone enhanced the expression of RGS2, CD200, and CRISPLD2 mRNAs by 2- to 4-fold via a mechanism that was enhanced by rolipram, but not siguazodan (Fig. 9A). In combination, siguazodan plus rolipram significantly augmented this expression of both RGS2 and CD200 over the effect of rolipram alone (Fig. 9A). A similar trend was observed for CRISPLD2 mRNA. Formoterol enhanced dexamethasone-induced gene expression at 2 hours in a concentration-dependent manner (Fig. 9B). In the further presence of siguazodan plus rolipram, the formoterol concentration-response relationship for each mRNA was displaced by approximately 1 log10 unit to the left in the absence of any change to maximum response (Fig. 9B; Table 2). In the presence of a low concentration of formoterol (10 μM), the enhancement of dexamethasone-induced gene expression at 2 hours was unaffected by siguazodan, but was significantly enhanced by rolipram (Fig. 9C). Moreover, the combination of rolipram plus siguazodan was as effective as rolipram alone, indicating that at 2 hours, these responses primarily occurred via PDE4 inhibition.

The above data contrast with the effects on gene expression observed at 12 hours (Fig. 9D). Although dexamethasone modestly enhanced RGS2, CD200, and CRISPLD2 mRNA expression, there was no apparent effect of rolipram. Conversely,
siguazodan resulted in small enhancements, significant for CRISPLD2, in the expression of all three genes (Fig. 9D, left panels). In the presence of siguazodan plus rolipram, the dexamethasone-induced gene expression of all three genes was significantly enhanced (Fig. 9D, left panels). This effect was significantly greater than the response to dexamethasone plus rolipram and, in each case, was more than the effect of dexamethasone plus siguazodan (although only significant for CRISPLD2).

With a maximally effective concentration of formoterol (1 nM), the dexamethasone-induced expression of all three genes was markedly increased (Fig. 9D, right panels). For both RGS2 and

![Image: Graphs showing gene expression levels with various treatments.]
CRISPLD2, siguazodan and rolipram each produced small but significant increases in mRNA expression. A similar trend was apparent for CD200. In each case, the combination of siguazodan plus rolipram significantly increased dexamethasone plus formoterol-induced gene expression, an effect that was significantly greater than that achieved by each PDE inhibitor alone (Fig. 9D, right panels). Thus, inhibition of both PDE3 and PDE4 is responsible for the longer-term enhancements in dexamethasone plus formoterol-dependent gene expression.

**Discussion**

The ability of glucocorticoids to induce effector gene expression is a fundamental component of their anti-inflammatory activity (Newton and Holden, 2007; Clark and Belvisi, 2012). Furthermore, the activity of a simple GRE-dependent glucocorticoid-induced reporter and glucocorticoid-induced gene transcription can be significantly enhanced by LABAs (Kaur et al., 2008). The ability to synergistically enhance glucocorticoid-dependent transcription may contribute to the superior efficacy of ICS/LABA combination therapies in asthma and COPD (Giembycz et al., 2008; Newton et al., 2010), although it is not applicable to all glucocorticoid-induced genes, including TSC22D3 (GILZ), FKBP5 (FKBP51), and ZFP36 (TTP) (Kaur et al., 2008). Conversely, although the acute \(\beta_2\)-adrenoceptor–mediated relaxation of ASM is a recognized, nongenomic response of the cAMP-PKA pathway (Giembycz and Newton, 2006), the therapeutic relevance of activating cAMP-dependent gene expression is less explored. Nevertheless, LABAs increase

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**TABLE 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Potency to Formoterol in the Presence of 1 (\mu)M Dexamethasone (pEC(_{50}))</th>
<th>Determinations</th>
<th>ANOVA with Bonferroni post Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>10.1 ± 0.1</td>
<td>8</td>
<td>***</td>
</tr>
<tr>
<td>+ siguazodan</td>
<td>10.2 ± 0.2</td>
<td>8</td>
<td>***</td>
</tr>
<tr>
<td>+ rolipram</td>
<td>11.2 ± 0.1</td>
<td>8</td>
<td>***</td>
</tr>
<tr>
<td>+ siguazodan + rolipram</td>
<td>11.1 ± 0.1</td>
<td>7</td>
<td>*** ***</td>
</tr>
</tbody>
</table>

***P < 0.001 for comparisons with naïve cells; ***P < 0.001 for comparisons with siguazodan-treated cells.
RGS2 expression to protect against bronchoconstriction and, potentially, the proinflammatory effects of mediators such as histamine and acetylcholine (Holden et al., 2011, 2014; Xie et al., 2012). Furthermore, RGS2 expression is synergistically enhanced by LABAs, or other cAMP-elevating compounds, in combination with glucocorticoids in human bronchial epithelial and ASM cells (Holden et al., 2011, 2014). Similarly, LABAs and glucocorticoids synergistically induce the expression of CD200, a gene product that reduces macrophage activation and inflammatory cytokine expression (Snelgrove et al., 2008), and the expression of CRISPLD2, which binds lipopolysaccharide and may attenuate Toll-like receptor 4–dependent responses during bacterial infections (Wang et al., 2009). The expression of DUSP1, a putative anti-inflammatory gene that is responsible for switching off MAPK activity and reducing expression of at least some inflammatory genes (Abraham et al., 2006; Shah et al., 2014), is induced by both LABAs and glucocorticoids (Kaur et al., 2008; Manetsch et al., 2012). However, these effects on DUSP1 mRNA were largely additive with the effects of LABAs essentially over within 2 hours. This effect is shown by our current data and explains why DUSP1 expression was not investigated further in this study. Nevertheless, the known functions of these various genes illustrates how enhancing glucocorticoid- and/or LABA-dependent gene expression could contribute to therapeutic activity. Importantly, such interactions are likely to be relevant when considering the effect of adding PDE inhibitors that also increase intracellular cAMP (Newton et al., 2010; Giembycz and Newton, 2014).

In patients with severe bronchitic COPD, the PDE4 inhibitor roflumilast provides therapeutic advantage to individuals taking LABAs, particularly those taking ICS/LABA combination therapy (reviewed in Giembycz and Field, 2010; Gross et al., 2010). Indeed, we previously documented that PDE4 inhibitors enhance glucocorticoid-dependent gene expression in human airway epithelial cells (Moodley et al., 2013; Holden et al., 2014). The current study extends these findings to show that PDE4 inhibitors sensitize LABA- and GR-dependent transcriptional responses. However, there is considerable interest in the utility of inhibiting PDEs other than PDE4 and in the inhibition of multiple PDEs (Giembycz and Newton, 2011). One candidate that could deliver therapeutic benefit is PDE3, for which inhibitors were developed for the treatment of dilated cardiomyopathy (Movsesian and Alharethi, 2002). Because PDE3 inhibitors are also bronchodilators, dual PDE3/PDE4 inhibition could produce both bronchodilator and anti-inflammatory activities (Banner and Press, 2009; Giembycz and Newton, 2011). Indeed, support for this concept was recently reported in trials using the dual PDE3/PDE4 inhibitor, RPL554, in healthy human volunteers and in patients with asthma or COPD (Franciosi et al., 2013). Our current data provide an explanation for these effects.
Like PDE4 inhibitors, PDE3 inhibition modestly, but significantly, sensitized BEAS-2B cells to formoterol-induced cAMP-dependent transcription. However, combining a PDE3 inhibitor, which was ineffective by itself, with a PDE4 inhibitor enhanced both the duration of cAMP-dependent transcription and the sensitization of formoterol-induced cAMP-dependent transcription relative to the effect of PDE4 inhibition alone. With similar effects observed on CREB phosphorylation and CRE-dependent transcription induced by formoterol or formoterol plus siguazodan and rolipram being prevented by PKIα overexpression, these data support a classic PKA-dependent process. However, because PDE3 can theoretically hydrolyze cAMP and cGMP (albeit with a lower $V_{\text{max}}$) (Zaccolo and Movsesian, 2007), a role for the soluble guanylyl cyclase/cGMP/PKG signaling pathway was explored with negative results. Thus, neither inhibitors (ODQ, KT5823), nor activators (8Br-cGMP, SNP) of this pathway modified CRE reporter activity. These findings argue against a major role for the cGMP-PKG pathway in the effects elicited by PDE3 inhibition. Importantly, the changes in CRE-dependent transcription were also reproduced on RGS2, CD200, and CRISPLD2 mRNA expression, which are also cAMP-dependent effectors. In respect of glucocorticoid-induced transcription, the PDE3 inhibitor siguazodan was without effect on, and failed to potentiate or extend, the enhancement of GRE-dependent transcription produced by PDE4 inhibition in the absence and presence of formoterol. Moreover, 2 hours after treatment, there was no effect of PDE3 inhibition on the expression of RGS2, CD200, or CRISPLD2 induced by dexamethasone. However, PDE4 inhibition markedly enhanced dexamethasone-induced gene expression and this was further increased by PDE3 inhibition. Nevertheless, at 2 hours, the sensitization of formoterol-enhanced glucocorticoid-induced gene expression was mimicked by multiple cAMP elevators and was also blocked by PKIα (Kaur et al., 2008; Moodley et al., 2013). Furthermore, the ability of LABAs to synergize with glucocorticoids on the induction of RGS2 expression was mimicked by various cAMP elevators and was also blocked by PKIα (Holden et al., 2014), suggesting that cAMP-PKA–dependent processes are the likely mechanisms of action for these effects. By contrast, longer treatment times (i.e., 12 hours after stimulation) with PDE3 plus PDE4 inhibitors revealed marked enhancements of dexamethasone-induced gene expression, particularly in the presence of formoterol. Thus, although peak RGS2 mRNA expression
induced by dexamethasone plus formoterol was unaffected, the subsequent rate of decline was strikingly reduced by combined PDE3/PDE4 inhibition. This effect extended to CD200 and CRISPLD2, required both PDE3 and PDE4 inhibition, and suggests that dual PDE3/PDE4 inhibitors may significantly prolong the effects of ICS/LABA combination therapies.

Overall, although the predominant effects on gene expression were due to PDE4 inhibition, there were clear benefits to adding a PDE3 inhibitor. These included potentiating the effect of the PDE4 inhibitor, further sensitizing BEAS-2B cells to LABA, and, in particular, extending the duration of action of glucocorticoid/LABA/PDE4 inhibitor–induced gene expression. In terms of modulating therapeutic effectiveness, PDE inhibitors could therefore enhance and/or sensitize target cells to LABA-induced transcription. Perhaps more clinically relevant is that PDE inhibitors may enhance and/or sensitize target cells to the ability of LABAs to enhance glucocorticoid-dependent transcription in vivo. This might be particularly relevant in the context of the ongoing phase 4 Rollumilast in the Prevention of COPD Exacerbations while Taking Appropriate Combination Treatment study, in which the effect of rollumilast and placebo on the exacerbation rates of patients with severe bronchitic COPD treated with ICS/LABA combination therapy, with or without a long-acting muscarinic receptor antagonist, are compared (Calverley et al., 2012).

In terms of understanding these effects, it is increasingly clear that different cellular pools of cAMP may subserve differential physiologic outcomes (Bailie et al., 2005; Houslay, 2010). In this context, the subcellular localization of different PDE enzymes, or their various isoforms, contributes to the functional compartmentalization of different cAMP pools (Houslay et al., 2007; Maurice et al., 2014). In terms of the current study, the sensitization of CRE-dependent transcription occurred after both PDE3 and PDE4 inhibition and these effects combine to produce enhanced effects. Conversely, the ability to sensitize the enhancement of CRE-dependent transcription only occurred with a PDE4 inhibitor and this suggests a functional distinction in the cAMP pools leading to each response. In respect to LABA- and glucocorticoid-inducible genes, this may lead to “mixed” effects depending on the exact contribution of each pathway to the overall gene expression. Interestingly, our data also reveal longer-term effects, whereby the role of the PDE3 inhibitor, relative to PDE4, appears to be enhanced. Although such effects may be explained by changes in the expression of different PDE isoforms, the mechanistic basis of this effect requires investigation.

One striking aspect of the current data is the clear demonstration that cAMP PDE inhibitors sensitized BEAS-2B cells to transcriptional outcomes produced by LABAs. In contrast, sensitization of BEAS-2B cells to transcriptional outcomes produced by LABAs. In contrast, sensitization of BEAS-2B cells was either absent or reduced by a PDE3 inhibitor. These included potentiating the effect of the PDE4 inhibitor, further sensitizing BEAS-2B cells to LABA, and, in particular, extending the duration of action of glucocorticoid/LABA/PDE4 inhibitor–induced gene expression. In terms of modulating therapeutic effectiveness, PDE inhibitors could therefore enhance and/or sensitize target cells to LABA-induced transcription. Perhaps more clinically relevant is that PDE inhibitors may enhance and/or sensitize target cells to the ability of LABAs to enhance glucocorticoid-dependent transcription in vivo. This might be particularly relevant in the context of the ongoing phase 4 Rollumilast in the Prevention of COPD Exacerbations while Taking Appropriate Combination Treatment study, in which the effect of rollumilast and placebo on the exacerbation rates of patients with severe bronchitic COPD treated with ICS/LABA combination therapy, with or without a long-acting muscarinic receptor antagonist, are compared (Calverley et al., 2012).

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One striking aspect of the current data is the clear demonstration that cAMP PDE inhibitors sensitized BEAS-2B cells to transcriptional outcomes produced by LABAs. In airway epithelial cells and ASM cells, which have a high β2-adrenoceptor density and/or efficient receptor-adenyl cyclase coupling (Giembycz, 2009; Giembycz and Newton, 2014), this interaction is reflected by an increase in the potency of the LABA in the absence of any change in the maximal response attained (see Figs. 2, 6, and 9) (Newton et al., 2010). However, nonuniform distribution combined with continual removal of inhibited drugs may lead to maximally effective responses not being attained, or maintained, across the whole lung. By sensitizing to the effects of inhaled LABA, PDE inhibitors may help preserve responsiveness. By contrast, inflammatory cells that have low β2-adrenoceptor density and/or weak receptor-effector coupling may reveal modest, even absent, responsiveness to LABA (Newton et al., 2010; Giembycz and Newton, 2014). In these cells, a PDE inhibitor is predicted to increase the magnitude of LABA-induced responses (without changing potency) (Giembycz and Newton, 2014). This may translate into improved clinical outcomes. Importantly, PDE inhibition will, in combination with LABAs, help maintain glucocorticoid-induced gene expression, a response that can be attenuated by inflammatory stimuli, respiratory viruses, cigarette smoke, and factors that promote airway remodeling (Hinze et al., 2011; Rider et al., 2011, 2013; Salem et al., 2012).

In conclusion, we use cAMP- and glucocorticoid-dependent gene expression in BEAS-2B cells as a functionally relevant model to provide proof of principle that concurrent PDE3 and PDE4 inhibition can impart superior benefits to PDE4 inhibition alone. Combined PDE3/PDE4 inhibition sensitizes to LABA-induced gene transcription to a greater extent than either inhibitor alone. Similarly, although sensitization of LABA-enhanced, glucocorticoid-dependent transcription was due to PDE4 inhibition, LABA- and glucocorticoid-induced gene expression revealed advantages of dual PDE3/PDE4 inhibition, including the significant prolongation of anti-inflammatory gene expression. Given that multiple genes, including RGS2, CD200, and CRISPLD2, have anti-inflammatory potential (Giembycz and Newton, 2014), we suggest that combined PDE3/PDE4 inhibition should be clinically evaluated as an add-on to existing ICS/LABA combination therapy in obstructive lung diseases.

Authorship Contributions

Participated in research design: Giembycz, Newton.

Conducted experiments: BinMahfouz, Borthakur, Yan, George, Newton.

Performed data analysis: BinMahfouz, Borthakur, Yan, George, Giembycz, Newton.

Wrote or contributed to the writing of the manuscript: BinMahfouz, Borthakur, Yan, George, Giembycz, Newton.

References


