

Superiority of Combined Phosphodiesterase PDE3/PDE4 Inhibition over PDE4 Inhibition Alone on Glucocorticoid- and Long-Acting β_2 -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 β_2 -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 β_2 -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 bronchitis, 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 β_2 -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 β_2 -adrenoceptor expression,

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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-((3-((dimethylamino)carbonyl)phenyl)sulfonyl)-8-methyl-4-((3-methyloxy)phenyl)amino)-3-quinolinecarboxamide; ICS, inhaled corticosteroid; KT5823, (9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-k']pyrrolo [3,4-j][1,6]benzodiazocine-10-carboxylic acid, methyl ester; LABA, long-acting β_2 -adrenoceptor agonist; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PCR, polymerase chain reaction; PDE, phosphodiesterase; PKA, protein kinase A; PKG, protein kinase G; SNP, sodium nitroprusside.

coupling to G_{α_s} , and reduce receptor desensitization (Mak et al., 1995a,b; Chong et al., 1997; Kalavantavanich and Schramm, 2000). Conversely, β_2 -adrenoceptor agonists elicit various proinflammatory responses, such as the enhancement of interleukin-8 release (Holden et al., 2010), which are inhibited by glucocorticoids (Giembycz 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 most 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 (Giembycz et al., 2008; Kaur et al., 2008). For example, regulator of G-protein signaling 2 (RGS2), which terminates signaling from the heterotrimeric G protein G_{α_q} (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 (Giembycz 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 (Giembycz 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; Giembycz 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 (Giembycz 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., 2008), 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 NaHCO_3 (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*-(*R,R*)-*N*-[2-hydroxy-5-[1-hydroxy-2-[1-(4-methoxyphenyl)propan-2-ylamino]ethyl]phenyl]formamide] (formoterol; AstraZeneca, Mölndal, Sweden), forskolin [(3*R*)-(3*α*,4*α*,5*β*,6*β*,6*α*,10*α*,10*αβ*,10*β*)-5-(acetyloxy)-3-ethenyl-10,11-dihydro-6,10,10*β*-trihydroxy-3,4,7,7,10*α*-pentamethyl-1*H*-naphtho[2,1-*b*]pyran-1-one), cilostazol (6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1*H*)-quinolinone), dexamethasone [(8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one], sodium nitroprusside (SNP) (Sigma-Aldrich, Oakville, ON, Canada), 8-bromo-cGMP (8Br-cGMP), KT5823 [(9*S*,10*R*,12*R*)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*kl*]pyrrolo[3,4-*i*][1,6]benzodiazocine-10-carboxylic acid, methyl ester], ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one), rolipram [(*R,S*)-4-(3-cyclopentyl-4-methoxyphenyl)pyrrolidin-2-one], siguazodan (*N*-cyano-*N'*-methyl-*N'*-[4-(1,4,5,6-tetrahydro-4-methyl-6-oxo-3-pyridazinyl)phenyl]guanidine), zardaverine (6-[4-(difluoromethoxy)-3-methoxyphenyl]-3(2*H*)-pyridazinone) (Tocris, Bristol, UK), GSK256066 [(6-((3-[(dimethylamino)carbonyl]phenyl)sulfonyl)-8-methyl-4[[3-methoxyphenyl]amino]-3-quinolinecarboxamide] (Gilead Sciences, Seattle, WA), and roflumilast *N*-oxide(3-(cyclopropylmethoxy)-*N*-(3,5-dichloro-1-oxido-4-pyridinyl)-4-(difluoromethoxy) benzamide and *N*-(3,5-dichloro-1-oxopyridin-4-yl)-4-difluoromethoxy-3-cyclopropyl-methoxybenzamide) (Nycomed, 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 6× CRE reporter (pADneo2-C6-BGL) or a 2× GRE reporter (pGL3.neo.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, 6× 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 infections (MOIs). Ad5-protein kinase (cAMP-dependent, catalytic) inhibitor α (PKI α /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 Biosciences, 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 $\pm$ 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 with a Bonferroni or Dunnett's post test or a paired t test as indicated. Where more than five simultaneous comparisons are made, Bonferroni should not be used due to the inappropriately high false negative rate (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, PDE3B, 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 $\times$ CRE reporter except for a small enhancement produced at the highest concentrations tested (10 and 30 μ M). 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 μ M, 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 μ M) or rolipram (10 μ M), 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

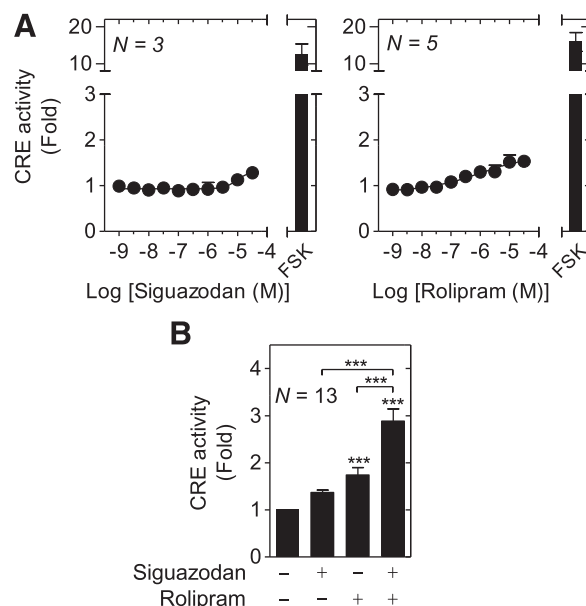


Fig. 1. Effect of PDE inhibitors on luciferase activity in 6 $\times$ CRE BEAS-2B reporter cells. (A) CRE BEAS-2B reporter cells were treated with various concentrations of siguazodan or rolipram and forskolin (FSK) (10 μ M). Cells were harvested after 6 hours for luciferase assay. Data are expressed as fold induction of luciferase relative to unstimulated cells and are plotted as mean $\pm$ S.E. (B) 6 $\times$ CRE BEAS-2B reporter cells were not stimulated or stimulated with siguazodan (10 μ M), rolipram (10 μ M), or a combination of both together (both 10 μ M). At 6 hours, the cells were harvested for luciferase assay. Data are expressed as fold induction relative to unstimulated cells and are plotted as mean $\pm$ S.E. Significance relative to unstimulated cells was assessed by analysis of variance with a Bonferroni post test. Additional comparisons are as indicated. *** P < 0.001.

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

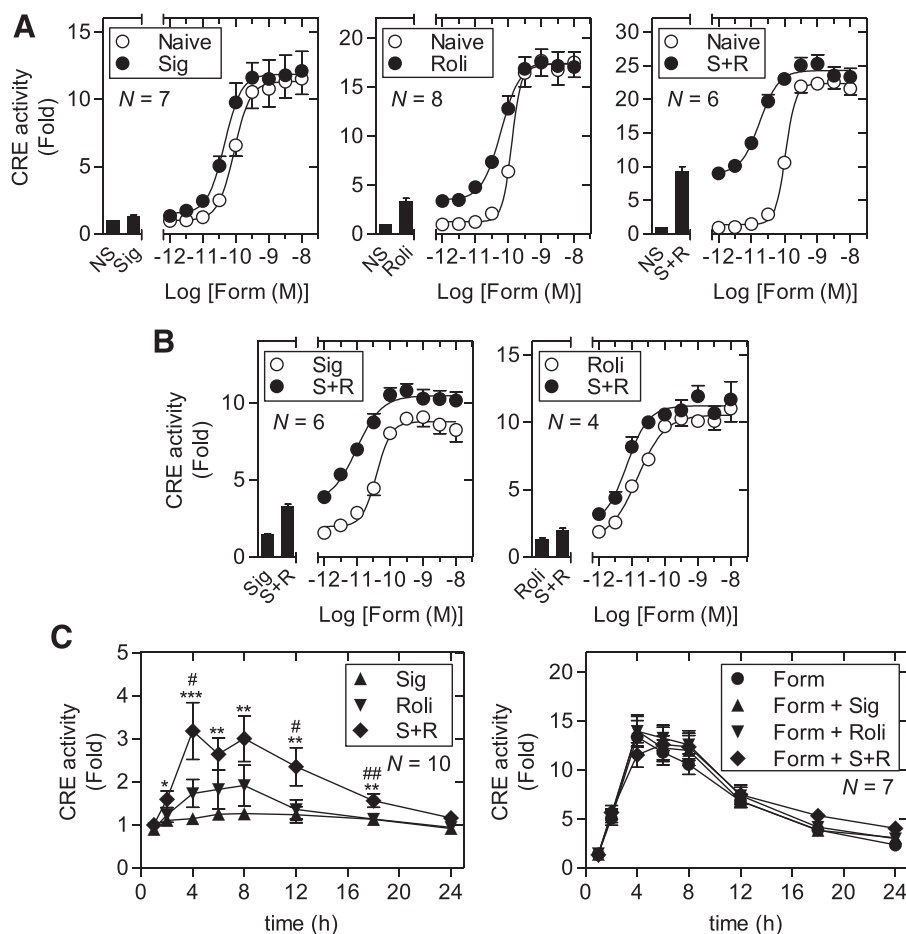


Fig. 2. Effect of PDE inhibitors and formoterol on luciferase activity and kinetics in 6x CRE BEAS-2B reporter cells. (A and B) BEAS-2B 6x CRE reporter cells were preincubated for 30 minutes with vehicle, siguazodan (10 μ M) (Sig), rolipram (10 μ M) (Roli), or a combination of both (both at 10 μ M) (S + R) before treatment with various concentrations of formoterol. After 6 hours, cells were harvested for luciferase assay. Data are expressed as fold induction relative to nonstimulated cells (NS) and are plotted as mean $\pm$ S.E. (C) BEAS-2B 6x CRE reporter cells were treated with vehicle, siguazodan (10 μ M), rolipram (10 μ M), or both together for the indicated times (left panel). Alternatively, cells were pretreated with vehicle, siguazodan (10 μ M), rolipram (10 μ M), or both together for 30 minutes before the addition of formoterol (1 nM) (Form) for the indicated times (right panel). After luciferase assay, data were expressed as fold induction over NS at each time point and plotted as mean $\pm$ S.E. Significance was assessed at each time by analysis of variance with a Bonferroni post test. The combination (S + R) at 2, 4, 6, 8, 12, and 18 hours was significantly enhanced relative to untreated cells (stars not shown). * P < 0.01; ** P < 0.01; *** P < 0.001 (significance relative to siguazodan). # P < 0.05; ## P < 0.01 (significance relative to rolipram).

substantially sensitized the response to formoterol (Supplemental 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 μ M) failed to promote the CRE-dependent transcription, rolipram (10 μ M) 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 μ M), 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 μ M) or siguazodan (10 μ M) alone or in combination (Fig. 2C, right panel).

Effects of PKI α 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 6x CRE reporter cells with an adenovirus expressing PKI α (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.

PDE3 is an enzyme that can hydrolyze cAMP and cGMP. However, under physiological conditions, PDE3 typically acts as a cGMP-inhibited, cAMP PDE (reviewed in Zaccolo and Movsesian, 2007; Maurice et al., 2014). Through this mechanism, an elevation in cGMP could indirectly promote CRE-dependent transcription by increasing cAMP through PDE3 inhibition. This possibility was tested by examining the effect of SNP (a nitric oxide donor that can activate soluble guanylyl cyclase) and 8Br-cGMP (a cell permeant analog of cGMP) on luciferase expression in 6x CRE BEAS-2B reporter cells. As shown in Supplemental Fig. 2A, neither SNP nor 8Br-cGMP affected CRE-dependent transcription in the absence or presence of rolipram. These data were consistent with the additional finding that activation of CRE reporter activity by siguazodan and rolipram in combination was not affected by ODQ (300 nM to 300 μ M), an inhibitor of soluble guanylyl cyclase (Supplemental Fig. 2B) (Hwang et al., 1998). Thus, the enhancement of rolipram-induced CRE-dependent transcription by siguazodan cannot be attributed to its ability to inhibit

TABLE 1

Effect of PDE inhibitors on the potency of formoterol in driving CRE-dependent transcription

pEC₅₀ values are derived from the data in Fig. 2 and Supplemental Fig. 1. Significance between pEC₅₀ values was compared by the paired *t* test. Data are presented as the mean $\pm$ S.E. unless otherwise indicated.

Treatment	Potency to Formoterol (pEC ₅₀)	Determinations	Paired <i>t</i> Test
	<i>M</i>	<i>n</i>	
Naïve	10.6 $\pm$ 0.0	7	***
+ siguazodan	10.3 $\pm$ 0.0	7	
Naïve	9.9 $\pm$ 0.0	8	***
+ rolipram	10.23 $\pm$ 0.1	8	
Naïve	9.97 $\pm$ 0.02	6	***
+ siguazodan + rolipram	10.75 $\pm$ 0.09	6	
+ siguazodan	10.54 $\pm$ 0.04	5	***
+ siguazodan + rolipram	11.05 $\pm$ 0.05	5	
+ rolipram	10.9 $\pm$ 0.1	4	*
+ rolipram + siguazodan	11.2 $\pm$ 0.1	4	
Naïve	10.5 $\pm$ 0.1	6	*
+ cilostazol	10.8 $\pm$ 0.1	6	
Naïve	10.3 $\pm$ 0.1	6	***
+ GSK256066	10.7 $\pm$ 0.1	6	
Naïve	10.3 $\pm$ 0.1	6	*
+ roflumilast <i>N</i> -oxide	10.6 $\pm$ 0.1	6	
Naïve	10.5 $\pm$ 0.14	5	***
+ zardaverine	11.2 $\pm$ 0.11	5	

P* < 0.05; **P* < 0.001.

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 $> 10 \mu\text{M}$ 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 detectable 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.

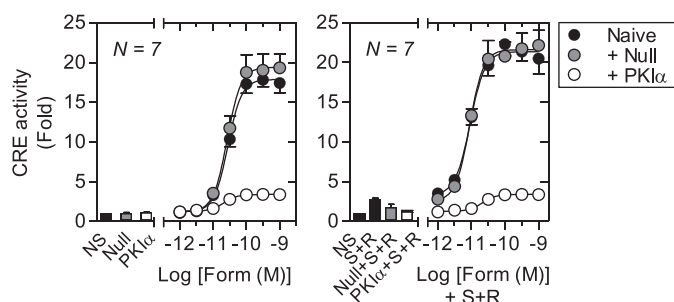


Fig. 3. Effect of PKI α overexpression on CRE-dependent transcription. $6\times$ CRE BEAS-2B cells were either not treated or infected at a MOI of 30 with either Ad5.CMV.PKI α or Ad5.CMV.Null for 48 hours. Thereafter, cells were left not stimulated (NS) or treated for 30 minutes with siguazodan ($10 \mu\text{M}$) plus rolipram ($10 \mu\text{M}$) (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 $\pm$ S.E.

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 ($10 \mu\text{M}$) or rolipram ($10 \mu\text{M}$) 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 $10 \mu\text{M}$) (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.

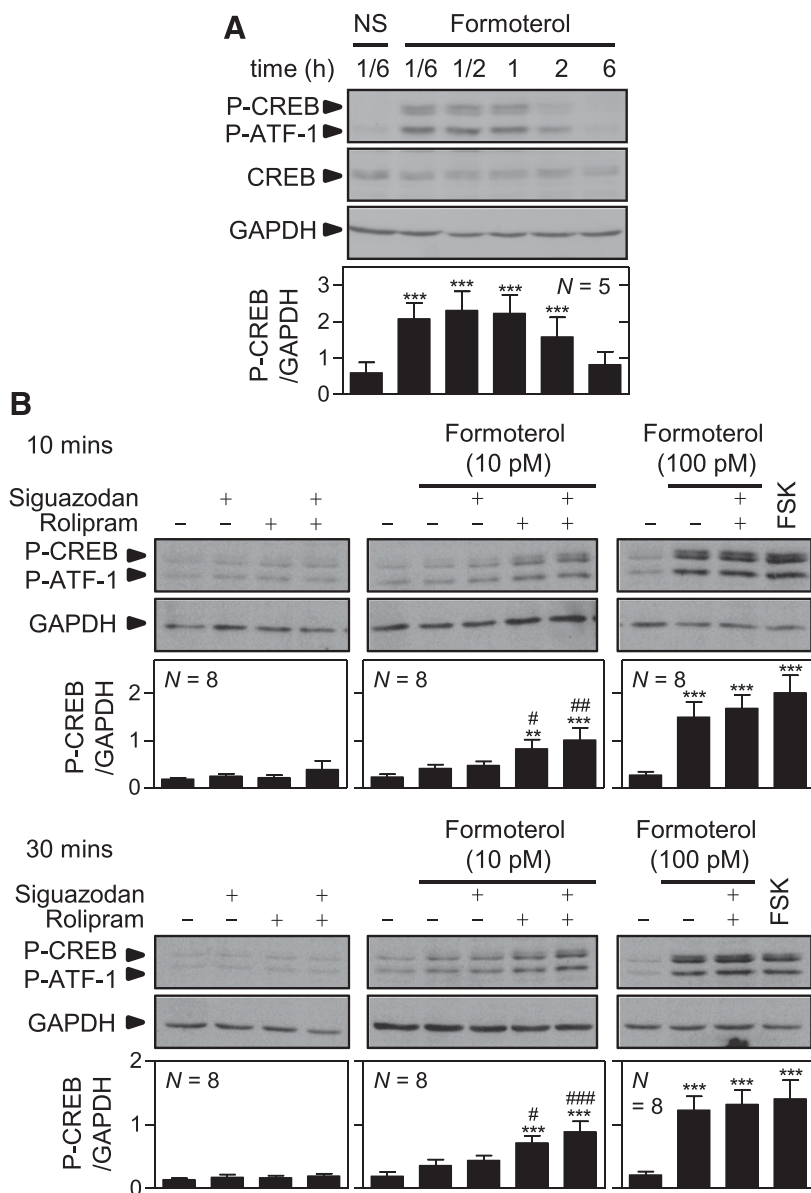


Fig. 4. Effect of cAMP-elevating agents on CREB phosphorylation. (A) BEAS-2B cells were either not stimulated (NS) or treated with formoterol (10 nM). Total protein was harvested after 10 minutes, 30 minutes, 1 hour, 2 hours, and 6 hours for Western blot analysis of phospho-CREB (P-CREB), total CREB, and GAPDH. After densitometric analysis, data expressed as a ratio of P-CREB/GAPDH were plotted as means $\pm$ S.E. Phosphorylation of CREB was analyzed using analysis of variance (ANOVA) with a Dunnett's post test. *** $P < 0.001$ (significance relative to NS). (B) BEAS-2B cells were either not stimulated or pretreated with either rolipram (10 μ M), siguazodan (10 μ M), or a combination of both for 30 minutes, then treated with formoterol (10 pM or 100 pM) or forskolin (10 μ M). Cells were harvested after 10 and 30 minutes for Western blot analysis of P-CREB and GAPDH. After densitometric analysis, data were expressed as a ratio of P-CREB/GAPDH and plotted as means $\pm$ S.E. Phosphorylation of CREB was analyzed using ANOVA with a Bonferroni post test. ** $P < 0.01$; *** $P < 0.001$ (significance relative to NS). # $P < 0.05$; ## $P < 0.01$, ### $P < 0.001$ (significance relative to 10 pM formoterol).

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 log₁₀ 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).

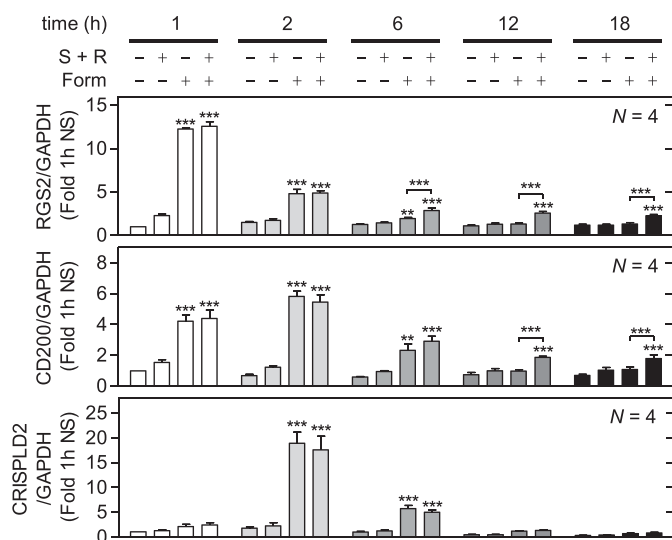


Fig. 5. Effect of LABA and PDE inhibitors on RGS2, CD200, and CRISPLD2 gene expression. BEAS-2B cells were preincubated for 30 minutes with vehicle, or a combination of siguazodan (10 μ M) plus rolipram (10 μ M) (S + R) and then exposed, or not, to formoterol (1 nM) (Form). Cells were harvested after 1, 2, 6, 12, and 18 hours for real-time PCR. Data were normalized to GAPDH, expressed as fold change relative to nonstimulated (NS) cells at 1 hour, and plotted as mean $\pm$ S.E. Significance, relative to NS at each time point, was tested by analysis of variance with a Bonferroni post test. Additional comparisons are indicated. ** $P < 0.01$; *** $P < 0.001$.

At 12 hours, there was no effect of the PDE inhibitors, either alone or together, on RGS2 mRNA expression, but modest elevations in CD200 and CRISPLD2 expression were apparent (Fig. 6D). These effects of siguazodan and rolipram appeared additive for CD200, whereas the enhancement was primarily driven by rolipram for CRISPLD2. Combining the 12-hour data from Figs. 5 and 6D showed that formoterol (1 nM) alone produced significant 1.17 ± 0.07 ($n = 9$), 1.62 ± 0.12 ($n = 10$), and 3.09 ± 0.24 ($n = 9$) fold increases in RGS2, CD200, and CRISPLD2 mRNA expression, respectively. This effect was not materially altered by siguazodan, but was enhanced by rolipram to a level that was similar to that achieved in the presence of both siguazodan and rolipram combined (Fig. 6D).

Effect of PDE3 and PDE4 Inhibitors on GRE-Dependent Transcription and the Enhancement by Formoterol. Whereas siguazodan and rolipram, whether alone or in combination, showed no effect on 2 $\times$ GRE reporter activation, a maximally effective concentration of dexamethasone (1 μ M) robustly activated the 2 $\times$ GRE reporter (Fig. 7). This response was markedly enhanced by rolipram (10 μ M), but not siguazodan (10 μ M) (Fig. 7A). However, siguazodan plus rolipram was no better than rolipram alone, indicating that this effect was predominantly regulated by PDE4. Neither SNP nor 8Br-cGMP had any effect on dexamethasone-induced GRE reporter activation (Supplemental Fig. 5). As previously described (Kaur et al., 2008), formoterol enhanced dexamethasone-induced 2 $\times$ GRE reporter activity in a concentration-dependent manner. In the presence of rolipram (10 μ M), but not siguazodan (10 μ M), 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 3). The magnitude of this leftward displacement was not modified by combining siguazodan with rolipram (Fig. 7A).

Kinetic analyses of luciferase activity accumulation showed that at early time points (2–8 hours), the enhancement of

dexamethasone-induced transcription by siguazodan plus rolipram was primarily due to rolipram (Fig. 7B, left panel). However, neither rolipram, either alone or in combination with siguazodan, affected the kinetics of luciferase activity accumulation induced by dexamethasone (1 μ M) plus formoterol (1 nM) (Fig. 7B, right panel).

Effect of PDE3 Plus PDE4 Inhibitors on Glucocorticoid-Induced Gene Expression and the Enhancement by Formoterol. RGS2, CD200, and CRISPLD2 mRNA expression was modestly induced by dexamethasone (1 μ M) (Fig. 8A), whereas DUSP1 expression was induced more highly (Supplemental Fig. 4B). With RGS2, CD200, and CRISPLD2, this expression was enhanced by siguazodan plus rolipram but, with the exception of RGS2 at 12 and 18 hours, did not reach significance. By contrast, there was no effect of siguazodan plus rolipram on DUSP1 mRNA expression induced by dexamethasone (Supplemental Fig. 4B). Formoterol, added with dexamethasone, substantially enhanced the increase in mRNA expression 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 5.3 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 μ M) 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 pM), 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,

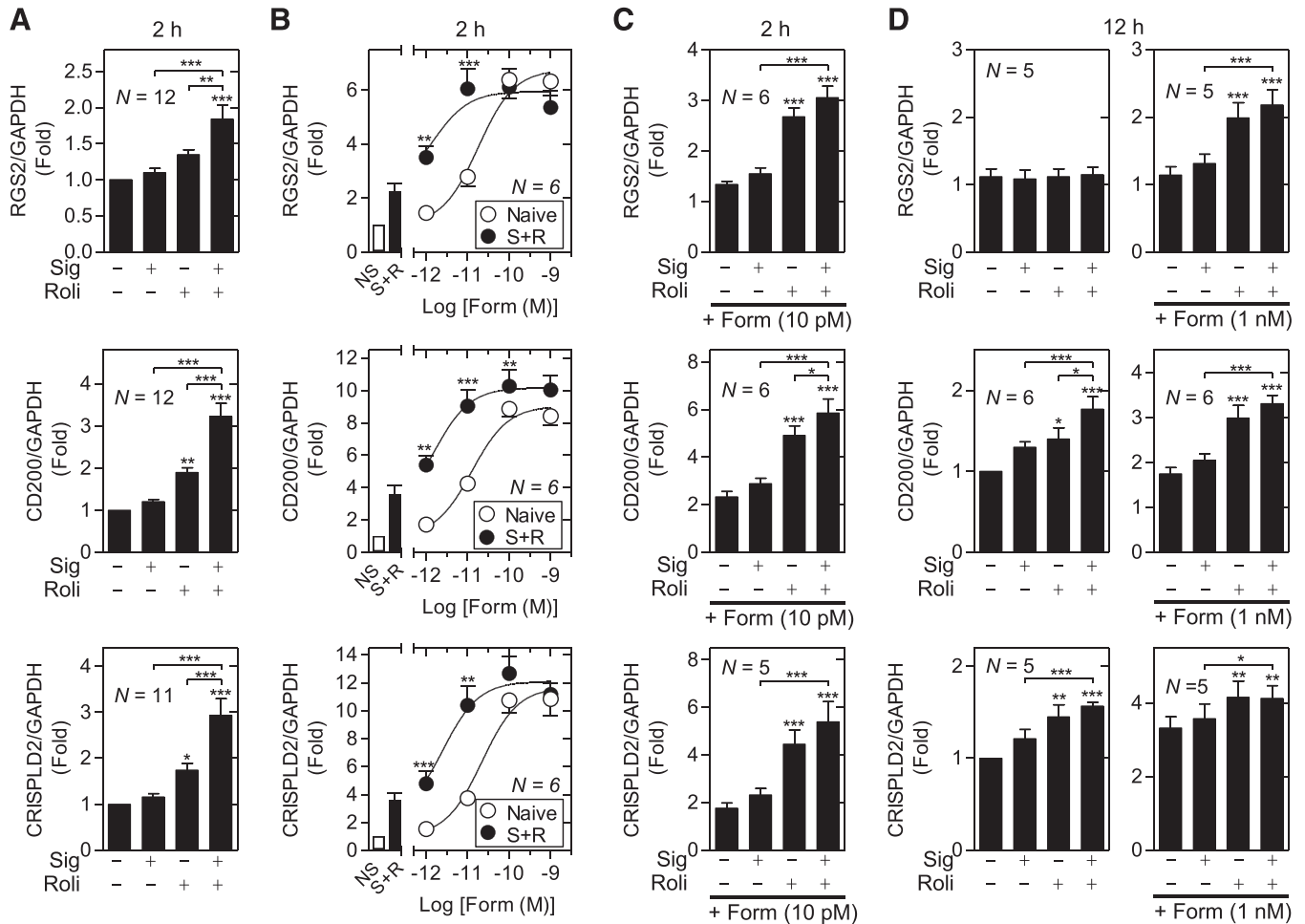


Fig. 6. Effect of PDE3 and PDE4 inhibitors on formoterol-induced gene expression. BEAS-2B cells were pretreated, or not, for 30 minutes with siguazodan (10 μ M) (Sig), rolipram (10 μ M) (Roli), or both where indicated (S + R). After either no stimulation (NS) or stimulation with the indicated concentrations of formoterol (Form), cells were harvested after 2 hours (A–C) or 12 hours (D) for mRNA extraction. SYBR Green real-time PCR was performed for RGS2 (upper panels), CD200 (middle panels), and CRISPLD2 (bottom panels). Data are normalized to GAPDH and expressed as fold of NS, and are plotted as means $\pm$ S.E. Significance, relative to NS at each time point (A and D, left panel), or relative to Form alone (C and D, right panel), was tested by analysis of variance with a Bonferroni post test. Additional comparisons are indicated. In (B), comparisons between naive and S + R treated in the presence of each different formoterol concentration were made using a paired *t* test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

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

TABLE 2

Effect of PDE inhibitors on the potency of formoterol in driving gene expression in the absence and presence of dexamethasone

pEC₅₀ values for the potency of formoterol at inducing gene expression in the absence or presence of siguazodan (10 mM) plus rolipram (10 mM) (Sig + Roli) or with dexamethasone (1 mM) in the absence or presence of siguazodan plus rolipram are derived from the data shown in Figs. 6B and 9B, respectively. Data are presented as the mean $\pm$ S.E. unless otherwise indicated.

Gene	Treatment	Potency to Formoterol (pEC ₅₀)	Determinations	Potency to Formoterol in the Presence of 1 μ M Dexamethasone (pEC ₅₀)	Determinations
		<i>M</i>	<i>n</i>	<i>M</i>	<i>n</i>
RGS2	Naïve	10.8 $\pm$ 0.4	6	10.9 $\pm$ 0.1	5
	+ Sig + Roli	11.8 $\pm$ 0.3	6	11.9 $\pm$ 0.2	5
CD200	Naïve	10.9 $\pm$ 0.1	6	10.9 $\pm$ 0.1	5
	+ Sig + Roli	11.8 $\pm$ 0.2	6	12.0 $\pm$ 0.1	5
CRISPLD2	Naïve	10.7 $\pm$ 0.2	6	11.0 $\pm$ 0.1	5
	+ Sig + Roli	11.6 $\pm$ 0.2	6	11.9 $\pm$ 0.3	5

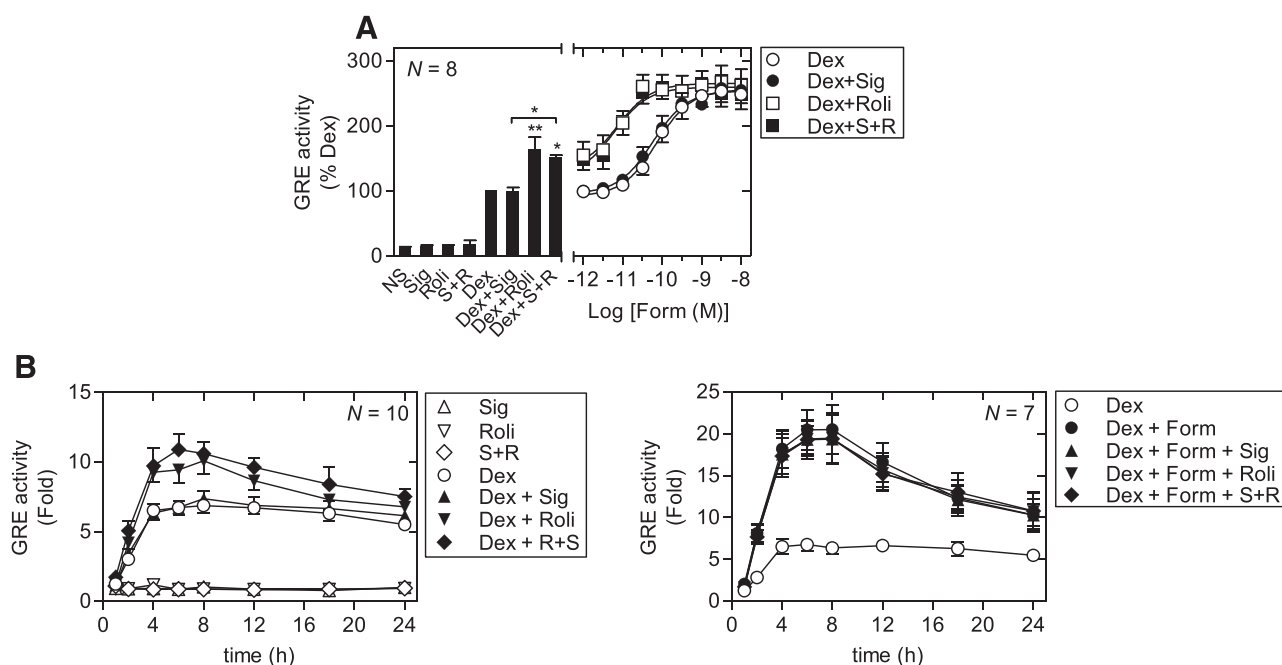


Fig. 7. Effect of cAMP-elevating agents on 2x GRE-dependent transcription. (A) BEAS-2B 2x GRE reporter cells were preincubated for 30 minutes with vehicle, siguazodan (10 μ M) (Sig), rolipram (10 μ M) (Roli), or both (S + R) before treatment with dexamethasone (1 μ M) (Dex) alone or with various concentrations of formoterol (Form). After 6 hours, the cells were harvested for luciferase assay. Data are expressed as a percentage of dexamethasone and plotted as mean $\pm$ S.E. Significance for Dex + Sig, Dex + Roli, and Dex + S + R relative to dexamethasone-treated cells, or as indicated, was assessed by analysis of variance (ANOVA) using a Bonferroni post test. * P < 0.5; ** P < 0.01. (B) BEAS-2B 2x GRE reporter cells were pretreated for 30 minutes with vehicle, siguazodan (10 μ M), rolipram (10 μ M), or both before treatment with dexamethasone (1 μ M) alone (left panel) or with dexamethasone (1 μ M) plus formoterol (1 nM) (Dex + Form) (right panel). Cells were harvested at the indicated times for luciferase assay. Data are expressed as fold induction over control at each time point, and are plotted as mean $\pm$ S.E. Significance for Dex + Sig, Dex + Roli, and Dex + S + R relative to dexamethasone-treated cells was assessed for each time point by ANOVA using a Bonferroni post test (stars not shown). Dex + S + R relative to Dex showed P < 0.05 for 2–24 hours. Dex + Sig was not different from Dex at any time. Dex + Roli relative to Dex showed P < 0.05 for 2–12 and 24 hours. Dex + S + R relative to Dex + Sig showed P < 0.05 for 2–12 and 18 hours.

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 β_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

TABLE 3

Effect of PDE inhibitors on the potency of formoterol in enhancing dexamethasone-driven GRE-dependent transcription
pEC₅₀ values for the enhancement of dexamethasone (1 mM)-induced 2 GRE-dependent transcription by formoterol in the absence or presence of siguazodan (10 mM) and/or rolipram (10 mM) are derived from the data presented in Fig. 7A. Significance between pEC₅₀ values was assessed by analysis of variance with a Bonferroni post test. Data are presented as the mean $\pm$ S.E. unless otherwise indicated.

Treatment	Potency to Formoterol in the Presence of 1 μ M Dexamethasone (pEC ₅₀)	Determinations	ANOVA with Bonferroni post Test
	<i>M</i>	<i>n</i>	
Naïve	10.1 $\pm$ 0.1	8	
+ siguazodan	10.2 $\pm$ 0.2	8	
+ rolipram	11.2 $\pm$ 0.1	8	***
+ siguazodan + rolipram	11.1 $\pm$ 0.1	7	***/###

*** P < 0.001 for comparisons with naïve cells; ### P < 0.001 for comparisons with siguazodan-treated cells.

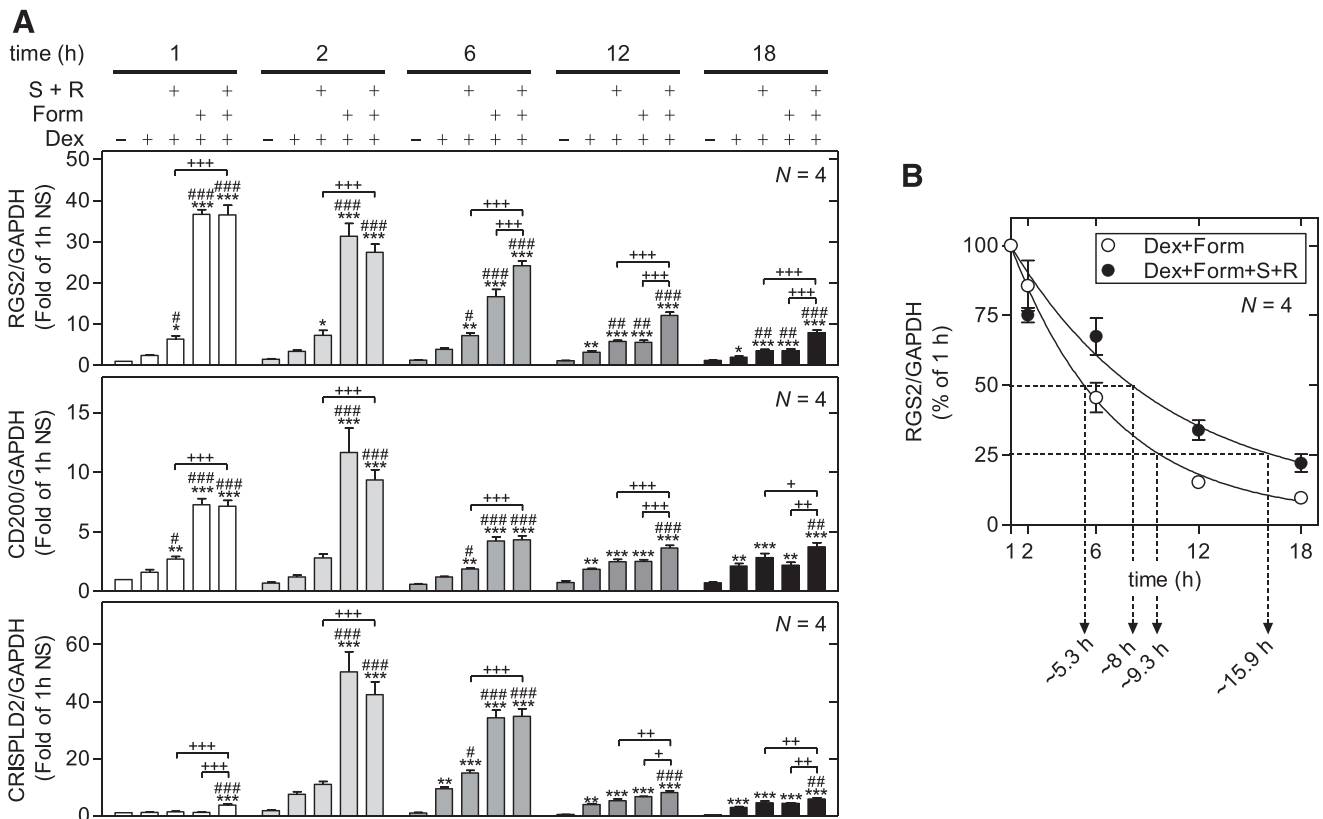


Fig. 8. Effect of siguazodan, rolipram, and formoterol in the presence of dexamethasone on gene expression. (A) BEAS-2B cells were pretreated with siguazodan (10 μ M) plus rolipram (10 μ M) (S + R) for 30 minutes, before stimulation, or not, with dexamethasone (1 μ M) (Dex) alone or with formoterol (1 nM) (Form) as indicated. Cells were harvested at the indicated times for RNA extraction. After cDNA synthesis, SYBR Green real-time PCR was performed for RGS2, CD200, and CRISPLD2. Data are normalized to GAPDH and expressed as fold of nonstimulated cells (NS) at 1 hour, and are plotted as mean $\pm$ S.E. (B) Data for RGS2 from the upper panel in (A) were replotted and the effect of each combination ($\circ$ = Dex + Form; $\bullet$ = Dex + Form + S + R) expressed as a percentage of the value at 1 hour. Times until 50 and 25% of the maximum response were reached are shown. * P < 0.05; ** P < 0.01; *** P < 0.001 (significance, using an analysis of variance with a Newman–Keuls post test is indicated, relative to NS at each time point). # P < 0.05; ## P < 0.01; ### P < 0.001 (relative to dexamethasone at each time point). Additional comparisons are as indicated. + P < 0.05; ++ P < 0.01; +++ P < 0.001.

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.

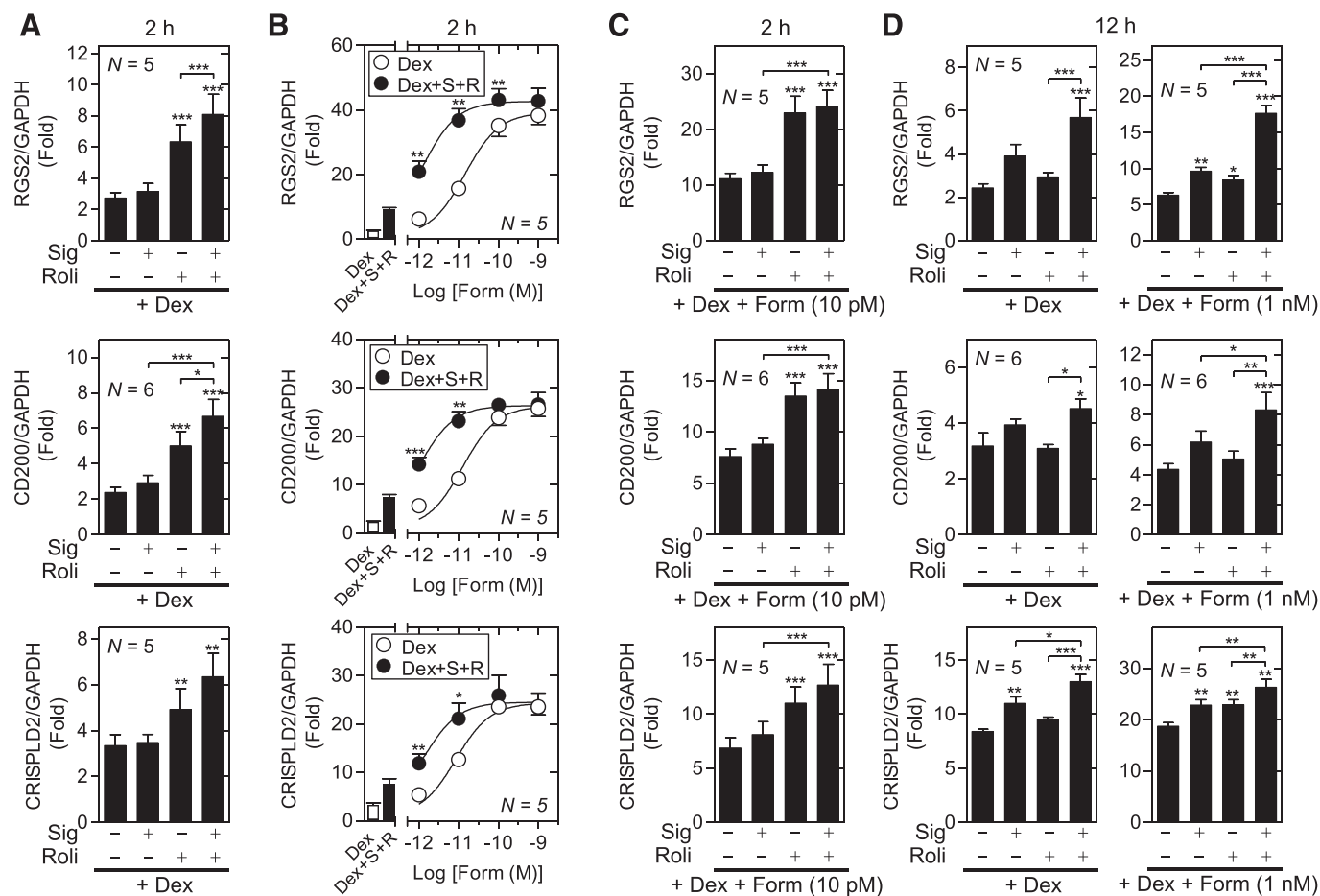


Fig. 9. Effect of siguazodan and rolipram on dexamethasone and dexamethasone plus formoterol-induced gene expression. BEAS-2B cells were pretreated for 30 minutes, or not, with siguazodan (10 μ M) (Sig) and/or rolipram (10 μ M) (Roli) before stimulation, or not, with dexamethasone (1 μ M) (Dex) or dexamethasone (1 μ M) plus the indicated concentrations of formoterol (Dex + Form). Cells were harvested after 2 hours (A–C) or 12 hours (D) for RNA extraction. SYBR Green real-time PCR was performed for RGS2, CD200, and CRISPLD2. Data are normalized to GAPDH and expressed as fold of nonstimulated cells (NS), and are plotted as means $\pm$ S.E. Significance, relative to dexamethasone-treated cells, was assessed by analysis of variance with a Bonferroni post test. Additional comparisons are indicated. In (B), comparisons between Dex and dexamethasone plus siguazodan plus rolipram (S + R) for each concentration of formoterol were performed using the paired *t* test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

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 PKI α overexpression, these data support a classic PKA-dependent process. However, because PDE3 can theoretically hydrolyze cAMP and cGMP (albeit with a lower V_{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 by combined PDE3/PDE4 inhibition was due primarily to PDE4 inhibition, an effect that, on the GRE reporter, was not mimicked by PKG activators and, as with LABAs, was blocked by PKI α (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 multiple cAMP elevators and was also blocked by PKI α (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 Roflumilast in the Prevention of COPD Exacerbations while Taking Appropriate Combination Treatment study, in which the effect of roflumilast 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 (Baillie 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 GRE-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 airway epithelial cells and ASM cells, which have a high β_2 -adrenoceptor density and/or efficient receptor-adenylyl 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 inhaled 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 β_2 -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 (Hinzey 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.

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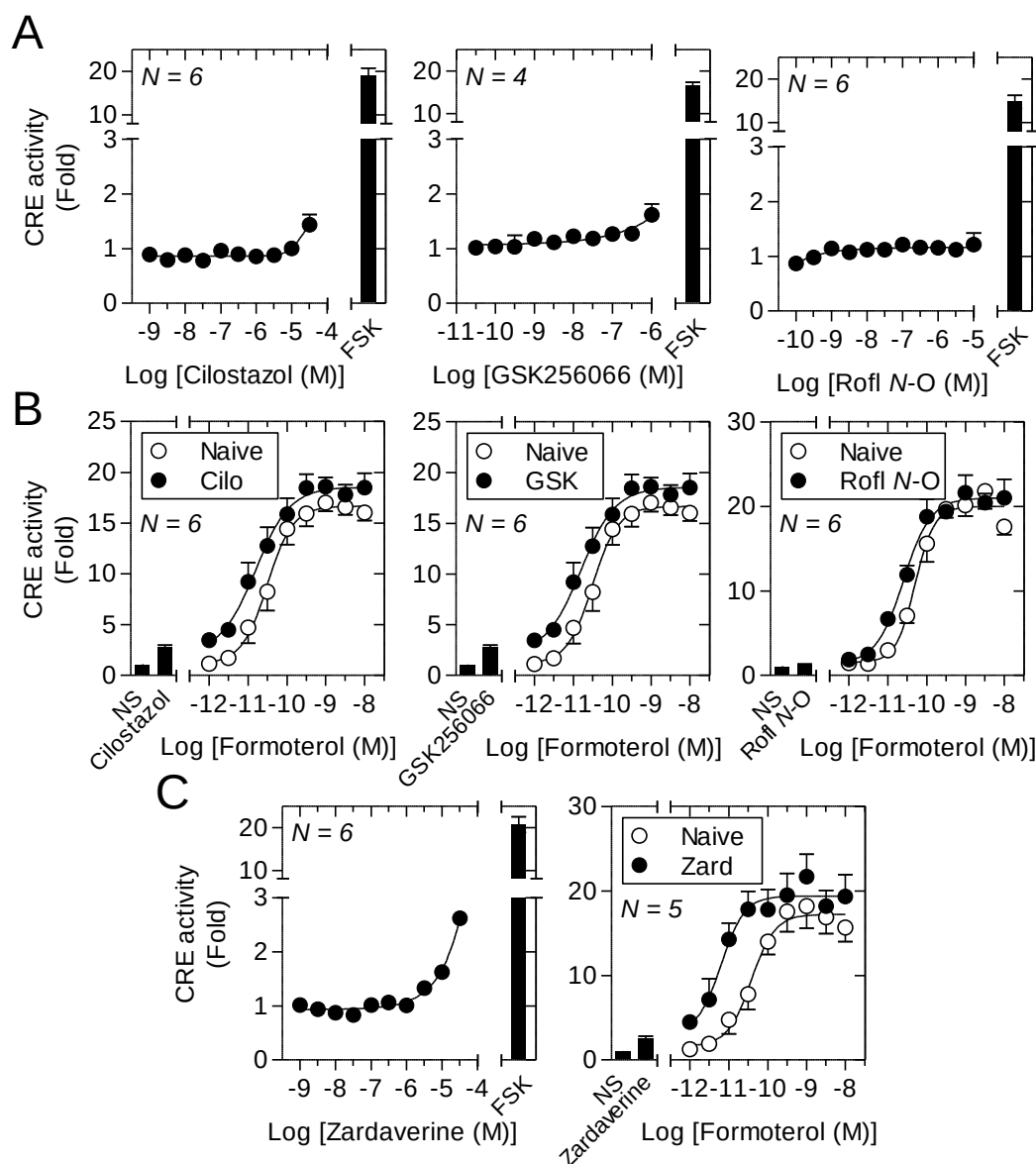
Supplemental data

Superiority of combined PDE3/PDE4 inhibition over PDE4 inhibition alone on glucocorticoid- and long-acting β_2 -adrenoceptor agonist-induced gene expression in human airway epithelial cells

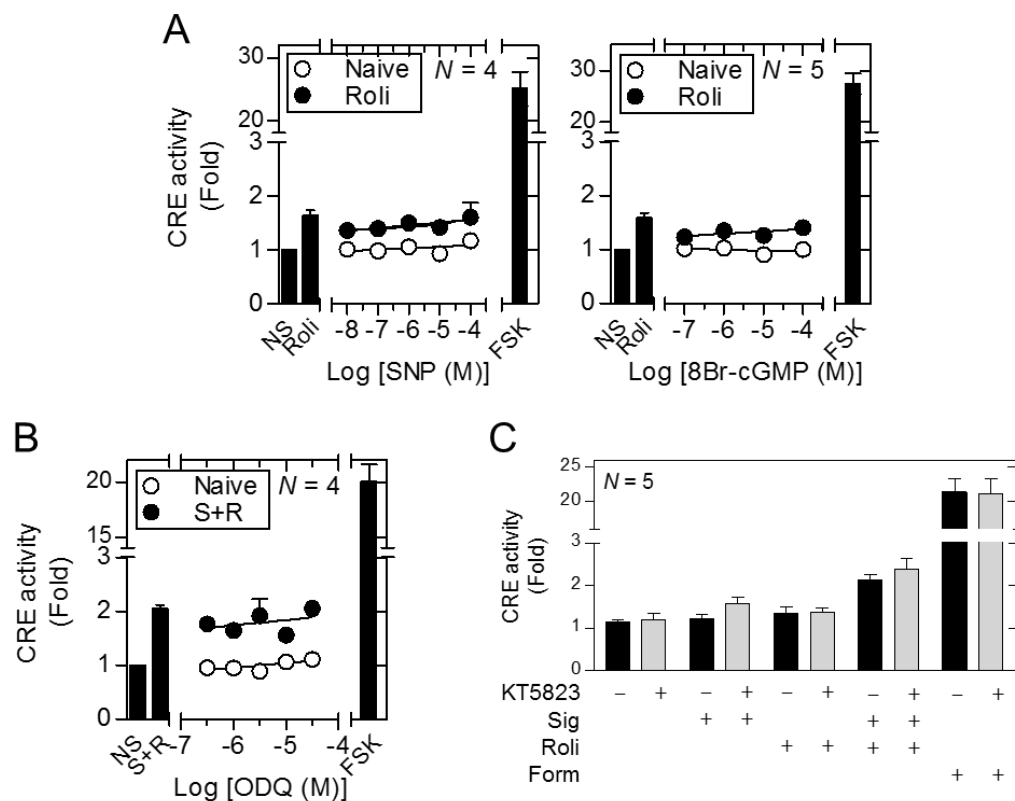
Hawazen BinMahfouz, Bibhusana Borthakur, Dong Yan, Tresa George, Mark A. Giembycz, Robert Newton

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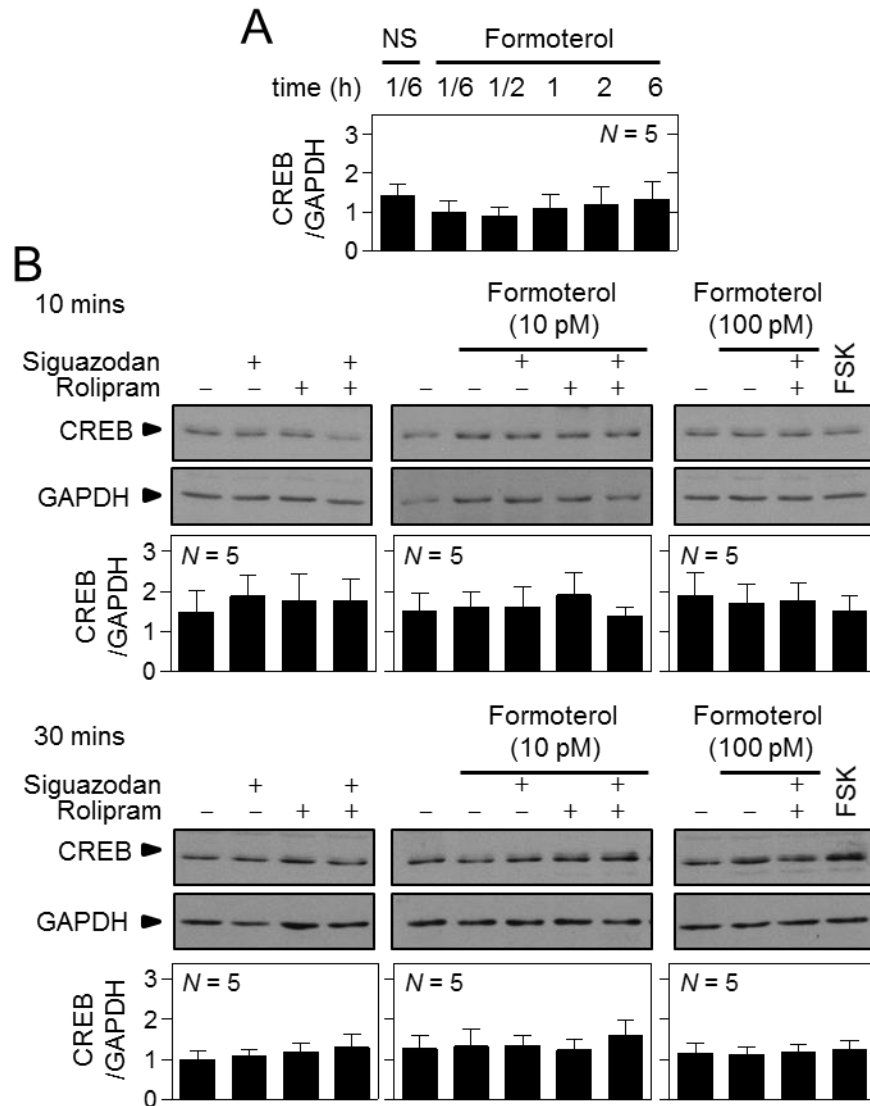
Journal: Molecular Pharmacology



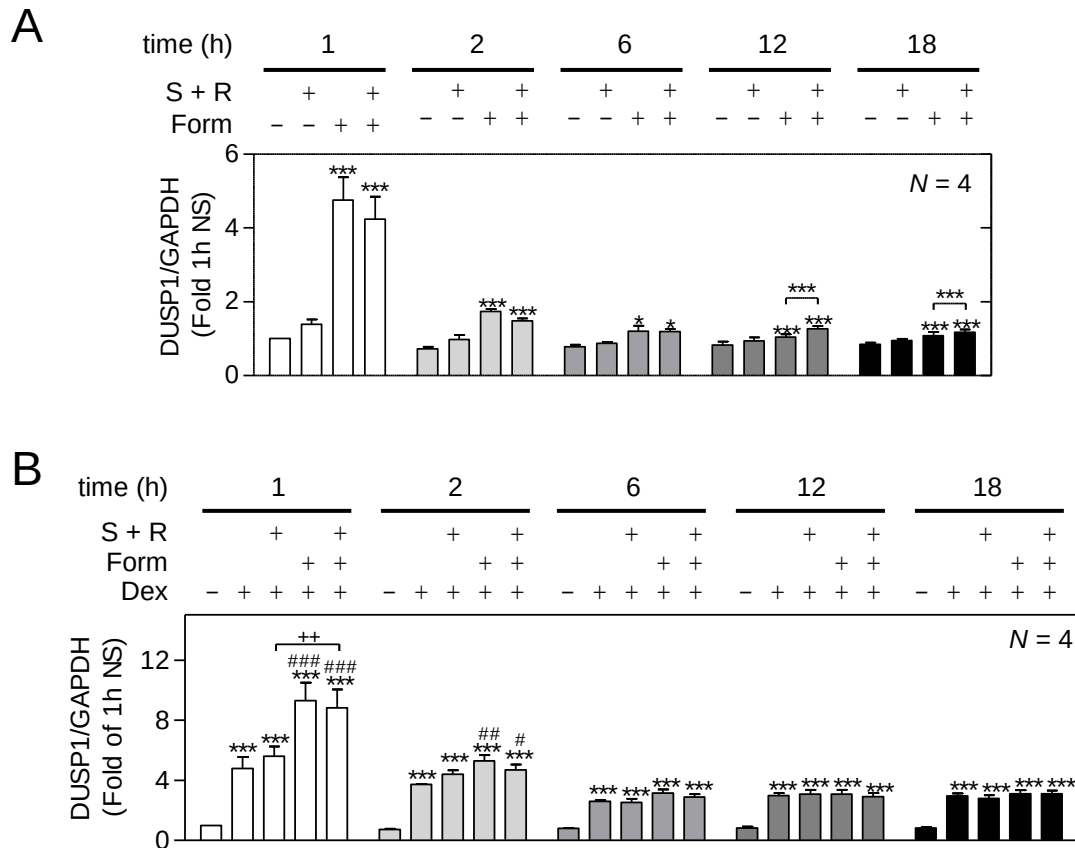
Supplemental Fig. 1. Effect of PDE inhibitors on luciferase activity in 6xCRE BEAS-2B reporter cells. A, CRE BEAS-2B reporter cells were treated with various concentrations of cilostazol, GSK256066 or roflumilast *N*-oxide (Rofl *N*-O) and forskolin (10 μ M) (FSK). Cells were harvested after 6 h for luciferase assay. B, 6xCRE BEAS-2B reporter cells were pre-treated, or not, for 30 mins with cilostazol (10 μ M), GSK256066 (10 nM), or roflumilast *N*-oxide (10 nM) before treating with various concentrations of formoterol. After 6 h, the cells were harvested for luciferase assay. C, 6xCRE BEAS-2B reporter cells were treated with various concentrations of zardaverine and forskolin (10 μ M) (FSK) (left panel). Alternatively, 6xCRE BEAS-2B reporter cells were pre-treated, or not, for 30 mins with zardaverine (10 μ M) before treating with various concentrations of formoterol. Cells were harvested after 6 h for luciferase assay. Data from *N* independent determinations, expressed as fold induction of luciferase relative to unstimulated cells, are plotted as mean $\pm$ S.E. Log EC₅₀ values for all treatments are shown in Table 1 of the main manuscript.



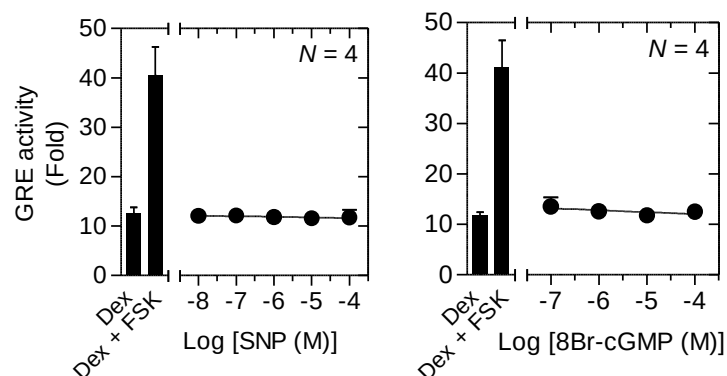
Supplemental Fig. 2. Effect of modulation of the cGMP/PKG pathway on CRE reporter activation. A, 6×CRE BEAS-2B reporter cells were treated with various concentrations of sodium nitroprusside (SNP) or 8Br-cGMP in the absence or presence of rolipram (10 μ M) (Roli) as indicated. Cells were also treated with forskolin (10 μ M) (FSK). After 6 h, the cells were harvested for luciferase assay. B, 6×CRE BEAS-2B reporter cells were treated with various concentrations of 1H-[1,2,4] oxadiazolo [4, 3-a] quinoxalin-1-one (ODQ) or 8Br-cGMP in the absence or presence of siguazodan (10 μ M) plus rolipram (10 μ M) (S + R) as indicated. Cells were also treated with forskolin (10 μ M) (FSK). After 6 h, the cells were harvested for luciferase assay. C, 6×CRE BEAS-2B reporter cells were pre-treated with KT5823 (1 μ M) for 30 mins prior to treatment with siguazodan (10 μ M) (Sig), rolipram (10 μ M) (Roli), both together or formoterol (10 nM) (Form). After 6 h, the cells were harvested for luciferase assay. Data from *N* independent determinations, expressed as fold induction of luciferase relative to unstimulated cells, are plotted as mean $\pm$ S.E.



Supplemental Fig. 3. Effect of cAMP-elevating agents on total CREB expression. A, BEAS-2B cells were either not stimulated or treated with formoterol (10 nM). Total protein was harvested after 1/6, 1/2, 1, 2 and 6 h for western blot analysis of total CREB (CREB) and GAPDH. A representative image is shown in Fig. 4A of the main manuscript and following densitometric analysis, data, expressed as a ratio of CREB/GAPDH, are plotted as means $\pm$ S.E. B, BEAS-2B cells were either not stimulated or pre-treated with either rolipram (10 μ M), siguazodan (10 μ M) or a combination of both for 30 min, then treated with formoterol (10 pM or 100 pM) or forskolin (10 μ M) (FSK). Cells were harvested after 10 and 30 min for western blot analysis of CREB and GAPDH. Representative images are shown and following densitometric analysis, data, expressed as a ratio of CREB/GAPDH, are plotted as means $\pm$ S.E.



Supplemental Fig. 4. Effect of LABA and PDE inhibitors in the absence and presence of glucocorticoid on DUSP1 expression. BEAS-2B cells were pre-incubated for 30 min with vehicle, or a combination of siguazodan (10 μ M) plus rolipram (10 μ M) (S + R) and then exposed, or not, to formoterol (1 nM) (Form), either (A) in the absence, or (B) in the presence of dexamethasone (1 μ M) (Dex). Cells were harvested after 1, 2, 6, 12 and 18 h for real-time PCR. Data were normalized to GAPDH, expressed as fold change relative to non-stimulated cells (NS) at 1 h and plotted as mean $\pm$ S.E. In A, significance, relative to NS at each time point, was tested by ANOVA with a Bonferroni post-test. Additional comparisons are as indicated. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. In B, significance, using an ANOVA with a Newman-Keuls post-test is indicated. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$, relative to NS at each time point. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$, relative to Dex at each time point. Additional comparisons are as indicated. + $P < 0.05$, ++ $P < 0.01$ and +++ $P < 0.001$.



Supplemental Fig. 5. Effect of sodium nitroprusside (SNP) and 8Br-cGMP on glucocorticoid-dependent transcription. 2×GRE BEAS-2B reporter cells were treated with various concentrations of sodium nitroprusside (SNP) or 8Br-cGMP in the presence of dexamethasone (1 μ M) (Dex) as indicated. Cells were also treated with Dex + forskolin (10 μ M) (FSK). After 6 h, the cells were harvested for luciferase assay. Data from *N* independent determinations, expressed as fold induction of luciferase relative to unstimulated cells, are plotted as mean $\pm$ S.E.

Supplemental Table 1

Probe	Set ID	MASS Output 1h (log ₂)	MASS Output 2h (log ₂)	MASS Output 6h (log ₂)	MASS Output 18h (log ₂)	Mean Fold Change 2h rt 1h	Mean Fold Change 6h rt 1h	Mean Fold Change 18h rt 1h	Mean Fold Change 2h rt 1h (log ₂)	Mean Fold Change 6h rt 1h (log ₂)	Mean Fold Change 18h rt 1h (log ₂)	Maximum Mean Fold Change	Transcript Expression Absent(A), Present (P), Marginal (M)	Gene Symbol
1558680_s_at		1.74	3.92	3.94	4.21	4.54	4.60	5.57	2.18	2.20	2.48	5.57	AAAAAAAAAAPPPAAP	PDE1A
208396_s_at		3.49	4.12	4.47	3.39	1.55	1.98	-1.07	0.64	0.99	-0.09	1.98	AAAAAAPAAPAAAA	PDE1A
231213_at		3.47	4.17	3.64	3.14	1.63	1.13	-1.26	0.70	0.17	-0.33	1.63	PAAAAPPAPPAAAA	PDE1A
233547_x_at		3.04	3.15	3.37	2.58	1.08	1.26	-1.38	0.11	0.33	-0.46	1.38	AAAAAAAAAPAAAA	PDE1A
236344_at		8.06	7.82	8.41	8.02	-1.18	1.28	-1.03	-0.23	0.36	-0.04	1.28	PPPPPPPPPPPPPP	PDE1C
216869_at		6.27	6.41	6.78	6.22	1.11	1.43	-1.03	0.15	0.51	-0.05	1.43	AAAAAAAAAAAAAAM	PDE1C
207303_at		6.42	6.54	6.80	6.38	1.08	1.30	-1.03	0.12	0.38	-0.04	1.30	PPPPPPPPPPPPPP	PDE1C
1554378_a_at		4.71	4.88	5.33	4.64	1.13	1.54	-1.05	0.17	0.62	-0.07	1.54	AAAAAPAAAAAAA	PDE1C
206389_s_at		5.89	5.96	5.94	6.42	1.05	1.04	1.44	0.07	0.05	0.53	1.44	AAAPPPPAAPPPPP	PDE3A
206388_at		6.22	6.21	6.44	5.97	-1.01	1.16	-1.19	-0.01	0.21	-0.25	1.19	PPAPPPPPPPAPPP	PDE3A
214582_at		4.58	4.52	4.49	4.95	-1.04	-1.07	1.29	-0.06	-0.10	0.36	1.29	AAPPAAPAPAAAMA	PDE3B
204735_at		7.94	8.03	8.03	7.68	1.07	1.06	-1.20	0.09	0.09	-0.26	1.20	PPPPPPPPPPPPPP	PDE4A
203708_at		8.70	8.76	8.83	7.89	1.05	1.09	-1.75	0.07	0.13	-0.81	1.75	PPPPPPPPPPPPPP	PDE4B
211302_s_at		7.85	8.11	7.92	7.22	1.20	1.05	-1.55	0.26	0.07	-0.63	1.55	PPPPPPPPPPPPPP	PDE4B
215671_at		3.79	2.57	2.26	2.79	-2.33	-2.88	-1.99	-1.22	-1.53	-0.99	2.88	AAAAAPAAAAAAA	PDE4B
206791_s_at		6.46	6.72	6.79	6.55	1.20	1.26	1.06	0.26	0.34	0.09	1.26	AAAPPAAPAAAAA	PDE4C
231478_at		1.88	2.45	3.15	2.80	1.49	2.41	1.89	0.57	1.27	0.92	2.41	AAAPAAAAAAA	PDE4C
211818_s_at		5.98	5.91	5.82	5.94	-1.05	-1.12	-1.02	-0.07	-0.16	-0.03	1.12	PMAPPPMAAAPPPA	PDE4C
228962_at		6.70	7.18	6.82	6.53	1.40	1.09	-1.13	0.48	0.12	-0.17	1.40	PPPPPPPPPPPPPP	PDE4D
210837_s_at		5.82	6.17	5.92	5.98	1.27	1.07	1.12	0.35	0.10	0.16	1.27	PPPPPPPPPPPPPP	PDE4D
210836_x_at		6.17	6.36	6.25	6.33	1.14	1.05	1.11	0.18	0.08	0.15	1.14	PPPPPPPPPPPPMP	PDE4D
204491_at		8.72	8.75	8.78	8.64	1.02	1.04	-1.06	0.03	0.05	-0.08	1.06	PPPPPPPPPPPPPP	PDE4D
1556787_s_at		4.19	4.57	5.10	4.94	1.29	1.87	1.67	0.37	0.90	0.74	1.87	AAAPAAAAAPAAP	PDE5A
1553175_s_at		5.62	5.01	3.77	5.47	-1.53	-3.62	-1.11	-0.61	-1.86	-0.16	3.62	PAAPPAAPAPPPPA	PDE5A
227088_at		6.54	6.64	6.18	6.49	1.07	-1.29	-1.03	0.10	-0.36	-0.05	1.29	PMPPPPPPPPPPPP	PDE5A
206757_at		5.24	5.20	4.74	5.11	-1.02	-1.41	-1.09	-0.03	-0.50	-0.12	1.41	PAAMPAPAMMPPPA	PDE5A
1562228_s_at		6.78	7.05	6.79	6.62	1.20	1.01	-1.12	0.27	0.01	-0.16	1.20	AAMMMMMPPAPAPAA	PDE5A
240088_at		4.38	4.57	4.18	3.31	1.14	-1.15	-2.11	0.18	-0.21	-1.07	2.11	AMAAAPPAAAAAAMA	PDE5A
206623_at		7.00	7.08	6.90	6.99	1.06	-1.07	-1.01	0.08	-0.10	-0.01	1.07	PPPPPPPPPMPPPP	PDE6A
231065_at		6.35	6.49	6.10	5.94	1.10	-1.19	-1.33	0.14	-0.25	-0.41	1.33	PPPPPPPPPPPPPP	PDE6D
216883_x_at		10.43	10.14	10.21	10.23	-1.23	-1.17	-1.16	-0.29	-0.22	-0.21	1.23	PPPPPPPPPPPPPP	PDE6D
223358_s_at		10.15	10.41	10.46	10.27	1.20	1.23	1.09	0.26	0.30	0.12	1.23	PPPPPPPPPPPPPP	PDE7A
224046_s_at		8.51	8.71	8.78	8.63	1.14	1.20	1.08	0.19	0.26	0.12	1.20	PPPPPPPPPPPPPP	PDE7A
1552343_s_at		6.09	6.61	5.37	5.98	1.43	-1.65	-1.08	0.52	-0.72	-0.12	1.65	MAPPMAAAPPPAPAM	PDE7A
230500_at		5.79	6.02	6.29	5.72	1.17	1.42	-1.05	0.23	0.50	-0.07	1.42	PPPPPPPPPPPPPP	PDE7A
230109_at		7.78	7.78	7.41	7.38	-1.00	-1.29	-1.32	0.00	-0.37	-0.40	1.32	AAAAPAAAAAAPAAA	PDE7B
220343_at		5.44	5.73	5.47	5.39	1.22	1.02	-1.03	0.29	0.03	-0.05	1.22	AAAAMAAAAAAAA	PDE7B
1552931_a_at		8.14	8.06	7.97	7.93	-1.06	-1.13	-1.16	-0.08	-0.17	-0.21	1.16	PPPPPPPPPPPPPP	PDE8A
212522_at		9.62	9.74	9.73	9.67	1.09	1.08	1.04	0.12	0.11	0.05	1.09	PPPPPPPPPPPPPP	PDE8A
205593_s_at		6.60	6.55	5.64	6.43	-1.04	-1.95	-1.13	-0.05	-0.96	-0.17	1.95	AAAAAAPAAPAAAA	PDE9A
205501_at		5.14	4.93	4.68	4.87	-1.16	-1.37	-1.21	-0.21	-0.46	-0.27	1.37	PPPPPPPPPPPPPP	PDE10A
211171_s_at		5.49	5.85	5.28	5.33	1.28	-1.15	-1.12	0.36	-0.21	-0.16	1.28	AAAAAAAAPAAAA	PDE10A
221110_x_at		4.99	5.74	5.48	5.24	1.68	1.41	1.20	0.75	0.49	0.26	1.68	APAAPAPAAPMPAA	PDE11A
237248_at		2.43	2.41	3.23	3.01	-1.01	1.74	1.50	-0.02	0.80	0.58	1.74	AAAAAPAAAAAAA	PDE11A
224224_s_at		3.37	3.59	3.21	3.28	1.17	-1.11	-1.07	0.22	-0.16	-0.10	1.17	AAAAAPAAPAAAA	PDE11A
224223_s_at		3.83	3.60	3.37	3.41	-1.17	-1.38	-1.34	-0.23	-0.46	-0.42	1.38	AAAAAPAAAAAAA	PDE11A
212581_x_at		13.61	13.55	13.70	13.54	-1.05	1.06	-1.05	-0.07	0.09	-0.07	1.06	PPPPPPPPPPPPPP	GAPDH
201049_s_at		13.72	13.62	13.75	13.63	-1.07	1.02	-1.07	-0.10	0.03	-0.09	1.07	PPPPPPPPPPPPPP	RPS18

Key
Column A: Affymetrix probe set unique identification number.
Columns B-E: log2 transformed MASS output from each gene chip averaged for each time point for each probe set.
Columns F-H: mean fold change (induced or repressed) in transcript expression for each probe set relative to the 1h time point expressed as anti-log2 ratios of the data in columns B-E.
Columns I-K: mean fold change in transcript expression for each probe set relative to the 1h time point expressed in log2 units.
Column L: maximum mean fold change in transcript expression for each probe set relative to the 1h time point. This value was derived from the relevant anti-log2 ratio of the data in columns B, C, D or E.
Column M: Transcript expression present (P), absent (A) or marginal (M) for each sample at each time point. 1h (samples 1, 5, 9 & 13); 2h (samples 2, 6, 10 & 14), 6h (samples 3, 7, 11 & 15) and 18h (4, 8, 12 & 16).
Column N to Z: as labelled.

Probe	Set ID	Gene Name	Representative Public ID	Archival Unigene Cluster	UNIGENE ID	Chromosome Location	ENSEMBL
1558680_s_at		phosphodiesterase 1A, calmodulin-dependent	BQ894022	Hs.383511	Hs.191046	chr2q32.1	ENSG00000115252
208396_s_at		phosphodiesterase 1A, calmodulin-dependent	NM_005019	Hs.41717	Hs.191046	chr2q32.1	ENSG00000115252
231213_at		phosphodiesterase 1A, calmodulin-dependent	AU146305	Hs.22467	Hs.191046	chr2q32.1	ENSG00000115252
233547_x_at		phosphodiesterase 1A, calmodulin-dependent	N53248	Hs.41717	Hs.191046	chr2q32.1	ENSG00000115252
236344_at		phosphodiesterase 1C, calmodulin-dependent 70kDa	AW299452	Hs.143821	Hs.655694	chr7p14.3	ENSG00000154678
216869_at		phosphodiesterase 1C, calmodulin-dependent 70kDa	U40372	Hs.41718	Hs.655694	chr7p14.3	ENSG00000154678
207303_at		phosphodiesterase 1C, calmodulin-dependent 70kDa	NM_005020	Hs.41718	Hs.655694	chr7p14.3	ENSG00000154678
1554378_a_at		phosphodiesterase 1C, calmodulin-dependent 70kDa	BC022479	Hs.41718	Hs.655694	chr7p14.3	ENSG00000154678
206389_s_at		phosphodiesterase 3A, cGMP-inhibited	NM_000921	Hs.777	Hs.591150	chr12p12	ENSG00000172572
206388_at		phosphodiesterase 3A, cGMP-inhibited	U36798	Hs.777	Hs.591150	chr12p12	---
214582_at		phosphodiesterase 3B, cGMP-inhibited	NM_000753	Hs.150741	Hs.445711	chr11p15.1	ENSG00000152270
204735_at		phosphodiesterase 4A, cAMP-specific (phosphodiesterase E2 dunce homolog, Drosophila)	NM_006202	Hs.89901	Hs.89901	chr19p13.2	ENSG00000065989
203708_at		phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	NM_002600	Hs.188	Hs.198072	chr1p31	---
211302_s_at		phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	L20966	Hs.188	Hs.198072	chr1p31	ENSG00000184588
215671_at		phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	AU144792	Hs.319540	Hs.198072	chr1p31	---
206791_s_at		phosphodiesterase 4C, cAMP-specific (phosphodiesterase E1 dunce homolog, Drosophila)	BF511742	Hs.189	Hs.132584	chr19p13.11	ENSG00000105650
231478_at		phosphodiesterase 4C, cAMP-specific (phosphodiesterase E1 dunce homolog, Drosophila)	AI051127	Hs.132584	Hs.132584	chr19p13.11	ENSG00000105650
211818_s_at		phosphodiesterase 4C, cAMP-specific (phosphodiesterase E1 dunce homolog, Drosophila)	U88712	Hs.189	Hs.132584	chr19p13.11	ENSG00000105650
228962_at		phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	BF507941	Hs.28482	Hs.117545	chr5q12	ENSG00000113448
210837_s_at		phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	AF012074	Hs.172081	Hs.117545	chr5q12	ENSG00000113448
210836_x_at		phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	AF012073	Hs.172081	Hs.117545	chr5q12	ENSG00000113448
204491_at		phosphodiesterase 4D, cAMP-specific (phosphodiesterase E3 dunce homolog, Drosophila)	R40917	Hs.172081	Hs.117545	chr5q12	ENSG00000113448
1556787_s_at		Phosphodiesterase 5A, cGMP-specific	AW975338	Hs.431074	Hs.587281	chr4q25-q27	---
1553175_s_at		phosphodiesterase 5A, cGMP-specific	AB015656	Hs.121852	Hs.587281	chr4q25-q27	ENSG00000138735
227088_at		phosphodiesterase 5A, cGMP-specific	BF221547	Hs.16578	Hs.647971	chr4q25-q27	ENSG00000138735
206757_at		phosphodiesterase 5A, cGMP-specific	NM_001083	Hs.139271	Hs.587281	chr4q25-q27	---
1562228_s_at		phosphodiesterase 5A, cGMP-specific	AF043732	Hs.121852	Hs.587281	chr4q25-q27	ENSG00000138735
240088_at		phosphodiesterase 5A, cGMP-specific	BG494416	Hs.116533	Hs.587281	chr4q25-q27	ENSG00000138735
206623_at		phosphodiesterase 6A, cGMP-specific, rod, alpha	NM_000440	Hs.182240	Hs.567314	chr5q31.2-q34	ENSG00000132915
231065_at		Phosphodiesterase 6D, cGMP-specific, rod, delta	AI655478	Hs.129450	Hs.516808	chr2q35-q36	---
216883_x_at		phosphodiesterase 6D, cGMP-specific, rod, delta	AJ001626	Hs.48291	Hs.516808	chr2q35-q36	---
223358_s_at		phosphodiesterase 7A	AW269834	Hs.150395	Hs.527119	chr8q13	ENSG00000205268
224046_s_at		phosphodiesterase 7A	U67932	Hs.150395	Hs.527119	chr8q13	ENSG00000205268
1552343_s_at		phosphodiesterase 7A	NM_002604	Hs.406325	Hs.527119	chr8q13	ENSG00000205268
230500_at		Phosphodiesterase 7A	AA651933	Hs.150395	Hs.527119	chr8q13	---
230109_at		phosphodiesterase 7B	AI638433	Hs.164230	Hs.594417	chr6q23-q24	ENSG00000171408
220343_at		phosphodiesterase 7B	NM_018945	Hs.283016	Hs.594417	chr6q23-q24	ENSG00000171408
1552931_a_at		phosphodiesterase 8A	NM_002605	Hs.78746	Hs.9333	chr15q25.3	ENSG00000073417
212522_at		phosphodiesterase 8A	W73272	Hs.78746	Hs.9333	chr15q25.3	ENSG00000073417
205593_s_at		phosphodiesterase 9A	NM_002606	Hs.18953	Hs.473927	chr21q22.3	ENSG00000160191
205501_at		phosphodiesterase 10A	AI143879	Hs.128291	Hs.348762	chr6q26	ENSG00000112541
211171_s_at		phosphodiesterase 10A	AB026816	Hs.128291	Hs.348762	chr6q26	ENSG00000112541
221110_x_at		phosphodiesterase 11A	NM_016953	Hs.272345	Hs.570273	chr2q31.2	ENSG00000128655
237248_at		phosphodiesterase 11A	AI919276	Hs.47208	Hs.570273	chr2q31.2	ENSG00000128655
224224_s_at		phosphodiesterase 11A	AB038041	Hs.272345	Hs.570273	chr2q31.2	ENSG00000128655
224223_s_at		phosphodiesterase 11A	AF281865	Hs.272345	Hs.570273	chr2q31.2	ENSG00000128655
212581_x_at		glyceraldehyde-3-phosphate dehydrogenase	BE561479	Hs.169476	Hs.544577	chr12p13	ENSG00000111640
201049_s_at		ribosomal protein S18	NM_022551	Hs.275865	Hs.627414	chr6p21.3	ENSG00000096150 /// ENSG00000223367 /// ENSG00000226225 /// ENSG0000023150

Probe ID	Set	REFSeq Transcript ID	Pathway
1558680_s_at		NM_001003683	/// NM_1G_Protein_Signaling // GenMAPP
208396_s_at		NM_001003683	/// NM_1G_Protein_Signaling // GenMAPP
231213_at		NM_001003683	/// NM_1G_Protein_Signaling // GenMAPP
233547_x_at		NM_001003683	/// NM_1G_Protein_Signaling // GenMAPP
236344_at		NM_005020	G_Protein_Signaling // GenMAPP
216869_at		NM_005020	G_Protein_Signaling // GenMAPP
207303_at		NM_005020	G_Protein_Signaling // GenMAPP
1554378_a_at		NM_005020	G_Protein_Signaling // GenMAPP
206389_s_at		NM_000921	---
206388_at		NM_000921	---
214582_at		NM_000922	---
204735_at		NM_001111307	/// NM_1G_Protein_Signaling // GenMAPP
203708_at		NM_001037339	/// NM_1G_Protein_Signaling // GenMAPP /// Smooth_muscle_contraction // GenMAPP
211302_s_at		NM_001037339	/// NM_1G_Protein_Signaling // GenMAPP /// Smooth_muscle_contraction // GenMAPP
215671_at		NM_001037339	/// NM_1G_Protein_Signaling // GenMAPP /// Smooth_muscle_contraction // GenMAPP
206791_s_at		NM_000923	/// NM_001G_Protein_Signaling // GenMAPP
231478_at		NM_000923	/// NM_001G_Protein_Signaling // GenMAPP
211818_s_at		NM_000923	/// NM_001G_Protein_Signaling // GenMAPP
228962_at		NM_001104631	/// NM_1G_Protein_Signaling // GenMAPP /// Smooth_muscle_contraction // GenMAPP
210837_s_at		NM_001104631	/// NM_1G_Protein_Signaling // GenMAPP /// Smooth_muscle_contraction // GenMAPP
210836_x_at		NM_001104631	/// NM_1G_Protein_Signaling // GenMAPP /// Smooth_muscle_contraction // GenMAPP
204491_at		NM_001104631	/// NM_1G_Protein_Signaling // GenMAPP /// Smooth_muscle_contraction // GenMAPP
1556787_s_at		NM_001083	/// NM_033- ---
1553175_s_at		NM_001083	/// NM_033- ---
227088_at		NM_001083	/// NM_033- ---
206757_at		NM_001083	/// NM_033- ---
1562228_s_at		NM_001083	/// NM_033- ---
240088_at		NM_001083	/// NM_033- ---
206623_at		NM_000440	---
231065_at		NM_002601	---
216883_x_at		NM_002601	---
223358_s_at		NM_002603	/// NM_002G_Protein_Signaling // GenMAPP
224046_s_at		NM_002603	/// NM_002G_Protein_Signaling // GenMAPP
1552343_s_at		NM_002603	/// NM_002G_Protein_Signaling // GenMAPP
230500_at		NM_002603	/// NM_002G_Protein_Signaling // GenMAPP
230109_at		NM_018945	G_Protein_Signaling // GenMAPP
220343_at		NM_018945	G_Protein_Signaling // GenMAPP
1552931_a_at		NM_002605	/// NM_173G_Protein_Signaling // GenMAPP
212522_at		NM_002605	/// NM_173G_Protein_Signaling // GenMAPP
205593_s_at		NM_001001567	/// NM_1 ---
205501_at		NM_001130690	/// NM_1 ---
211171_s_at		NM_001130690	/// NM_1 ---
221110_x_at		NM_001077196	/// NM_1 ---
237248_at		NM_001077196	/// NM_1 ---
224224_s_at		NM_001077196	/// NM_1 ---
224223_s_at		NM_001077196	/// NM_1 ---
212581_x_at		NM_002046	Glycolysis_and_Gluconeogenesis // GenMAPP
201049_s_at		NM_022551	Ribosomal_Proteins // GenMAPP /// Ribosomal_Proteins // GenMAPP