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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

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Running Title: PDE3/4 inhibition on CRE- and GRE-dependent transcription

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ABBREVIATIONS: ASM, airway smooth muscle; CD200, cluster of differentiation 200; COPD, chronic obstructive pulmonary disease; CRE, cAMP response element; CREB, cAMP response element-binding protein; CRISPLD2, cysteine-rich secretory protein LCCL domain containing 2; DMEM, Dulbecco's modified Eagle's medium; DUSP1, dual-specificity phosphatase 1; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; GRE, glucocorticoid response element; ICS, inhaled corticosteroid; LABA, long-acting β_2-adrenoceptor agonist; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; ODQ, 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one; PDE, phosphodiesterase; PKA, protein kinase A; RGS, regulator of G protein signaling; SNP, sodium nitroprusside.

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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 ICS 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 to inhibiting either PDE alone. In respect of 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 fides* 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 pro-inflammatory 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.

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Introduction

Glucocorticoids taken in an inhaled form, and referred to as inhaled corticosteroids (ICS), are the mainstay anti-inflammatory treatment for mild/moderate asthma and work by suppressing airway inflammation (Barnes, 2006). However, in severe asthma, ICS 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, are 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 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, coupling to $G\alpha_s$ and reduce receptor desensitization (Mak et al., 1995b; Mak et al., 1995a; Chong et al., 1997; Kalavantavanich and Schramm, 2000). Conversely, β_2-adrenoceptor agonists elicit various pro-inflammatory responses, for example the enhancement of IL8 release (Holden et al., 2010), which are inhibited by glucocorticoids (Giembycz and Newton, 2006; Newton et al., 2010). While 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 effect, it is clear that the transactivation of glucocorticoid-sensitive genes is important (Newton, 2000; Newton and Holden, 2007; Clark and Belvisi, 2012). Thus,

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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, 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). As this also occurs with glucocorticoid-induced genes, *in vivo* correlates may account for the benefits of ICS/LABA therapy (Kaur et al., 2008; Giembycz et al., 2008). For example, regulator of G-protein signaling 2 (RGS2), which terminates signaling from the heterotrimeric G protein, $G\alpha_q$ (Heximer, 2004; Kimple et al., 2009), is bronchoprotective *in vivo* and may also attenuate cytokine release (Holden et al., 2011; Xie et al., 2012; Holden et al., 2014). 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; Holden et al., 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, in 2012, approved for severe COPD patients with chronic bronchitis and frequent exacerbations (Giembycz and Field, 2010). While improvements in lung function may occur in the absence of ICS (Calverley et al., 2009), the additional benefit of adding-on roflumilast to patients already taking ICS fits with the fact

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that PDE4 inhibitors, like LABAs, also enhance glucocorticoid-dependent transcription (Miller et al., 2002; Rennard et al., 2011; Kaur et al., 2008; 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 when compared to a PDE4 inhibitor alone (Giembycz and Newton, 2011; Abbott-Banner and Page, 2014). In the present study, we have used human bronchial epithelial cells, which express PDE3 and PDE4 (Wright et al., 1998; Dent et al., 1998; Rabe et al., 1993), 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.

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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 (DMEM)/Ham's F12 medium (Invitrogen, Burlington, ON, Canada), supplemented with 10% fetal calf serum (FCS), 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, Sweden), forskolin ([3R-(3α,4aβ,5β,6β,6aα,10α,10aβ,10bα)]-5-(acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-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) (all Sigma-Aldrich, Oakville, ON, Canada), 8-bromo-cGMP (8Br-cGMP), 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'-kl]pyrrolo [3,4-i] [1,6] benzodiazocine-10-carboxylic acid, methyl ester), 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), rolipram ((*RS*)-4-(3-cyclopentyloxy-4-methoxy-phenyl)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) (all Tocris, Bristol, UK), GSK256066 (6-({3-[(dimethylamino) carbonyl] phenyl} sulphonyl)-8-methyl-4-{[3-methyloxy) phenyl] amino}-3-quinolinecarboxamide) (Gilead Sciences, Seattle, Washington, USA) and roflumilast *N*-oxide (3-(cyclopropylmethoxy)-N-(3,5-dichloro-1-oxido-4-pyridinyl)-4-(difluoromethoxy) benzamide; *N*-(3,5-dichloro-1-oxopyridin-4-yl)-4-difluoromethoxy-3-cyclopropyl-methoxybenzamide)

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(Nycomed, Konstanz, Germany) were dissolved in dimethylsulphoxide (DMSO). Final concentrations of DMSO 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 (Meja et al., 2004; Chivers et al., 2004). Confluent cells were incubated in serum-free medium overnight before treatment with drugs. Cells were harvested after 6 h, 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, USA); cAMP response element-binding protein (CREB) (9197); phospho-CREB (P-CREB) (9191) (Cell Signaling Technology, Danvers, MA, USA). Immunodetection was by enhanced chemiluminescence (GE Healthcare Bio-Sciences Inc) and visualization was by autoradiography. For adenoviral infections, 6×CRE reporter BEAS-2B cells at ~70% confluence were incubated for 24 h in DMEM/F12 + 10 % FCS with adenoviral (Ad5) expression vectors at the indicated multiplicity of infections (MOI). Ad5-PKIα and the empty Ad5 vector (null) were as described (Meja et al., 2004).

RNA isolation, cDNA synthesis and SYBR green real-time PCR. Total RNA (0.5 µg), extracted by the RNeasy mini kit method (Qiagen), was reverse transcribed using QScript reverse transcriptase (Quanta Biosciences, Gaithersburg, MD). SYBR GreenER (Invitrogen) real-time PCR was performed using a StepOne Plus real-time PCR machine (Applied Biosystems Inc, Foster City, CA, USA) 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,

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CD200, 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 (GraphPad Software, San Diego, CA). Comparisons between groups were performed using one-way analysis of variance (ANOVA) with a Bonferroni or Dunnett's post-test or a paired t-test as indicated. Where greater than 5 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-Keul multiple comparison test was used.

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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 have been previously identified in human airway epithelial cells and their ability to mediate anti-inflammatory activity is well studied (Wright et al., 1998; Dent 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 6 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). In contrast, rolipram, a PDE4 inhibitor, increased CRE-dependent transcription in a concentration-dependent manner (EC_{50} ~960 nM). However, the maximal induction was modest (<2-fold) when compared to forskolin, which increased luciferase activity by almost 20-fold (Fig. 1A). Siguazodan, in combination with rolipram, both at 10 μM, produced a ~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

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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 PDE3/4 inhibitor, zardaverine, modestly increased CRE reporter activity and significantly and 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 μM) failed to promote the CRE-dependent transcription, rolipram (10 μM) produced a

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modest, but transient, activation of the reporter that peaked between 4 – 8 h (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 h, 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 - 8 h (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 PKIα over-expression and modulation of the cGMP/protein kinase G (PKG) 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). As null virus at this MOI was inactive, the sensitization of formoterol-induced CRE-dependent transcription by PDE3 plus PDE4 inhibitors appears to involve the classical 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 8-Br-cGMP, a cell permeant analog of cGMP, on luciferase expression in 6×CRE BEAS-2B reporter cells.

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As shown in supplemental figure 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 μM) 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 the hydrolysis of cGMP generated constitutively by soluble guanylyl cyclase. For completeness, the role of cGMP-dependent protein kinase (PKG) was evaluated using the inhibitor, KT5823, which is approx. 50-fold selective for PKG over PKA (K_i = 0.2 and >10 μM respectively). As shown in supplemental figure 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 min and was maintained for at least 1 h post-treatment (Fig. 4A). Two hours post-formoterol treatment, CREB phosphorylation had begun to wane and, by 6 h, this had approached basal levels. It is important to note that because of apparent blocking of signal produced by the total CREB antibody following prior immunodetection of phospho-CREB, even following 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. While the phosphorylation of ATF-1 was also detected and increased in parallel with CREB phosphorylation (Fig. 4), total ATF-1 expression was not assessed and therefore changes in ATF-1 expression cannot be excluded.

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There was little or no effect of either siguazodan (10 μM) or rolipram (10 μM) 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 min post-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 μM) (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.

Effect of PDE3 plus PDE4 inhibitors on formoterol-induced gene expression. Treatment of BEAS-2B cells with siguazodan plus rolipram produced modest, non-significant, increases in RGS2, CD200, CRISPLD2 and DUSP1 mRNA expression (Fig. 5, Supplemental Fig. 4A). In contrast, formoterol (1 nM) significantly, but transiently, increased the mRNA expression of all four genes. RGS2 and DUSP1 expression peaked at 1 h, while CD200 and CRISPLD2 were maximal at 2 h 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 h and at all times thereafter (Fig. 5). Similar, but considerably more modest effects were apparent for CD200 and DUSP1 mRNA at 12 and 18 h (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 h (Fig. 6). However, due to the low fold enhancement by formoterol, or formoterol plus siguazodan plus rolipram, DUSP1

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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 h. Rolipram produced modest increases in RGS2, CD200 and CRISPLD2 mRNA at 2 h (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 h, the concentration-response curves that described formoterol-induced RGS2, CD200 and CRISPLD2 expression were displaced to the left by approximately 1 $\log_{10}$ 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).

At 12 h, 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). In respect of CD200, these effects of siguazodan and rolipram appeared additive, whereas for CRISPLD2 the enhancement was primarily driven by rolipram. Combining the 12 h data from figures 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×GRE reporter activation, a maximally effective concentration of dexamethasone (1 μM) robustly activated the 2×GRE reporter (Fig. 7A & B). This response was markedly enhanced by rolipram (10

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μM), but not siguazodan (10 μM) (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×GRE reporter activity in a concentration-dependent manner. In the presence of rolipram (10 μM), but not siguazodan (10 μM), the formoterol concentration-response curve was displaced to the left by approximately 1 $\log_{10}$ 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 h), 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 μM) 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 μM) (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 h, did not reach significance. In 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 - 2 h 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 h (Fig. 8A). However, at 6, 12 and 18 h, when dexamethasone plus formoterol-induced expression of

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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 h maximum was extended from ~5.3 to ~8 h 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 h maximum value was extended from ~9.3 to almost 16 h. Qualitatively similar data were obtained for CD200 and CRISPLD2, but not for DUSP1, which was not studied further (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 h, dexamethasone enhanced the expression of RGS2, CD200 and CRISPLD2 mRNAs by 2 - 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 h 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 one $\log_{10}$ unit to the left in the absence of any change to maximum response (Fig. 9B) (Table 2). In the presence of a low concentration formoterol (10 pM), the enhancement of dexamethasone-induced gene expression at 2 h 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 h these responses primarily occurred via PDE4 inhibition.

The above data contrast with the effects on gene expression observed at 12 h (Fig. 9D). While dexamethasone modestly enhanced RGS2, CD200 and CRISPLD2 mRNA expression, there was no apparent effect of rolipram. Conversely, siguazodan resulted in small enhancements, significant for

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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 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 are responsible for the longer-term enhancements in dexamethasone plus formoterol-dependent gene expression.

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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-induce reporter and glucocorticoid-induced gene transcription can be significantly enhanced by LABAs (Kaur et al., 2008). While not applicable to all glucocorticoid-induced genes, including; *TSC22D3* (*GILZ*), *FKBP5* (*FKBP51*) and *ZFP36* (*TTP*) (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). Conversely, while the acute β_2-adrenoceptor-mediated relaxation of ASM is a recognized, non-genomic 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 RGS2 expression to protect against bronchoconstriction and, potentially, the pro-inflammatory effects of mediators, such as histamine and acetylcholine (Holden et al., 2011; Xie et al., 2012; Holden et al., 2014). 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; Holden et al., 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 (LPS) 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

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DUSP1 mRNA were largely additive with the effects of LABA essentially over within two hours. This effect is shown by our current data and explains why DUSP1 expression was not investigated further in the current 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-on 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 on 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). As 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, RPL 554, 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

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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 over-expression, these data support a classical PKA-dependent process. However, as 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 signalling 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 h post-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 h the sensitization of formoterol-enhanced glucocorticoid-induced gene expression by combined PDE3/4 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 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 multiple cAMP-elevators and also blocked by PKIα (Holden et al., 2014), suggesting that cAMP-PKA-dependent processes the likely mechanisms of action for these effects. In contrast, longer treatment times (i.e. 12 h post-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

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dexamethasone plus formoterol was unaffected, the subsequent rate of decline was strikingly reduced by combined PDE3/4 inhibition. This effect extended to CD200 and CRISPLD2, required both PDE3 and PDE4 inhibition and suggests that dual PDE3/4 inhibitors may significantly prolong the effects of ICS/LABA combination therapies.

Overall, while the predominant effects on gene expression were due to PDE4 inhibition, there were clear benefits to adding-on 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 REACT (**R**oflumilast in the prevention of COPD **E**xacerbations while taking **A**ppropriate **C**ombination **T**reatment) study in which the effect of roflumilast and placebo on the exacerbation rates of severe bronchitic COPD patients 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 sub-serve differential physiological 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 following both PDE3 and PDE4 inhibition and that 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

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respect of 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-terms effects, whereby the role of the PDE3 inhibitor, relative to the PDE4 appears to be enhanced. While 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 & 9) (Newton et al., 2010). However, non-uniform 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. In 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 airways remodeling (Rider et al., 2011; Hinzey et al., 2011; Rider et al., 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 PDE3/4 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 PDE3/4 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 PDE3/4 inhibition should be clinically evaluated as an add-on to existing ICS/LABA combination therapy in obstructive lung diseases.

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Authorship Contributions

Participated in research design and obtained funding: Giembycz, Newton.

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

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

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

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Footnotes

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Figure legends

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

Fig. 2. Effect of PDE inhibitors and formoterol on luciferase activity and kinetics in 6×CRE BEAS-2B reporter cells. A and B, BEAS-2B 6×CRE reporter cells were pre-incubated for 30 min with vehicle, siguazodan (10 μM) (Sig), rolipram (10 μM) (Roli), or a combination of both together (both at 10 μM) (S + R) before treating with various concentrations of formoterol. After 6 h, cells were harvested for luciferase assay. Data, expressed as fold induction relative to non-stimulated cells (NS), are plotted as mean ± S.E. C, BEAS-2B 6×CRE reporter cells were treated with vehicle, siguazodan (10 μM) (Sig), rolipram (10 μM) (Roli) or both together (S + R) for the indicated times (left panel). Alternatively, cells were pre-treated with vehicle, siguazodan (10 μM) (Sig), rolipram (10 μM) (Roli) or both together (S + R) for 30 min before the addition of formoterol (1 nM) (Form) for the indicated times (right panel). Following luciferase assay, data, expressed as fold induction over NS at each time point, are plotted as

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mean ± S.E. Significance was assessed at each time by ANOVA with a Bonferroni post-test. S + R at 2, 4, 6, 8, 12 and 18 h was significantly enhanced relative to untreated (stars not shown). Significance relative to Sig is indicated where: * $P < 0.01$, ** $P < 0.01$, *** $P < 0.001$. Significance relative to Roli is indicated where: # $P < 0.05$, ## $P < 0.01$.

Fig. 3. Effect of PKIα over-expression on CRE-dependent transcription. 6×CRE BEAS-2B cells were either not treated or infected at a MOI of 30 with either Ad5.CMV.PKIα or Ad5.CMV.Null for 48 h. Thereafter, cells were left not stimulated (NS) or treated for 30 min with siguazodan (10 µM) plus rolipram (10 µM) (S+R), before being exposed to various concentrations of formoterol (Form). After 6 h, cells were harvested luciferase assay. Data, expressed as fold induction, relative to NS, are plotted as means ± S.E.

Fig. 4. Effect of cAMP-elevating agents on CREB phosphorylation. 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 phospho-CREB (P-CREB), total CREB and GAPDH. Following densitometric analysis, data, expressed as a ratio of P-CREB/GAPDH, are plotted as means ± S.E. Phosphorylation of CREB was analysed using ANOVA with a Dunnett's post-test. Significance relative to NS is indicated; *** $P < 0.001$. B, BEAS-2B cells were either not stimulated or pre-treated with either rolipram (10 µM), siguazodan (10 µM) or a combination of both for 30 min, then treated with formoterol (10 pM or 100 pM) or forskolin (10 µM). Cells were harvested after 10 and 30 min for western blot analysis of P-CREB and GAPDH. Following densitometric analysis, data, expressed as a ratio of P-CREB/GAPDH, are plotted as means ± S.E. Phosphorylation of CREB was analysed using

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ANOVA with a Bonferroni post-test. Significance relative to NS is indicated; ** $P < 0.01$ and *** $P < 0.001$. Significance relative to formoterol (10 pM) is indicated; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$.

Fig. 5. Effect of LABA and PDE inhibitors on RGS2, CD200 and CRISPLD2 gene expression. BEAS-2B cells were pre-incubated for 30 min with vehicle, or a combination of siguazodan (10 μM) plus rolipram (10 μM) (S + R) and then exposed, or not, to formoterol (1 nM) (Form). 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 ± S.E. Significance, relative to NS at each time point, was tested by ANOVA with a Bonferroni post-test. Additional comparisons are indicated. ** $P < 0.01$ and *** $P < 0.001$.

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

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Fig. 7. Effect of cAMP elevating agents on 2×GRE-dependent transcription. A, BEAS-2B 2×GRE reporter cells were pre-incubated for 30 min with vehicle, siguazodan (10 μM) (Sig), rolipram (10 μM) (Roli), or both together (S + R) before treating with dexamethasone (1 μM) (Dex) alone or with various concentrations of formoterol (Form). After 6 h, the cells were harvested for luciferase assay. Data, expressed as a percentage of Dex, are plotted as mean ± S.E. Significance for Dex + Sig, Dex + Roli and Dex + S + R relative to dexamethasone-treated, or as indicated, was assessed by ANOVA using a Bonferroni post test. * $P < 0.5$, ** $P < 0.01$. B, BEAS-2B 2×GRE reporter cells were pre-treated for 30 min with vehicle, siguazodan (10 μM) (Sig), rolipram (10 μM) (Roli), or both together (S + R) before treating with Dex (1 μM) alone (left panel) or with Dex (1 μM) + formoterol (1 nM) (Dex + Form) (right panel). Cells were harvested at the indicated times for luciferase assay. Data expressed as fold induction over control at each time point, are plotted as mean ± S.E. Significance for Dex + Sig, Dex + Roli and Dex + S + R relative to dexamethasone-treated 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 h. Dex + Sig was not different from Dex at any time. Dex + Roli relative to Dex showed $P < 0.05$ for 2 - 12 & 24 h. Dex + S + R relative to Dex + Sig showed $P < 0.05$ for 2 - 12 and 18 h.

Fig. 8. Effect of siguazodan, rolipram and formoterol in the presence of dexamethasone on the gene expression. A, BEAS-2B cells were pre-treated with siguazodan (10 μM) plus rolipram (10 μM) (S + R) for 30 min, before simulation, or not, with dexamethasone (1 μM) (Dex) alone or with formoterol (1 nM) (Form) as indicated. Cells were harvested at the indicated times for RNA extraction. Following

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cDNA synthesis, SYBR green real-time PCR was performed for RGS2, CD200 and CRISPLD2. Data, normalized to GAPDH and expressed as fold of non-stimulated cells (NS) at 1 h, are plotted as mean ± S.E. B, Data for RGS2 from Fig. 8A (upper panel) were re-plotted and the effect of each combination (○ = Dex + Form; ● = Dex + Form + S + R) expressed as a percentage of the value at 1 h. Times until 50% and 25% of the maximum response were reached are shown. A, 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 +++ $P < 0.001$.

Fig. 9. Effect of siguazodan and rolipram on dexamethasone and dexamethasone plus formoterol-induced gene expression. BEAS-2B cells were pre-treated for 30 min, or not, with siguazodan (10 μM) (Sig) and/or rolipram (10 μM) (Roli) before stimulation, or not, with dexamethasone (1 μM) (Dex) or dexamethasone (1 μM) plus the indicated concentrations of formoterol (Dex + Form). Cells were harvested after 2 h (A, B & C) or 12 h (D) for RNA extraction. SYBR green real-time PCR was performed for RGS2, CD200 and CRISPLD2. Data, normalized to GAPDH and expressed as fold of not stimulated cells (NS), are plotted as means ± S.E. Significance, relative to dexamethasone-treated, was assessed by ANOVA with a Bonferroni post-test. Additional comparisons are indicated. In panel B, comparisons between Dex and Dex + S + R for each concentration of formoterol were using Student's paired t-test. * = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$.

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Tables

TABLE 1

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

Treatment	Potency to formoterol pEC_{50} (M) (± S.E.)	*N*	Paired t test
Naïve	10.6 (±0.0)	7	***
+ siguazodan	10.3 (±0.0)	7	
Naïve	9.9 (±0.0)	8	***
+ rolipram	10.23 (±0.1)	8	
Naïve	9.97 (±0.02)	6	***
+ siguazodan + rolipram	10.75 (±0.09)	6	
+ siguazodan	10.54 (±0.04)	5	***
+ siguazodan + rolipram	11.05 (±0.05)	5	
+ rolipram	10.9 (±0.1)	4	*
+ rolipram + siguazodan	11.2 (±0.1)	4	
Naïve	10.5 (±0.1)	6	*
+ cilostazol	10.8 (±0.1)	6	
Naïve	10.3 (±0.1)	6	***
+ GSK256066	10.7 (±0.1)	6	
Naïve	10.3 (±0.1)	6	*
+ roflumilast *N*-oxide	10.6 (±0.1)	6	
Naïve	10.5 (±0.14)	5	***
+ zardaverine	11.2 (±0.11)	5	

pEC_{50} values are derived from the data in Fig. 2 and Supplemental Fig. 1. Significance between pEC_{50} values was compared by paired t test. * $P < 0.05$, *** $P < 0.001$.

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TABLE 2

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

Gene	Treatment	Potency to formoterol pEC_{50} (M) (± S.E.)	*N*	Potency to formoterol in the presence of 1 μM dexamethasone pEC_{50} (M) (± S.E.)	*N*
RGS2	Naïve	10.8 (±0.4)	6	10.9 (±0.1)	5
	+ Sig + Roli	11.8 (±0.3)	6	11.9 (±0.2)	5
CD200	Naïve	10.9 (±0.1)	6	10.9 (±0.1)	5
	+ Sig + Roli	11.8 (±0.2)	6	12.0 (±0.1)	5
CRISPLD2	Naïve	10.7 (±0.2)	6	11.0 (±0.1)	5
	+ Sig + Roli	11.6 (±0.2)	6	11.9 (±0.3)	5

pEC_{50} values for the potency of formoterol at inducing gene expression in the absence or presence of siguazodan (10 μM) plus rolipram (10 μM) (Sig + Roli) or with dexamethasone (1 μM) in the absence or presence of siguazodan plus rolipram are derived from the data shown in Fig. 6B and Fig. 9B respectively.

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TABLE 3

Effect of PDE inhibitors on the potency of formoterol in enhancing dexamethasone driven GRE-dependent transcription.

Treatment	Potency to formoterol in the presence of 1 μM dexamethasone pEC_{50} (M) (± S.E.)	*N*	ANOVA with Bonferroni post test
Naïve	10.1 (±0.1)	8	
+ siguazodan	10.2 (±0.2)	8	
+ rolipram	11.2 (±0.1)	8	***
+ siguazodan + rolipram	11.1 (±0.1)	7	*** / ###

pEC_{50} values for the enhancement of dexamethasone (1 μM)-induced 2×GRE-dependent transcription by formoterol in the absence or presence of siguazodan (10 μM) and/or rolipram (10 μM) are derived from the data presented in Fig. 7A. Significance between pEC_{50} values was assessed by ANOVA with a Bonferroni post test. *** $P < 0.001$ for comparisons with naïve and ### $P < 0.001$ for comparison with siguazadan-treated.

Fig. 1

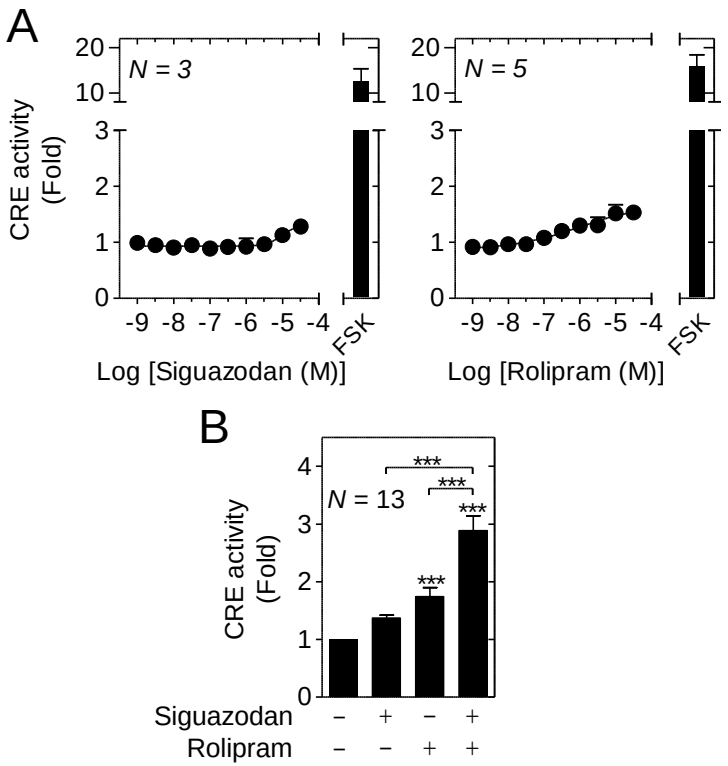


Fig. 2

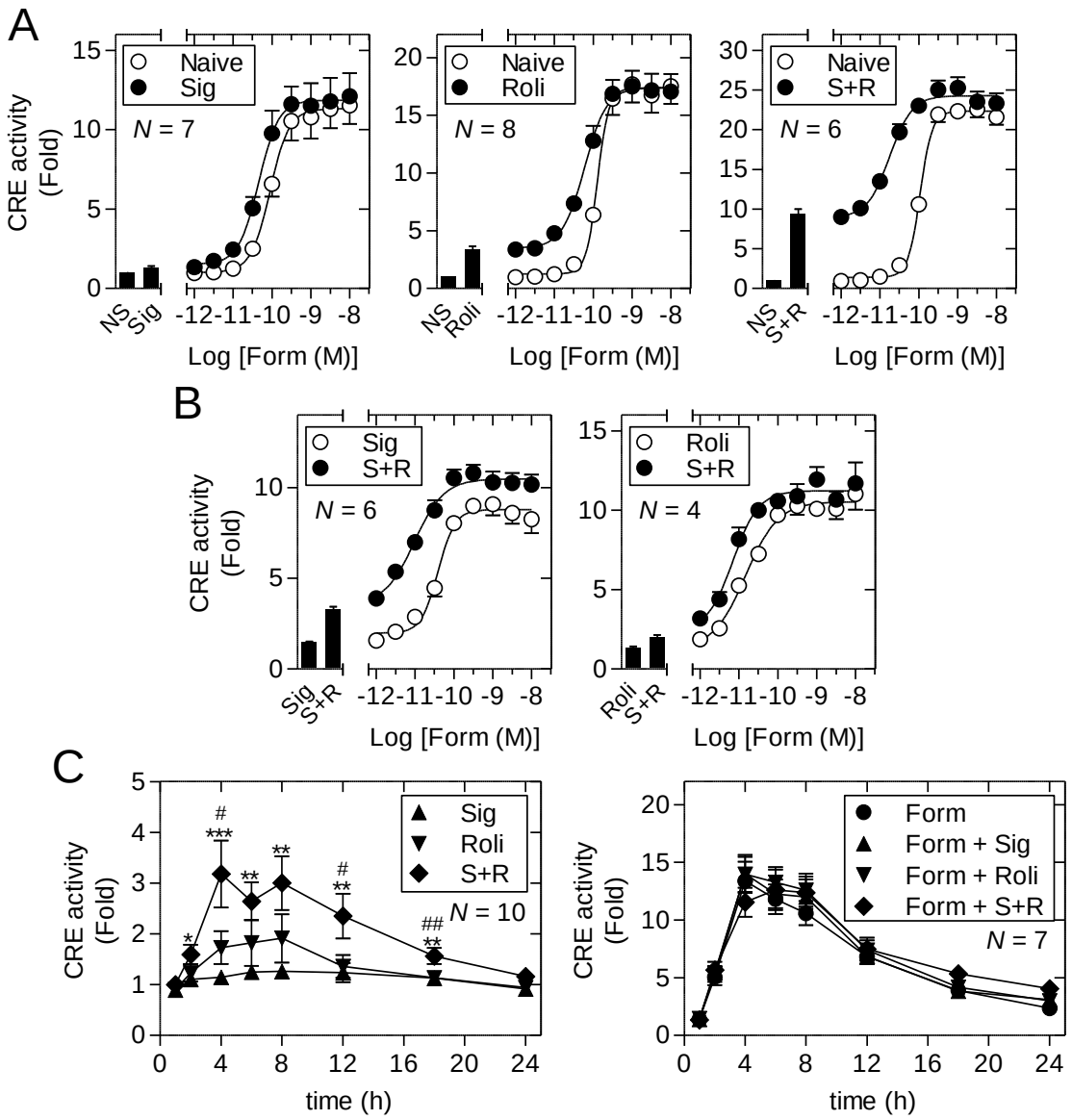


Fig. 3

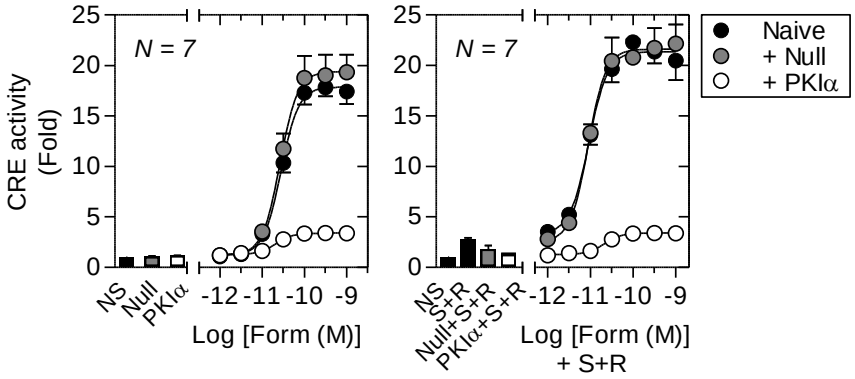


Fig. 4

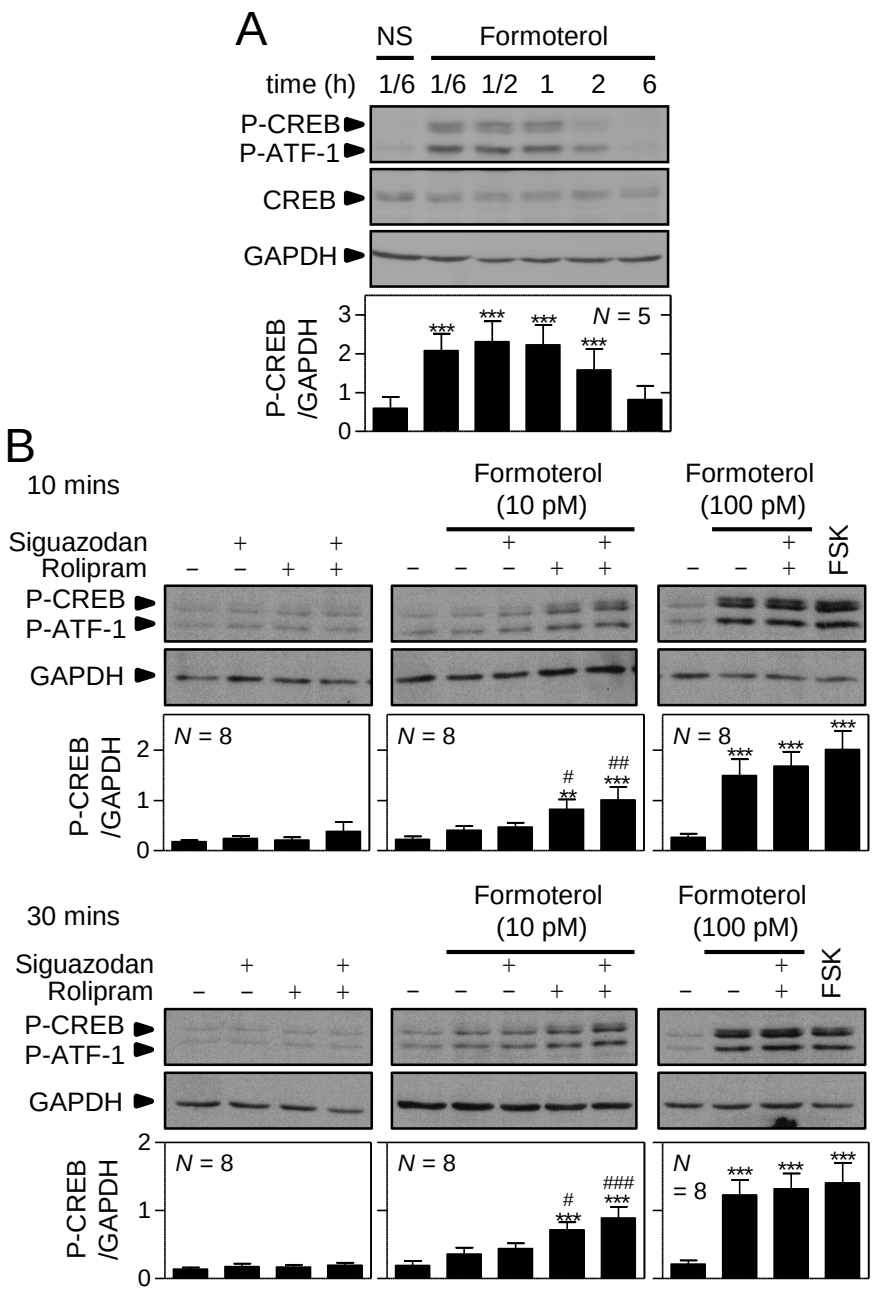


Fig. 5

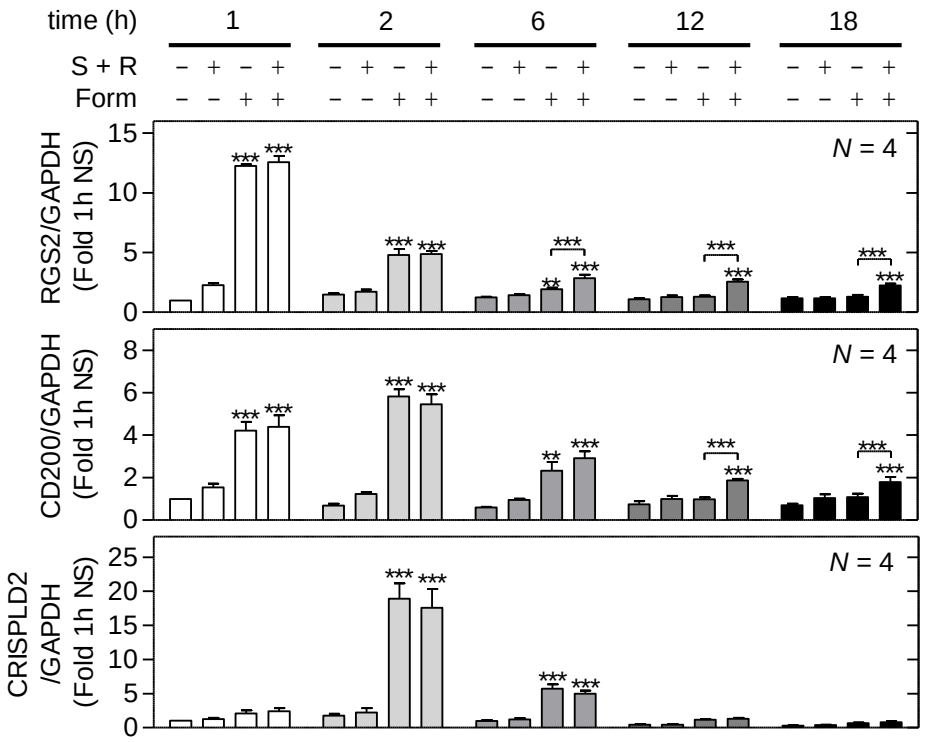


Fig. 6

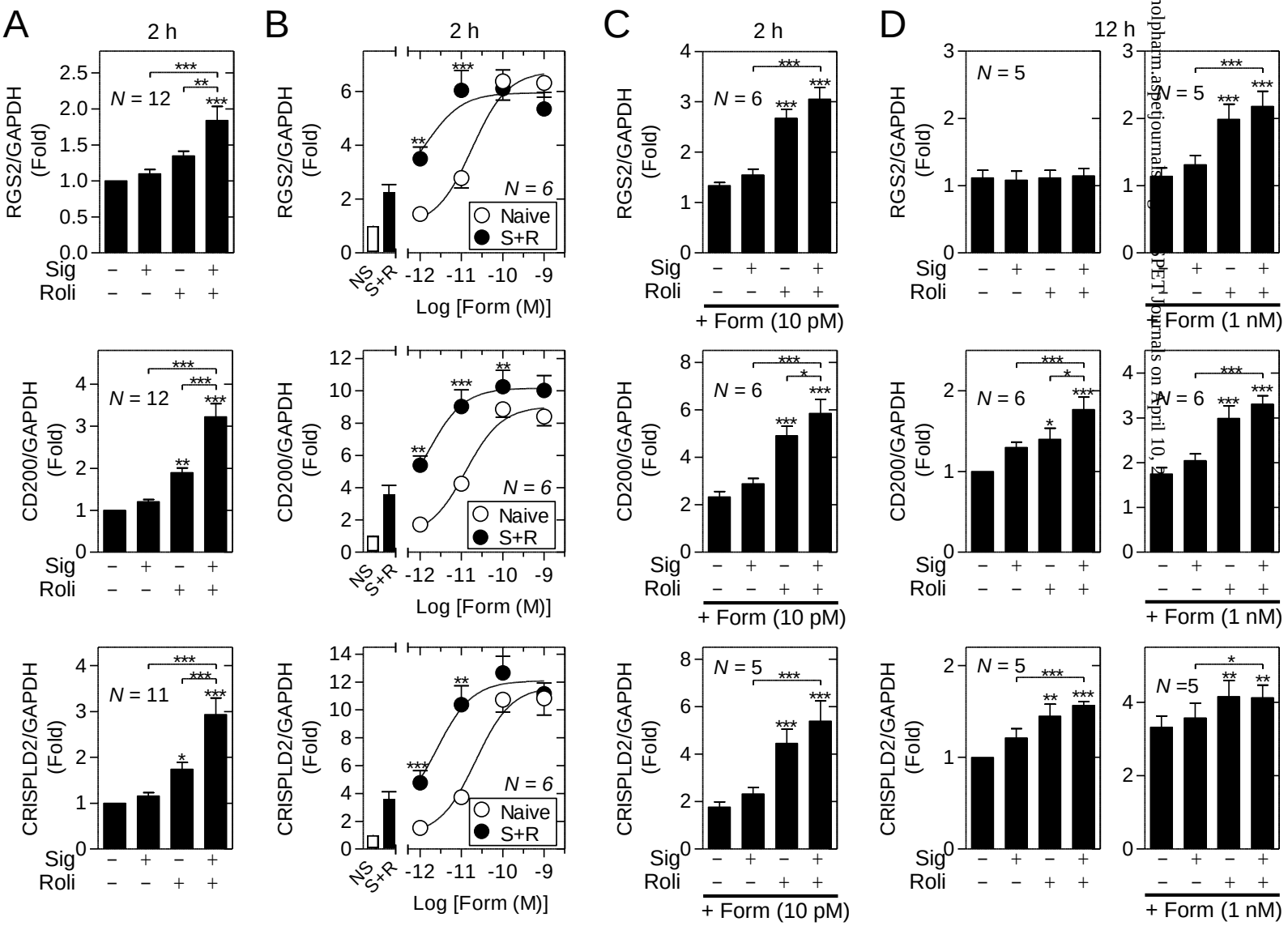


Fig. 7

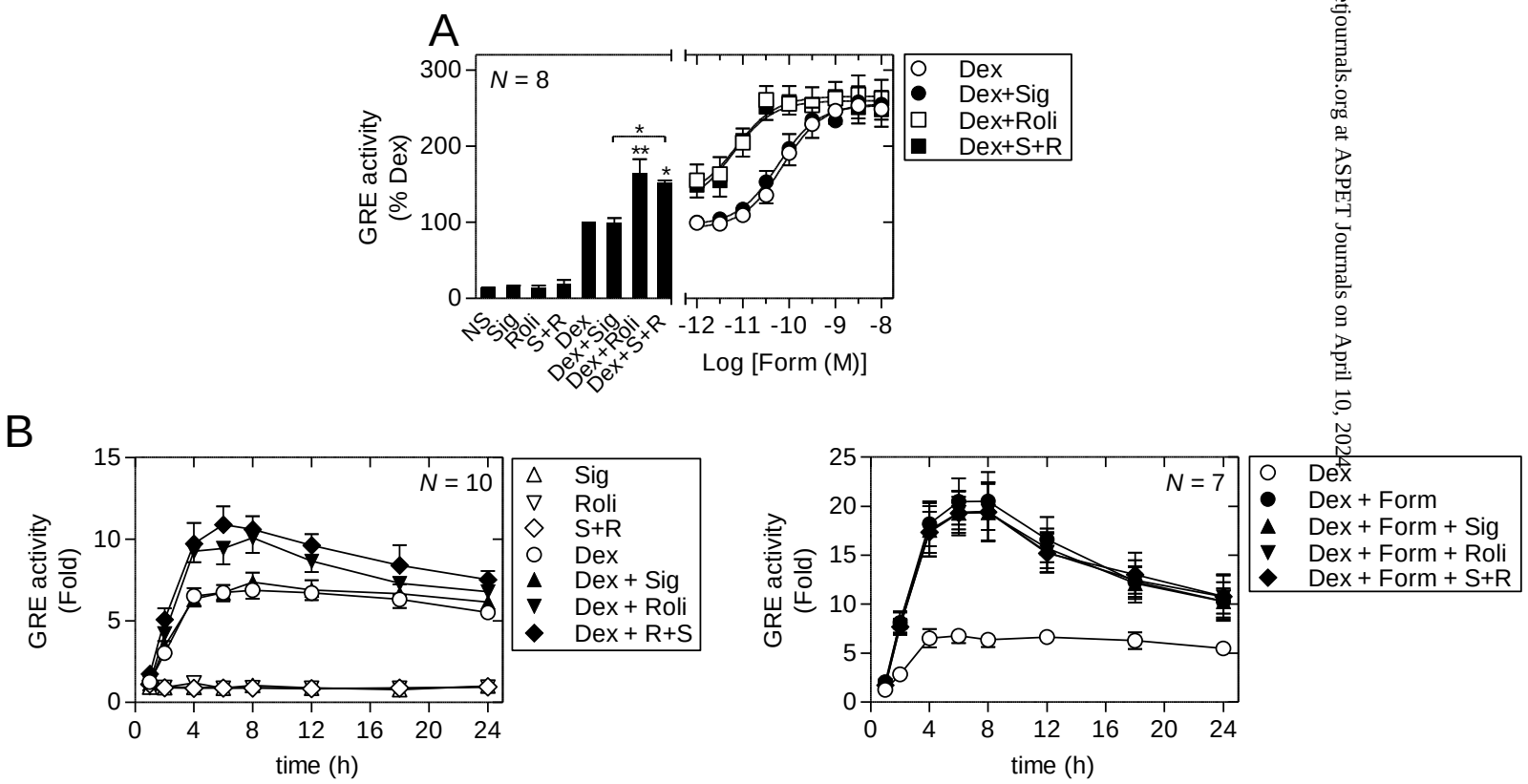
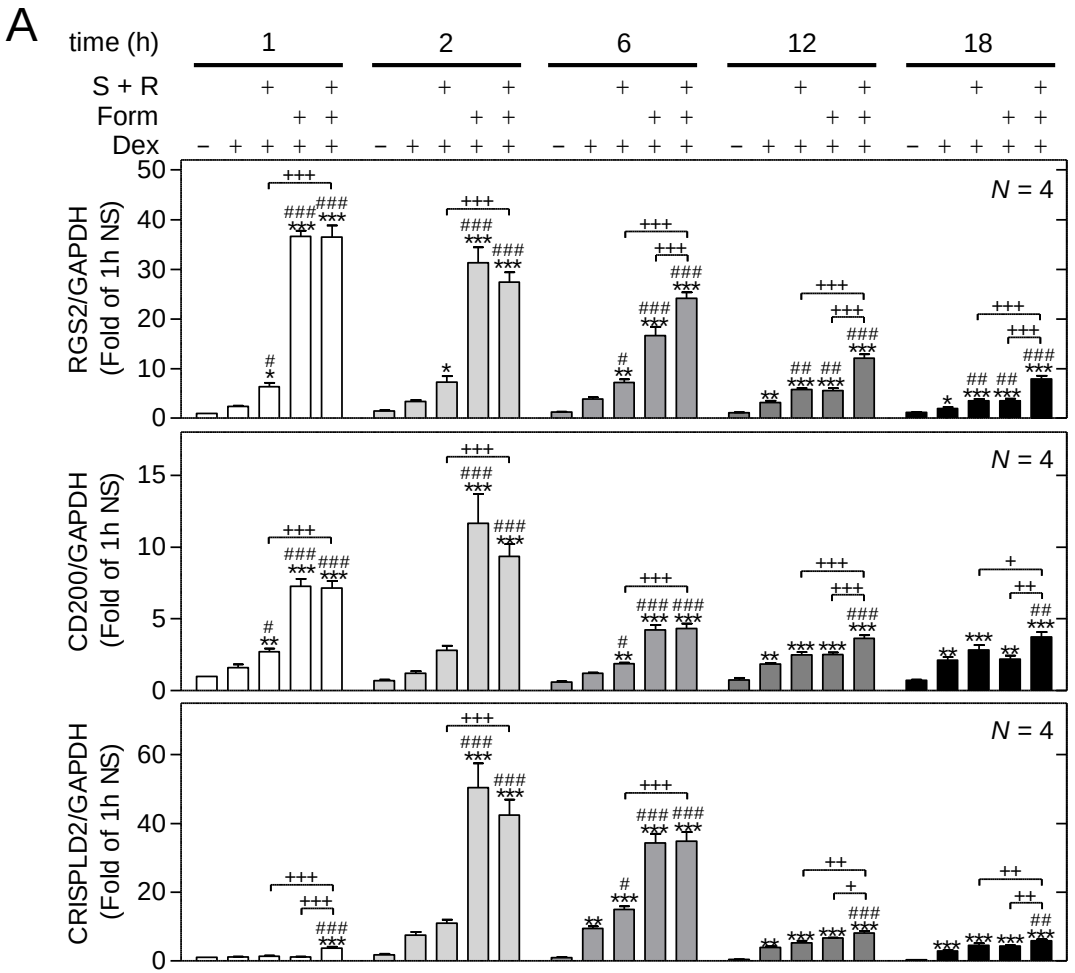


Fig. 8



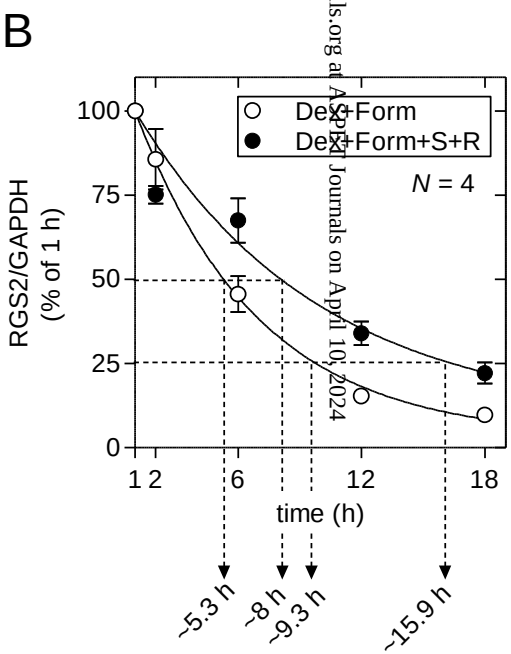


Fig. 9