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Long-Acting β_2-Adrenoceptor Agonists Synergistically Enhance Glucocorticoid-Dependent Transcription in Human Airway Epithelial and Smooth Muscle Cells[S]

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ABSTRACT

Addition of an inhaled long-acting β_2-adrenoceptor agonist (LABA) to an inhaled corticosteroid (ICS) is more effective at improving asthma control and reducing exacerbations than increasing the dose of ICS. Given that LABA monotherapy is not anti-inflammatory, pathways may exist by which LABAs enhance ICS actions. In the current study, the glucocorticoid dexamethasone had no effect on β_2-adrenoceptor agonist-induced cAMP-response element-dependent transcription in the human bronchial epithelial cell line BEAS-2B. In contrast, simple glucocorticoid response element (GRE)-dependent transcription induced by dexamethasone, budesonide,and fluticasone was synergistically enhanced by β_2-adrenoceptor agonists, including salmeterol and formoterol, to a level that could not be achieved by glucocorticoid alone. This enhancement was mimicked by other cAMP-elevating agents, and a cAMP mimetic, and was blocked by an inhibitor of cAMP-dependent protein kinase (PKA). Thus, β_2-adrenoceptor agonists synergistically enhance simple GRE-dependent transcription via the classical cAMP-PKA pathway. Consistent with the clinical situation, the addition of a β_2-adrenoceptor agonist to a glucocorticoid is steroid-sparing in that maximal GRE-dependent responses, evoked by glucocorticoid, are achieved at ~10-fold lower concentrations in the presence of β_2-adrenoceptor agonist. Finally, analysis of dexamethasone-inducible genes, including glucocorticoid-inducible leucine zipper (GILZ), aminopeptidase N, FKBP51, PAI-1, tristetraprolin, DNB5, p57KIP2, metallothionein 1X, and MKP-1, revealed enhanced inducibility of some genes by glucocorticoid/β_2-adrenoceptor agonist combinations in a manner that was consistent with the GRE-reporter. Because such effects also occur in primary human airway smooth muscle cells, we propose that enhancement of glucocorticoid-inducible gene expression may contribute to the superior efficacy of LABA/ICS combination therapies, over ICS alone, in asthma treatment.

Asthma is a chronic inflammatory disease that is characterized by reversible airways obstruction, airways remodelling, and nonspecific airways hyper-responsiveness. As with many inflammatory diseases, glucocorticoids (corticosteroids) represent the most effective therapy currently available for asthma control (Barnes, 2006). These drugs, typically in an inhaled form and referred to as inhaled corticosteroids (ICSs), control symptoms, reduce exacerbations, and improve health status in most asthmatics irrespective of disease severity (Barnes, 2006). Accordingly, ICSs are recommended as first-line therapy for patients who use a β_2-adrenoceptor agonist inhaler more than once a day (Barnes, 2006). The efficacy of ICSs is due primarily to the suppression of airways inflammation and associated airways hyper-responsiveness. Acting via the glucocorticoid receptor (GR), glucocorticoids repress the expression of inflammatory cytokines, their re-

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ABBREVIATIONS: ICS, inhaled corticosteroid; GR, glucocorticoid receptor; LABA, long-acting β_2-adrenoceptor agonist; PKA, cAMP-dependent protein kinas; GRE, glucocorticoid response element; HASM, human airway smooth muscle; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SFM, serum-free medium; ICI 118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride; PG, prostaglandin; 8-Br-cAMP, 8-bromo-cAMP; MOI, multiplicity of infection; PCR, polymerase chain reaction; ICI 118,551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride; PKI, protein kinase inhibitor; GILZ, glucocorticoid-inducible leucine zipper; TTP, tristetraprolin; MT1X, metallothionein 1X; MKP, mitogen-activated protein kinase phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FKBP51, FK508 binding protein 51.

ceptors, adhesion molecules and other disease-inducing mediators (Barnes, 2006; Newton and Holden, 2007). These effects, combined with the promotion of apoptosis in many cell types, including the eosinophil, reduce the pulmonary leukocyte burden and attenuate airway inflammation.

Despite most patients being well controlled by ICSs, a significant proportion taking low to moderate doses remain symptomatic. In this context, clinical evidence shows that the addition of an inhaled long-acting β_2-adrenoceptor agonist (LABA) to a regular ICS is more effective at improving asthma control, lung function, and reducing exacerbation frequency than increasing, even quadrupling, the dose of ICS (Greening et al., 1994; Woolcock et al., 1996; Pauwels et al., 1997; Shrewsbury et al., 2000; O'Byrne et al., 2005). Thus, by augmenting the effects of ICS, LABAs are glucocorticoid-sparing (Barnes, 2002). On this basis, patients on ICS with uncontrolled asthma are recommended treatment options that stress the addition of a LABA in fixed LABA/ICS combination inhaler devices such as Symbicort (formoterol/budesonide) and Advair (salmeterol/fluticasone) (Barnes, 2006). More recently, similar clinical benefit has been reported in chronic obstructive pulmonary disease, suggesting that LABA/ICS combination therapies may have general efficacy in controlling airways inflammation (Calverley et al., 2003, 2007). Furthermore, recent data suggest that the combination of formoterol/budesonide in a single inhaler can be used as a maintenance *and* rescue therapy, the so-called Symbicort Maintenance And Reliever Therapy (SMART) approach to asthma management (O'Byrne et al., 2005). Indeed, the SMART approach to the treatment of moderate to severe asthma is gaining general acceptance and is already recommended as a treatment option in Canada, Australia, and the European Union (O'Byrne, 2007).

Despite the therapeutic advantage of LABA/ICS combination therapies, a mechanistic basis for their superior efficacy over ICS alone remains vague. According to traditional dogma, agonism of β_2-adrenoceptors by LABAs augments the activity of adenylyl cyclase, leading to cAMP accumulation, activation of cAMP-dependent protein kinase (PKA), and the induction of functional responses such as airway smooth muscle relaxation (Giembycz and Newton, 2006). It is well established that glucocorticoids can augment β_2-adrenoceptor-mediated signaling and the mechanisms involved are well studied (Giembycz and Newton, 2006). In contrast, how LABAs enhance GR-dependent responses is largely unexplored. Given that the induction of anti-inflammatory effector genes is believed to a play an important role in the anti-inflammatory effects of glucocorticoids (Abraham and Clark, 2006; Newton and Holden, 2007), it is possible that LABAs may somehow enhance glucocorticoid response element (GRE)-dependent transcription. In the present study, we have investigated this potential interaction using human bronchial epithelial cells and primary human airway smooth muscle (HASM) cells as model systems. These cell types were selected on the basis that they elaborate a plethora of bioactive lipids, chemokines, cytokines and profibrotic mediators, and, accordingly, are primary targets for the anti-inflammatory actions of ICSs (Schwiebert et al., 1996; Barnes, 2006).

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 supplemented with 10% fetal calf serum (FCS). Culture of airway smooth muscle cells was established from normal human lung tissue, which was obtained via a tissue retrieval service and with local ethics board approval. In brief, bronchi were dissected to expose the smooth muscle bundles. Smooth muscle was cut from the surrounding connective tissue and placed into 1 ml of DMEM supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 20 μg/ml penicillin, 20 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 1 mg/ml collagenase (all Sigma, Oakville, Ontario) before incubation at 37°C in 5% CO_2/95% air for 30 to 45 min. The enzymatically separated cells were then placed in flasks containing 3 ml of DMEM supplemented with 10% FCS, 2 mM L-glutamine, 20 μg/ml penicillin, 20 μg/ml streptomycin, and 2.5 μg/ml amphotericin B and incubated at 37°C 5% CO_2/95% air. A further 2 ml of medium was added after 3 to 4 days; thereafter, the medium was replaced every 5 days. HASM cells between passages 3 and 8 were used for experiments. In all cases, the cells (BEAS-2B and HASM) were incubated for 24 h in serum-free medium (SFM) before treatments.

Dexamethasone (9α-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) as a 2-hydroxypropyl-β-cyclodextrin complex (Sigma, St. Louis, MO) was dissolved in Hanks' balanced salt solution (Sigma). Forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one), salmeterol [(±)-4-hydroxy-a1-[[[6-(4-phenylbutoxy)hexyl]amino]*m*-ethyl]-1,3-benzenedimethanol xinafoate], salbutamol (α-([*t*-butylamino]methyl)-4-hydroxy-*m*-xylene-α, α'-diol), formoterol ((R*,R*)-*N*-[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxyphenyl)-1-methylethyl]amino]ethyl]phenyl]formamide fumarate), rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone), ICI 118,551, budesonide (16,17-butylidenebis(oxy)-11,21-dihydroxypregna-1,4-diene-3,20-dione), fluticasone propionate ((6α,11β,16α,17α)-6,9-difluoro-11-hydroxy-16-methyl-3-oxo-17-(1-oxopropoxy)androsta-1,4-diene-17-carbothioic acid *S*-(fluoromethyl) ester) (fluticasone) (all from Sigma) were dissolved in dimethyl sulfoxide (Sigma). Prostaglandin (PG) E_2 (Sigma) and 8-bromo-cAMP (8-Br-cAMP) (Sigma) were dissolved in ethanol or distilled H_2O, respectively. Final concentrations of dimethyl sulfoxide or ethanol did not exceed 0.1% (v/v) and at this concentration, there was no effect on either GRE-dependent transcription or CRE-dependent transcription (data not shown).

Reporter Cell Lines and Luciferase Assay. Stable transfection was used to generate CRE and GRE BEAS-2B reporter cell lines as described previously (Chivers et al., 2004). BEAS-2B cells containing the CRE reporter, pADneo2-C6-BGL, which contains six tandem CRE motif repeats upstream of a minimal β-globin site, have been previously characterized (Meja et al., 2004). The GRE dependent reporter pGL3.neo.TATA.2GRE contains two copies of a consensus GRE site (sense strand, 5′-TGT ACA GGA TGT TCT-3′) positioned upstream of a minimal β-globin promoter driving a luciferase gene and a separate neomycin gene to confer resistance to G-418 (Chivers et al., 2004). In addition, the parent plasmid, pGL3.neo.TATA, which contains no GRE sites, and a reporter, pGL3.neo.TATA2GRE(mut), containing two copies of a mutated GRE consensus (mutations are indicated by underlined lower case letters) (sense strand, 5′-T<u>ca</u> ACA GGA T<u>ca</u> TCT-3′) were as described previously (Chivers et al., 2004). In each case, BEAS-2B cells at ~70% confluence in T162 flasks were transfected with 8 μg of plasmid DNA and 20 μl of Lipofectamine 2000 (Invitrogen, Burlington, ON, Canada). After 24 h, G-418 selection (0.1 mg/ml) was commenced until foci of stable transfectants appeared. The 2× GRE reporter pGL3.neo.TATA.2GRE was introduced into HASM cells by electroporation. Approximately 1×10^6 HASM cells in DMEM supplemented with 10% FCS were placed with 10 μg of plasmid DNA on ice for 10 min in 4-mm Gene Pulser cuvettes (Bio-Rad Laboratories, Mississauga, ON, Canada). Cells were electroshocked at 200 V and 950 μF for 1 s in a Gene Pulser II

electroporator connected to a capacitance extender (both Bio-Rad) before seeding in T75 cell culture flasks containing 15 ml of media (DMEM, 10% FCS). After 24 h, the media was replaced with fresh media supplemented with 0.4 mg/ml G-418 until foci of stable transfectants appeared. For both BEAS-2B and HASM cells, the foci were harvested to create heterogeneous populations of cells in which the site of integration was randomized. For experiments, confluent cells in 24-well plates were incubated in SFM, without G-418, for 24 h before treatments. Cells were harvested 6 h after treatments in 1× reporter lysis buffer (Promega, Madison, WI), and luciferase activity was measured using a BD Monolight Luminometer (BD Biosciences, San Diego, CA).

Adenoviral Infection. Subconfluent (~70%) BEAS-2B cells were infected at a multiplicity of infection (MOI) of 30 with either an empty Ad5 viral vector (null) or an Ad5 vector that expresses PKIα from a CMV promoter (Meja et al., 2004). After 24 h, media was changed to SFM for a further 24 h before experiments. At this MOI, PKIα was expressed in >95% of cells and activation of CRE-dependent transcription by salbutamol, 8-Br-cAMP, or rolipram was prevented (Meja et al., 2004).

Microarray Analysis. Total RNA was prepared as described previously and assayed for quality using RNA LabChips (Agilent Technologies, Palo Alto, CA) (Chivers et al., 2006). RNA (5 μg) was reverse-transcribed to generate cDNA and subsequently transcribed in vitro to generate biotin-labeled cRNA before fragmentation and hybridization with the GeneChip expression arrays (human genome U95Av2 and B arrays) as specified by the manufacturer (Affymetrix Inc., Santa Clara, CA). The array was subsequently washed and stained with a streptavidin-phycoerythrin–conjugated anti-biotin to visualize hybridized cRNA, and the GeneChip was scanned to quantify gene expression. After global normalization, analysis was performed using the P-FOLD algorithm for Bayesian estimation of -fold changes (Theilhaber et al., 2001).

RNA Isolation, Reverse Transcription and Real-Time PCR. Total RNA was extracted using the RNeasy Mini Kit (QIAGEN, Mississauga, ON, Canada). RNA (1 μg) was reverse-transcribed as described previously (Chivers et al., 2004). Resultant cDNA was diluted 1:5 in RNase-free water and stored at 4°C. Real-time PCR analysis was performed with an ABI 7900HT instrument (Applied Biosystems Inc., Foster City, CA) on 2.5 μl of cDNA in 20-μl reactions using Syber GreenER chemistry (Invitrogen) according to manufacturer's guidelines. Relative cDNA concentrations were determined from a cDNA standard curve that was analyzed simultaneously with the test samples. Amplification conditions were: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C, 15 s; 60°C, 1 min. Dissociation (melt) curves (95°C, 15 s; 60°C, 20 s; with ramping to 95°C over 20 min; 95°C, 15 s) were constructed to confirm primer specificity. Primers (Table 1) were designed using primer express software (Applied Biosystems).

TABLE 1

Primers for real-time PCR

Forward and reverse primers for each gene are listed. Common gene names are provided and gene symbols appear in brackets.

MKP-1 (DUSP1)	
Forward	5′-GCT CAG CCT TCC CCT GAG TA-3′
Reverse	5′-GAT ACG CAC TGC CCA GGT ACA-3′
p57KIP2 (CDKN1C)	
Forward	5′-CGG CGA TCA AGA AGC TGT C-3′
Reverse	5′-GGC TCT AAA TTG GCT CAC CG-3′
Dnb5 (SLC45A)	
Forward	5′-TGC TGG GGC ATG TGT ATC TA-3′
Reverse	5′-AGA GCA GCG AGT AAG GCA AG-3′
Metallothionein 1X (MT1X)	
Forward	5′-GAT CGG GAA CTC CTG CTT CT-3′
Reverse	5′-CTT GTC TGA CGT CCC TTT GC-3′
GAPDH	
Forward	5′-TTC ACC ACC ATG GAG AAG GC-3′
Reverse	5′-AGG AGG CAT TGC TGA TGA TCT-3′

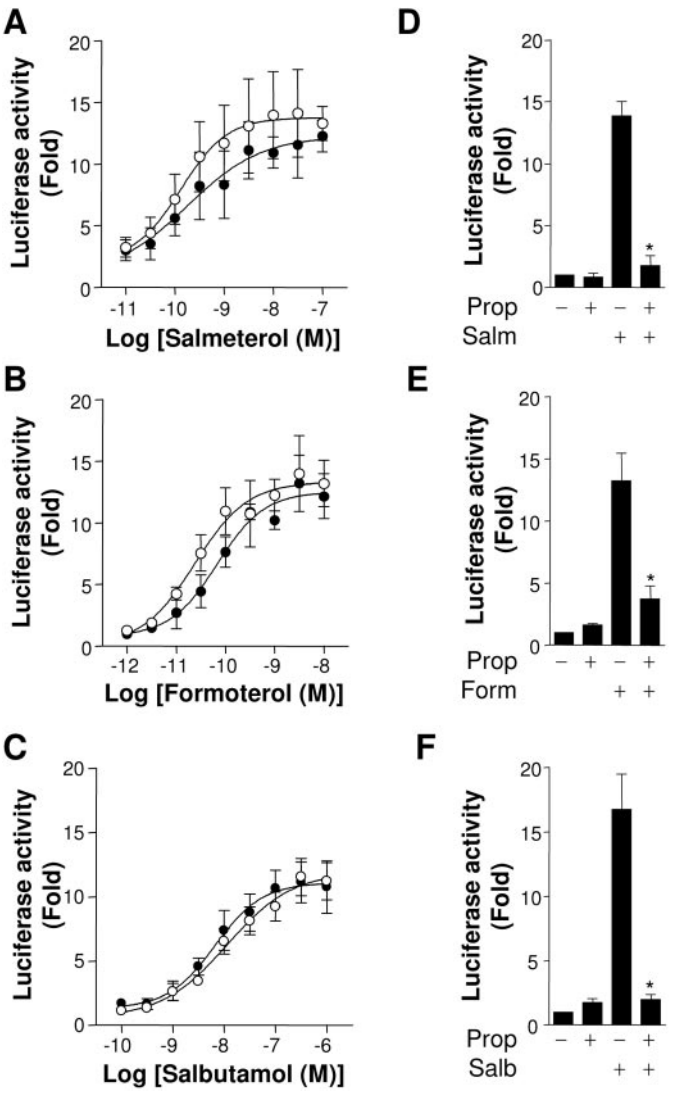

Fig. 1. Effect of dexamethasone on β_2-adrenoceptor-mediated activation of CRE-dependent transcription in human BEAS-2B bronchial epithelial cells. BEAS-2B 6×CRE reporter cells were treated with various concentrations of salmeterol (A), formoterol (B), and salbutamol (C) in the absence (○) and presence of dexamethasone (1 μM) (●). Alternatively, cells were preincubated for 30 min with 0.5 μM propranolol (Prop) before treating with salmeterol (D; 100 nM) (Salm), formoterol (E; 10 nM) (Form), and salbutamol (F; 1 μM) (Salb). Cells were harvested after 6 h for luciferase activity determination. Data (n = 6–7 for A–C; n = 3 for D–F) expressed as -fold induction are plotted as mean ± S.E.

TABLE 2

Effect of β_2-adrenoceptor agonist/glucocorticoid combinations on CRE-dependent transcription

As described in figure 1, CRE BEAS-2B reporter cells were treated with salmeterol (10 pM to 100 nM), salbutamol (100 pM to 1 μM) and formoterol (1 pM to 10 nM) in the absence and presence of dexamethasone (1 μM). After 6 h, cells were harvested for luciferase activity determination. pEC_{50} values and the -fold activation (over basal) were determined for each β_2-adrenoceptor agonist in the presence or absence of dexamethasone. See Figure 1 and text for further details.

Treatment	N	pEC_{50}	CRE Activation
		M	*-fold*
Salmeterol	6	−9.88 ± 0.30	12.5 ± 1.43
Salmeterol + Dexamethasone	6	−9.87 ± 0.50	10.7 ± 1.70
Formoterol	7	−10.62 ± 0.14	13.3 ± 2.02
Formoterol + Dexamethasone	7	−10.16 ± 0.32	12.5 ± 1.36
Salbutamol	6	−7.94 ± 0.28	11.9 ± 1.68
Salmeterol + Dexamethasone	6	−8.18 ± 0.32	11.1 ± 1.73

Curve Fitting and Statistical Analysis. Agonist concentration-response curves were fitted by least-squares, nonlinear iterative regression to the following form of the Hill equation [using Prism 4 (GraphPad Software Inc., San Diego, CA)]: $E = E_{min} + [(E_{max} - E_{min})/1 + 10^{(pEC_{50}) - p[A])^n}]$, where E is the effect, E_{min} and E_{max} are the lower and upper asymptote (i.e., the basal response and maximum agonist-induced response, respectively), p[A] is the log molar concentration of agonist, pEC_{50} is a location parameter equal to the log molar concentration of agonist producing $E_{max}/2$, and n is the gradient of the concentration-response curve at the pEC_{50} level.

Data are presented as mean ± S.E. Multiple comparisons were analyzed by analysis of variance with a Bonferroni post test. Alternatively, paired t test was used as appropriate. The null hypothesis was rejected when $P < 0.05$. Significance is indicated as *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

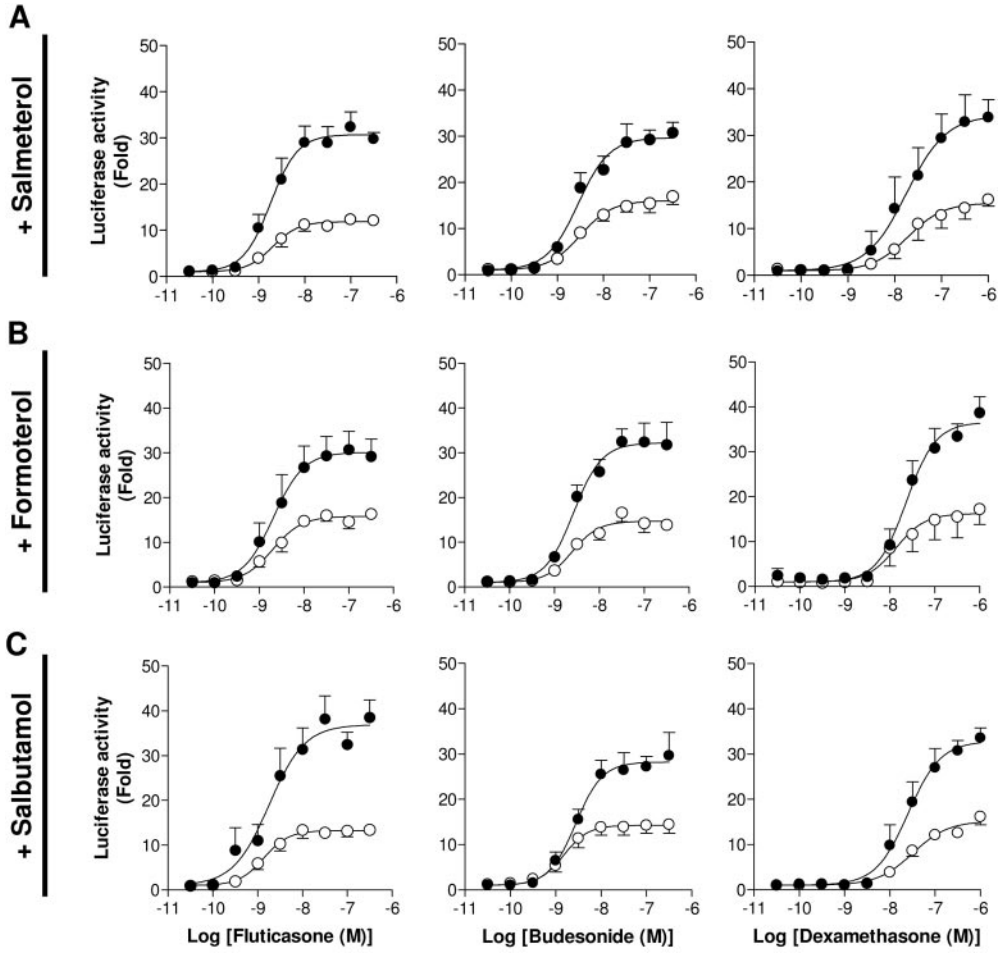

Fig. 2. β_2-Adrenoceptor agonists enhance GRE-dependent transcription. BEAS-2B 2×GRE reporter cells were treated with various concentrations of fluticasone, budesonide or dexamethasone in the absence (○) or presence (●) of the β_2-adrenoceptor agonists salmeterol (A; 100 nM), formoterol (B; 10 nM) and salbutamol (C; 1 μM). Cells were harvested after 6 h for luciferase activity determination. Data (n = 4–6) expressed as -fold induction are plotted as mean ± S.E.

TABLE 3

Effect of β_2-adrenoceptor agonist/glucocorticoid combinations on GRE-dependent transcription

As described in Fig. 2, GRE BEAS-2B cells were treated with various concentrations of fluticasone (30 pM to 300 nM), budesonide (30 nM to 300 nM) and dexamethasone (30 nM to 1 μM) in the absence and presence of fixed concentrations of salmeterol (100 nM), salbutamol (1 μM), and formoterol (10 nM). After 6 h, cells were harvested for luciferase activity determination. pEC_{50} values were calculated for each glucocorticoid in the presence or absence of each β_2-adrenoceptor agonist. Overall activation of the GRE reporter and the enhancement evoked by each β_2-adrenoceptor over that produced by the glucocorticoid alone are shown. See Fig. 2 and text for further details.

Treatments		N	pEC_{50} ± S.E.	GRE Activation	Enhancement
Glucocorticoid	β_2-AdrenoceptorAgonist				
			M	*-fold*	*-fold*
Fluticasone		18	−8.69 ± 0.08	13.8	
	+Salmeterol	6	−8.74 ± 0.19	31.8**	2.30
	+ Formoterol	6	−8.70 ± 0.19	29.9*	2.16
	+Salbutamol	6	−8.65 ± 0.17	37.1**	2.68
Budesonide		17	−8.65 ± 0.05	15.4	
	+Salmeterol	6	−8.56 ± 0.09	30.0*	1.94
	+ Formoterol	6	−8.57 ± 0.05	34.7*	2.25
	+Salbutamol	5	−8.54 ± 0.05	28.3***	1.84
Dexamethasone		16	−7.73 ± 0.07	14.5	
	+Salmeterol	6	−7.75 ± 0.11	34.4**	2.38
	+ Formoterol	4	−7.57 ± 0.10	37.7*	2.61
	+Salbutamol	6	−7.60 ± 0.15	32.9*	2.27

Results

Effect of β_2-Adrenoceptor Agonists and Glucocorticoids on CRE-Dependent Transcription. To examine the effect of glucocorticoids on CRE-dependent transcription, concentration-response curves were constructed to a panel of β_2-adrenoceptor agonists, using BEAS-2B cells stably transfected with a 6×CRE reporter, in the absence and presence of a concentration (1 μM) of dexamethasone that maximally promotes GRE-dependent transcription (Fig. 1). In each case, salmeterol, formoterol, and salbutamol activated CRE-dependent transcription. This effect was concentration-dependent and resulted in a 11- to 13-fold induction of luciferase activity over basal levels (Table 2). Induction of CRE reporter activity by maximally effective concentrations of salmeterol, formoterol, and salbutamol was significantly repressed by the antagonist, propranolol (0.5 μM), and confirms that these effects are β-adrenoceptor-mediated (Fig. 1, D–F). In each case, the EC_{50} values and maximum-fold induction of luciferase evoked by salmeterol, formoterol, and salbutamol were the same irrespective of whether the experiments were conduced in the absence or presence of dexamethasone (Table 2; Fig. 1, A–C)

Enhancement of Glucocorticoid-Dependent Transcription by β_2-Adrenoceptor Agonists. To examine GRE-dependent transcription, BEAS-2B cells were stably transfected with a 2×GRE reporter (pGL3.neo.TATA.2GRE), a reporter with two mutated GRE sites (pGL3.neo.TATA.2GREmut) and the parent construct containing just a TATA box driving luciferase expression (pGL3.neo.TATA). After treatment with dexamethasone, the 2×GRE reporter was activated in a concentration-dependent manner (Fig. 2). In contrast, dexamethasone activated neither the basal TATA construct nor the mutated 2×GRE construct, indicating that responsiveness was dependent on the presence of the GRE sites (data not shown).

To test the effect of β_2-adrenoceptor agonists on GRE-dependent transcription, 2×GRE reporter cells were treated with various concentrations of the glucocorticoids, fluticasone, budesonide, and dexamethasone in the absence and presence of maximally effective concentrations of salmeterol (100 nM), formoterol (10 nM), or salbutamol (1 μM) (Fig. 2). Whereas salmeterol, formoterol, and salbutamol alone were inactive, fluticasone, budesonide, and dexamethasone induced GRE-dependent transcription to a similar degree (13- to 15-fold) with EC_{50} values of 2, 2.2, and 18 nM, respectively (Fig. 2; Table 3). In contrast, concurrent treatment of 2×GRE BEAS-2B cells with a glucocorticoid and a β_2-adrenoceptor agonist significantly enhanced GRE-dependent transcription without changing the potency of glucocorticoid (Fig. 2; Table 3). This effect was not restricted to LABAs nor was it peculiar to the β_2-adrenoceptor agonist/glucocorticoid combination therapies that are used clinically (i.e., Advair and Symbicort). Indeed, as shown in Fig. 2, nine different β_2-adrenoceptor agonist/glucocorticoid combinations were tested; in every case, GRE-dependent transcription was augmented by approximately 2-fold (see Table 3 for full details). Thus, β_2-adrenoceptor agonists synergistically enhance GRE-dependent transcription in BEAS-2B cells.

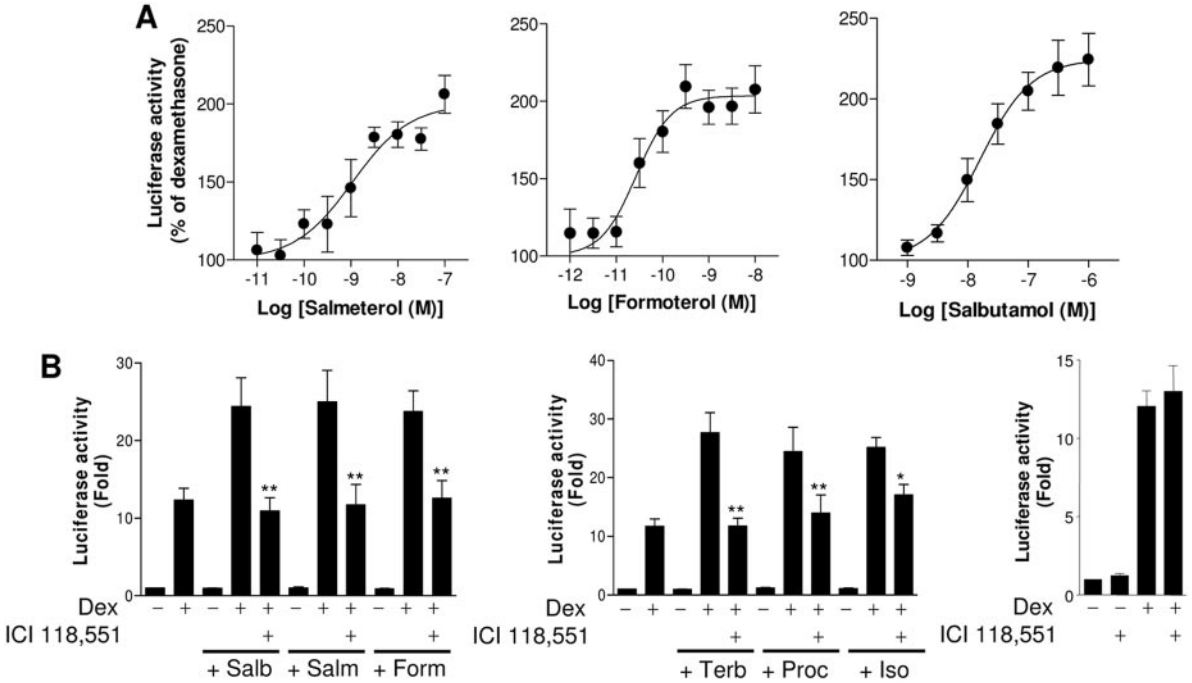

Fig. 3. Enhancement of GRE-dependent transcription by β_2-adrenoceptor agonists is concentration-dependent and antagonized by ICI 118,551. A, BEAS-2B 2×GRE reporter cells were stimulated with dexamethasone (1 μM) in the presence of various concentrations of salmeterol, formoterol, or salbutamol. Cells were harvested after 6 h for luciferase activity determination. Data (n = 5–10) expressed as a percentage of dexamethasone treatment alone and are plotted as mean ± S.E. B, BEAS-2B 2xGRE cells were pretreated for 30 min with ICI 118,551 (2 μM) before stimulation with dexamethasone (1 μM) in the absence and presence of salbutamol (1 μM; Salb), salmeterol (100 nM; Salm), formoterol (10 nM; Form), terbutaline (10 μM; Terb), procaterol (1 μM; Proc), and isoprenaline (1 μM; Iso) as indicated. The effect of ICI 118,551 on unstimulated and dexamethasone-induced reporter activity is shown (right). Cells were harvested as before for luciferase activity determination. Data (left; n = 5, middle; n = 4, right; n = 9) expressed -fold induction are plotted as mean ± S.E. Significance between glucocorticoid alone and glucocorticoid + β_2-adrenoceptor agonist is indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

In the presence of a maximally effective concentration of dexamethasone (1 μM), salmeterol, formoterol, and salbutamol enhanced GRE-dependent transcription in a concentration-dependent manner with EC_{50} values of 1.1 nM, 31.8 pM, and 166 nM, respectively (Fig. 3A). For each β_2-adrenoeceptor agonist/dexamethasone combination, the maximum induction of GRE-dependent transcription was approximately twice that evoked by the glucocorticoid alone (Fig. 3B), and this general effect was confirmed with terbutaline (10 μM), procaterol (1 μM), and isoprenaline (1 μM) (Fig. 3B). The ability of all β_2-adrenoceptor agonists tested to enhance dexamethasone-induced GRE-dependent transcription was abolished in cells pretreated with the selective β_2-adrenoceptor antagonist ICI 118,551 (2 μM) (Fig. 3B).

Effect of Other cAMP-Elevating Agents and a cAMP Mimetic on Glucocorticoid-Induced, GRE-Dependent Transcription. Treatment of BEAS-2B cells with PGE_2 (1 μM), rolipram (30 μM), forskolin (10 μM), and 8-Br-cAMP (1 mM) did not activate GRE-dependent transcription at concentrations that were near maximal for stimulating a CRE reporter construct (Meja et al., 2004). In contrast, treatment of 2×GRE BEAS-2B cells with PGE_2, rolipram, forskolin, or 8-Br-cAMP concurrently with dexamethasone (1 μM) enhanced GRE-dependent transcription by 2.5-, 1.8-, 3.0-, and 1.8-fold, respectively (Fig. 4A). This effect was concentration-dependent for both 8-Br-cAMP and forskolin with EC_{50} values of 1.03 ± 0.15 mM and 310 ± 28 nM, respectively (Fig. 4B). Thus, other cAMP-elevating drugs also interacted synergistically with glucocorticoids to potentiate GRE-dependent transcription.

A Role for PKA in the Enhancement of GRE-Dependent Transcription by Formoterol. To examine a possible role for PKA in the enhancement of GRE-dependent transcription by β_2-adrenoceptor agonists, we have taken advantage of the adenoviral vector Ad5.CMV.PKIα. This construct directs overexpression of the highly selective inhibitor of PKA, PKIα, which has no effect on cGMP-dependent protein kinase (Meja et al., 2004). At an MOI of 30, PKIα is expressed in >95% of BEAS-2B cells and CRE-dependent transcription induced by 8-Br-cAMP, forskolin, rolipram, PGE_2, or salbu-

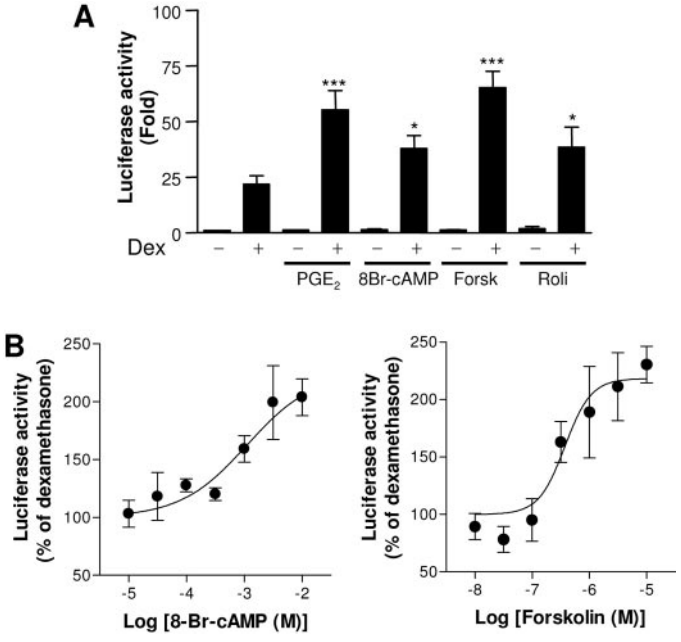

Fig. 4. Enhancement of glucocorticoid-induced GRE-dependent transcription by other cAMP-elevating agents and a cAMP mimetic. A, BEAS-2B 2xGRE reporter cells were stimulated with dexamethasone (1 μM) in the absence and presence of PGE_2 (1 μM), 8-Br-cAMP (1 mM), forskolin (10 μM) (Forsk), or rolipram (30 μM) (Roli). Cells were harvested after 6 h for luciferase activity determination. Data (n = 8), expressed as a percentage of the stimulation by dexamethasone are plotted as mean ± S.E. B, BEAS-2B 2×GRE reporter cells were stimulated with dexamethasone (1 μM) in the presence of various concentrations of 8-Br-cAMP or forskolin. Cells were harvested after 6 h for luciferase activity determination. Data (left panel, n = 2–4; right panel, n = 5), expressed as a percentage of the stimulation by dexamethasone, are plotted as mean ± S.E. Significance between glucocorticoid alone and glucocorticoid + β_2-adrenoceptor agonist is indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

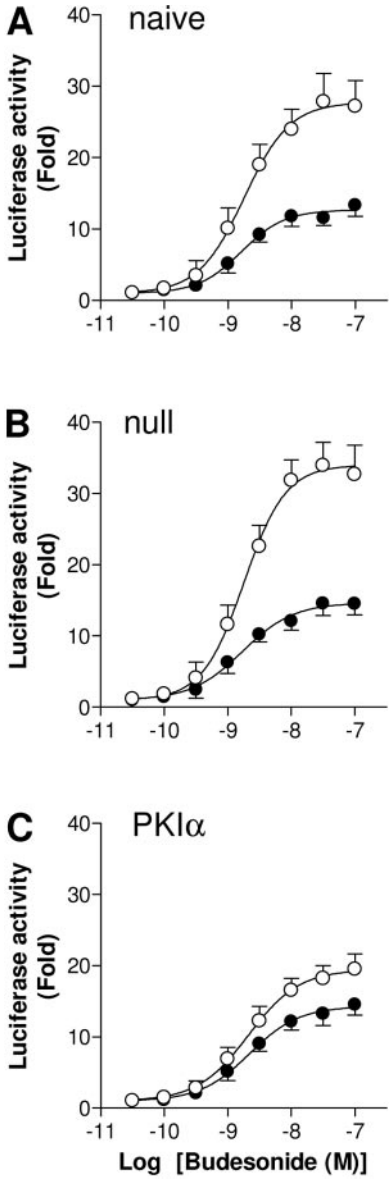

Fig. 5. Effect of PKIα on the enhancement of budesonide-induced GRE-dependent transcription by formoterol. BEAS-2B 2xGRE reporter cells were either not incubated with adenoviral expression vector (naive) (A), incubated with empty Ad5 vector at 30 MOI (null) (B), or incubated with Ad5.CMV.PKIα at 30 MOI (PKIα) (C). Cells were subsequently stimulated with various concentrations of budesonide in the absence and presence of formoterol (10 nM). After 6 h, cells were harvested for luciferase activity determination. Data (A, n = 8; B, n = 9; C, n = 9), expressed as -fold induction, are plotted as mean ± S.E.

tamol is prevented (Meja et al., 2004). As described in Fig. 2, GRE-dependent transcription induced by budesonide (300 nM) was enhanced 2.4-fold by the addition of formoterol (Fig. 5, Table 4). In BEAS-2B cells infected with the null virus, a significant 2.3-fold enhancement was still observed. However, in cells expressing the PKIα transgene, the ability of formoterol to enhance budesonide-induced transcription was reduced by >90%, and the residual effect was not significantly different from the effect of budesonide alone (Fig. 5, Table 4). It is noteworthy that the viral vector alone had no significant effect on activation of the 2×GRE reporter induced by glucocorticoid.

Effect of Salmeterol and Forskolin on Glucocorticoid-Induced Gene Expression. The above data indicate that β_2-adrenoceptor agonists and other agents that elevate intracellular cAMP can enhance glucocorticoid-dependent transcription from a conventional 2×GRE reporter. To examine this effect with respect to real genes, we have taken advantage of a prior microarray analysis in which dexamethasone-inducible genes were identified in pulmonary type II A549 cells (data not shown). Depending on the time point, this analysis revealed some 100 to 350 Affymetrix probe sets that showed greater than 2-fold inducibility by dexamethasone (data not shown). Of these, 9 genes, represented by 14 well annotated probe sets and showed robust induction by dexamethasone (Table 5), were confirmed as being dexamethasone-inducible (data not shown). The expression of these genes was then tested using real-time RT-PCR on cDNA from BEAS-2B cells that had been treated with dexamethasone (1 μM) in the absence or presence of forskolin (10 μM) or salmeterol (100 nM) as a representative LABA.

As shown in Supplemental Fig. 1, dexamethasone did not induce aminopeptidase N in the first 2 h after dexamethasone treatment, but by 6 and 18 h, gene inductions were 3.1- and 5.3-fold, respectively. Likewise, FK508 binding protein 51 (FKBP51) was not induced after a 1-h dexamethasone treatment, whereas stimulations of 3.9- to 11.7-fold were detected at 2, 6, and 18 h. Glucocorticoid-inducible leucine zipper (GILZ) and plasminogen activator inhibitor 1 revealed inductions by dexamethasone of 20.1- to 54.0-fold and 5.6- to 6.8-fold, respectively, at all times tested. Finally, dexamethasone-induced tristetraprolin (TTP) mRNA by 5.5-fold at 1 h before declining to 2.6-fold at 18 h. However, the induction of these genes by dexamethasone was unaffected by either forskolin or salmeterol (Supplemental Fig. 1).

In contrast, deleted in neuroblastoma 5 (DNB5), the cyclin-

TABLE 4

Effect of PKIα on the enhancement by formoterol of budesonide-induced GRE-dependent transcription

As shown in Fig. 5, GRE BEAS-2B cells were infected with Ad5. CMV. PKIα (PKIα), an empty vector, Ad5. CMV (null) or left untreated (naive). Cells were treated with various concentrations of budesonide (30 pM–100 nM) in the absence and presence of a fixed concentration of formoterol (10 nM). After 6 h, cells were harvested for luciferase activity determination. pEC_{50} values were calculated for budesonide in the presence or absence of formoterol. Overall 2×GRE reporter activation and the β_2-adrenoceptor-dependent enhancement over the glucocorticoid alone was also determined.

Treatment		N	pEC_{50} ± S.E.	GRE Activation	Enhancement
Virus	Drugs				
			M	*-fold*	*-fold*
Naive	Bud	4	−8.62 ± 0.12	12.2	
	Bud+form	4	−8.58 ± 0.10	30.1*	2.4
Null	Bud	5	−8.78 ± 0.23	17.1	
	Bud+form	5	−8.82 ± 0.20	39.7***	2.3
PKIα	Bud	5	−8.75 ± 0.23	15.7	
	Bud+form	5	−8.74 ± 0.21	18.4	1.1

TABLE 5

Selected dexamethasone-inducible genes identified in A549 cells

A549 cells were either not treated or treated with dexamethasone (1 μM) for 2, 4, 6 or 18 h. RNA was prepared and microarray analysis performed using recommended Affymetrix methods. Data (n = 3), analyzed using the P-fold algorithm, are presented as relative fold induction (R), verses not treated at each time point. P values are indicated (P).

Gene symbol (common name)	Accession No. (Ref Seq)	Affymetrix ID	2 h		4 h		6 h		18 h	
			-fold	P	-fold	P	-fold	P	-fold	P
TSC22D3 (GILZ)	NM_001015881 NM_004089 NM_198057	36629_AT	6.7	<0.001	10.2	<0.001	18.9	<0.001	3.47	0.0022
FKBP5 (FKBP51)	NM_004116	46271_AT	4.6	0.0025	9.16	<0.001	10.8	<0.001	9.46	<0.001
FKBP5 (FKBP51)	NM_004116	34721_AT	2.15	0.177	5.71	0.0011	10	0.0003	12	<0.001
SERPINE1 (PAI-1)	NM_000602	672_AT	2.81	0.112	2.86	0.0237	9.41	0.0038	2.92	0.154
SERPINE1 (PAI-1)	NM_000602	38125_AT	1.54	0.126	1.96	0.0442	3.09	0.0047	3.3	0.0039
CDKN1C (p57KIP2)	NM_000076	39545_AT	1.3	0.369	4.56	0.0034	8.73	<0.001	7.97	<0.001
CDKN1C (p57KIP2)	NM_000076	1787_AT	1.28	0.418	4.02	0.0523	8.65	0.0026	5.73	0.0149
DUSP1 (MKP-1)	NM_004417	1005_AT	9.07	<0.001	4.91	<0.001	7.7	0.001	3.03	0.0047
SLC45A1 (DNB5)	XM_001129279 XM_937695	47478_AT	1.93	0.273	5.93	0.0257	6.11	0.0092	4.99	0.0304
TSC22D3 (GILZ)	NM_001015881 NM_004089 NM_198057	36630_AT	3.94	0.0441	2.52	0.168	3.95	0.014	5.29	0.0082
ANPEP (Aminopeptidase N, CD13)	NM_001150	39385_AT	1.04	0.487	2.1	0.231	3.59	0.104	5.4	0.0094
CDKN1C (p57KIP2)	NM_000076	38673_S_AT	1.33	0.404	3.12	0.0999	3.68	0.0656	3.98	0.0529
MT1X (metallothionein 1X)	NM_005952	39120_AT	2.62	0.0148	2.62	0.0148	4.68	<0.001	4.68	<0.001
ZFP36 (TTP, TIS11)	NM_003407	40448_AT	3.5	0.0021	3.32	0.0032	3.06	0.0053	2.07	0.0331

SLC45A1, solute carrier family 45, member 1; TIS, TPA-inducible sequence.

dependent kinase inhibitor, p57KIP2 (p57^{Kip2}), metallothionein 1X (MT1X), and mitogen-activated protein kinase phosphatase (MKP)-1 all showed some form of combinatorial interaction between dexamethasone and the cAMP-elevating agents (Fig. 6A). Thus, DNB5 was modestly induced by dexamethasone at 1 and 2 h. This reached a maximum of 5.0-fold at 6 h and was maintained at 18 h. There was no effect of forskolin or salmeterol at any time point except 18 h, at which the induction by dexamethasone (5.6-fold) was augmented to 12.4- and 9.5-fold, respectively (although this latter effect did not reach statistical significance). Likewise, the expression of MT1X was unaffected by forskolin or salmeterol acting alone. In addition, the expression induced by dexamethasone at 1 and 2 h, 2.0- and 3.9-fold, respectively, was also unaffected by either forskolin or salmeterol. However, at 6 h, dexamethasone increased MT1X expression by 9.6-fold and this response was significantly enhanced by both forskolin (17.6-fold) and salmeterol (16.7-fold). Despite a slight decline in the overall induction, this enhancement was maintained to 18 h. In contrast, the induction of p57KIP2 by dexamethasone was synergistically enhanced by both forskolin and salmeterol. This supra-additive effect was most profound at 1 and 2 h, lower in magnitude at 6 h until, at 18 h, the response was solely dexamethasone-dependent. Finally, MKP-1 was dexamethasone-inducible (6.6- –11.0-fold) at all times tested. Unlike the other genes examined, both forskolin and salmeterol induced MKP-1 expression 9.0- and 7.1-fold, respectively, at 1 h and this effect had waned dramatically by 2 h. In combination, dexamethasone and either forskolin or salmeterol produced a significant enhancement of MKP-1 expression over any drug alone at 1 h (21.3- and 20.5-fold, respectively, versus 11.0-fold for dexamethasone alone). However, this interaction was of a purely additive nature and was lost by 2 h after treatment.

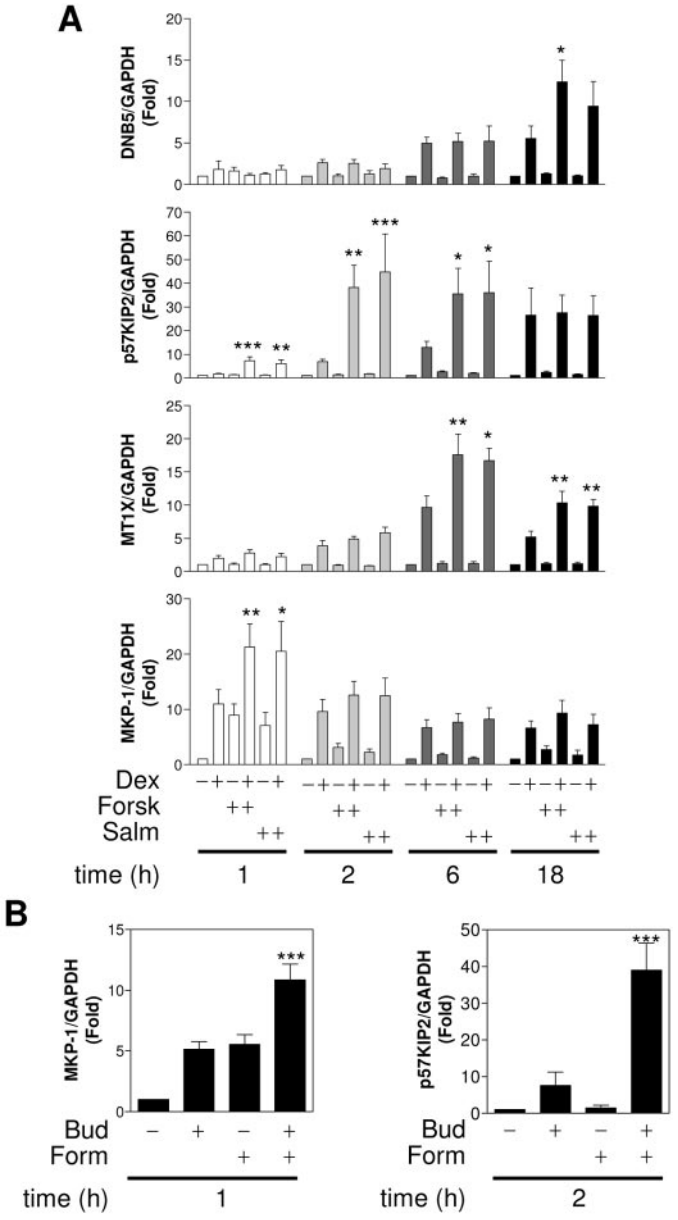

Fig. 6. Effect of LABAs and forskolin on the expression of glucocorticoid-inducible genes. A, BEAS-2B cells were treated with dexamethasone (1 μM) (Dex) in the absence and presence of forskolin (10 μM) (Forsk) or salmeterol (100 nM) (Salm). Cells were harvested at the times indicated and RNA was extracted. After cDNA synthesis real-time RT-PCR was performed for DNB5, p57KIP2, MT1X, MKP-1, and GAPDH. Data (n = 4 - 7), normalized to GAPDH and expressed as -fold stimulation at each time point, are plotted as means ± S.E. See main text for abbreviations. B, BEAS-2B cells were treated with budesonide (1 μM) in the absence or presence of formoterol (10 nM). Cells were harvested after 1 or 2 h as indicated and RNA was extracted. After cDNA synthesis, real-time RT-PCR was performed for MKP-1 or p57KIP2 and GAPDH. Data (n = 6 for MKP-1 and 4 for p57KIP2), normalized to GAPDH and expressed as -fold stimulation at each time-point, are plotted as means ± S.E.

To confirm that these findings are representative for multiple β_2-adrenoceptor agonist/glucocorticoid combinations, BEAS-2B cells were treated with budesonide, formoterol or the combination of both (Fig. 6B). After a 1-h treatment, MKP-1 mRNA expression was strongly induced by either budesonide or formoterol and, in combination, these compounds produced an additive increase in MKP-1 expression. Likewise, the expression of p57KIP2 was examined 2 h after treatment. As was described for dexamethasone and salmeterol (above), budesonide induced p57KIP2 expression by 7.6-fold, yet formoterol was without effect (Fig. 6B). In combination, budesonide plus formoterol resulted in a 39.0-fold increase in p57KIP2 expression and confirming synergy with a further β_2-adrenoceptor agonist/glucocorticoid combination.

Enhancement of Glucocorticoid-Dependent Transcription by β_2-Adrenoceptor Agonists and Forskolin in HASM. The above data suggest that β_2-agonists, and other cAMP-elevating agents, can enhance GRE-dependent transcription in BEAS-2B bronchial epithelial cells. To examine whether this observation applies more generally to other cell types, a stable GRE-dependent reporter cell line was generated in primary HASM cells. As described for BEAS-2B cells, the 2×GRE reporter revealed robust activation by dexamethasone (1 μM), but was unaffected by salbutamol (1 μM), salmeterol (100 nM), formoterol (10 nM), terbutaline (10 μM), isoprenaline (1 μM), procaterol (1 μM), or forskolin (10 μM) (Fig. 7A). In combination with dexamethasone each β_2-adrenoceptor agonist and forskolin resulted in significant ~2-fold enhancements of the response to dexamethasone. Although ICI 118,551 showed no effect on the response to dexamethasone alone, the synergistic enhancement by each β_2-adrenoceptor agonist was prevented, and this indicates specificity for the β_2-adrenoceptor.

To further confirm transcriptional synergy, the expression of p57KIP2 was examined in primary HASM cells that had

been treated with dexamethasone (1 μM), salmeterol (100 nM), forskolin (10 μM), or combinations thereof (Fig. 7B). In these experiments, dexamethasone-inducibility of p57KIP2 was less pronounced than in BEAS-2B cells. However, the addition of either forskolin or salmeterol to dexamethasone led to significant increases in the expression of p57KIP2 at all time points analyzed. Once again, this effect is one of synergy and confirms the prior finding from BEAS-2B cells.

Discussion

Greening et al. (1994), demonstrated that poorly controlled asthmatics on low-dose ICS (400 μg/day beclomethasone) showed little improvement when the dose of ICS was increased to 1000 μg/day, yet the addition of salmeterol to the ICS markedly improved lung function and asthma control. Confirmation of this response in more severe asthmatic subjects (Woolcock et al., 1996), and a *meta*-analysis of nine independent studies support this finding and indicate the existence of a genuine biological phenomenon (Shrewsbury et al., 2000). Explanations for this effect include two generic possibilities: 1) LABAs and ICS activate distinct processes to produce additive effects; 2) LABAs and ICS activate processes that combine to produce common synergistic effects. In this context, LABAs, as a monotherapy in vivo are not considered to be anti-inflammatory, which argues against mechanisms that combine to elicit additive effects (Roberts et al., 1999; Howarth et al., 2000). In contrast, exacerbation rates and asthma severity are reduced to a greater extent in asthmatics taking formoterol/budesonide combination than in those patients taking budesonide alone (Pauwels et al., 1997). This is consistent with synergistic interactions, whereby LABAs enhance the efficacy of ICSs to a level that cannot be achieved by ICS alone. Thus, rather than masking the underlying inflammation by increasing symptomatic relief, LABAs enhance the anti-inflammatory effectiveness of ICS (Pauwels et al., 1997).

Given that airway epithelial cells are a key target for the anti-inflammatory effects of ICS, the effect of β_2-adrenoceptor agonists and glucocorticoids was initially investigated on human bronchial epithelial BEAS-2B cells. Although glucocorticoid-dependent enhancement of β_2-adrenoceptor agonist signaling is widely reported (Giembycz and Newton, 2006), this may not apply in the epithelium as β_2-adrenoceptor number is not increased by dexamethasone (Aksoy et al., 2002). Thus, CRE-dependent transcription induced by β_2-adrenoceptor agonists was unaffected by dexamethasone (Fig. 1). In contrast, both LABAs and glucocorticoids can repress the expression of inflammatory genes (for example, granulocyte macrophage–colony-stimulating factor, interleukin-8, eotaxin) in cell culture systems (Pang and Knox, 2000, 2001; Korn et al., 2001). However, there is generally little evidence for enhanced effects other than simple additivity (but see Edwards et al., 2006). Although potentially helpful with respect to improving anti-inflammatory effectiveness, these effects are, nevertheless, difficult to reconcile with clinical observations regarding LABA/ICS therapies. Conversely, given evidence for anti-inflammatory effects of glucocorticoids acting via inducible genes (Abraham and Clark, 2006; Newton and Holden, 2007), we examined the hypothesis that β_2-adrenoceptor agonists synergistically enhance glucocorticoid-dependent transcription.

In the above studies, β_2-adrenoceptor agonists, including salmeterol and formoterol, enhanced simple GRE-dependent transcription by between 200 and 300% without affecting the potency of the glucocorticoid. Because β_2-adrenoceptor agonists alone did not activate GRE-dependent transcription, this was a case of synergy and was consistent with the clinical observations. Furthermore, the effect was class-specific because enhancement was observed with six different long- and short-acting β_2-adrenoceptor agonists and three different glucocorticoids. In addition, steroid sparing is evident, as shown for both the formoterol/budesonide and sal-

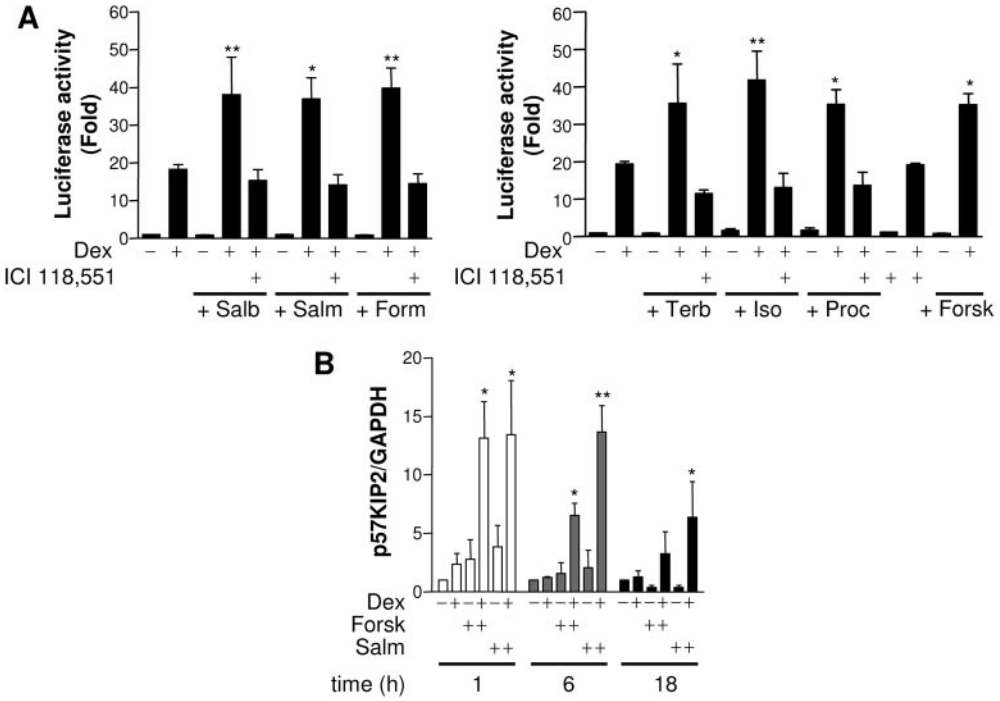

Fig. 7. Enhancement of glucocorticoid-induced transcription by β_2-adrenoceptor agonists and forskolin in HASM cells. A, HASM 2×GRE reporter cells were pretreated for 30 min with ICI 118,551 (2 μM) before stimulation with dexamethasone (1 μM) (Dex) in the absence and presence of salbutamol (1 μM; Salb), salmeterol (100 nM; Salm), formoterol (10 nM; Form), terbutaline (10 μM; Terb), isoprenaline (1 μM; Iso), procaterol (1 μM; Proc), or forskolin (10 μM; Fosk) as indicated. The effect of ICI 118,551 on unstimulated and dexamethasone-induced reporter activity is shown (right). Cells were harvested after 6 h for luciferase activity determination. Data (n = 3 or 4) expressed -fold induction are plotted as mean ± S.E. B, primary HASM cells were treated with dexamethasone (1 μM) in the absence and presence of forskolin (10 μM) (Forsk) or salmeterol (100 nM) (Salm). Cells were harvested at the times indicated and RNA was extracted. After cDNA synthesis, real-time RT-PCR was performed for p57KIP2 and GAPDH. Data (n = 4), normalized to GAPDH and expressed as -fold stimulation at each time-point, are plotted as means ± S.E.

meterol/fluticasone combinations (Fig. 8). Thus, budesonide activates GRE-dependent transcription with an EC_{90} of 18.8 nM. However, in the presence of a maximally effective concentration of formoterol, this level of response was elicited by 2.2 nM budesonide (a ~10-fold lower concentration) (Fig. 8A). Given similar observations for salmeterol/fluticasone (Fig. 8B), these data may explain, if physiological responses in vivo are regulated similarly, both the steroid sparing and the enhanced clinical efficacy of LABA/ICS combination therapies. Furthermore, the fact that β_2-adrenoceptor agonists also enhance dexamethasone-driven transcription in HASM cells argues for a general mechanism found in multiple cell types that are relevant to lung inflammation and asthma.

Like β_2-adrenoceptor agonists, PGE_2, 8-Br-cAMP, forskolin, and rolipram all activate the cAMP/PKA pathway in BEAS-2B cells (Meja et al., 2004). Likewise, these agents enhance dexamethasone-induced, GRE-dependent transcription, indicating a central role for cAMP and confirming the ability of cAMP-elevating drugs to increase the efficacy of glucocorticoids (Nordeen et al., 1994). This proposal is supported by the ability of adenovirus-delivered PKIα to prevent the enhancement of budesonide-induced GRE-dependent transcription by formoterol. Thus, specificity of PKIα for PKA and not cGMP-dependent protein kinase in BEAS-2Bs supports a conventional β_2-adrenoceptor-cAMP-PKA effector pathway that enhances GRE-dependent transcription (Meja et al., 2004).

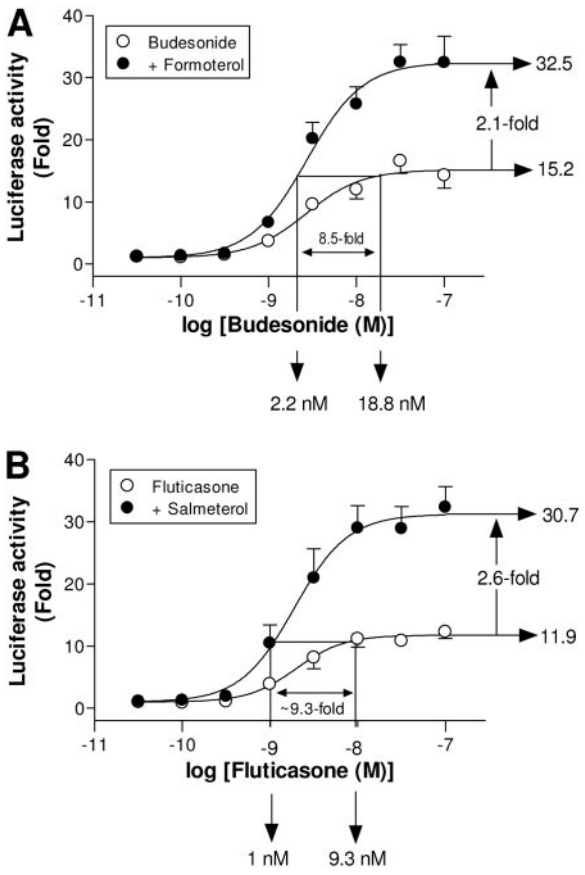

Fig. 8. LABA/ICS combinations are both steroid-sparing and enhance GRE-dependent transcription. The data in Fig. 2 showing the effect of formoterol/budesonide and salmeterol/fluticasone combinations on the activity of a simple 2×GRE reporter construct stably expressed in BEAS-2B cells are redrawn to illustrate that the addition of a LABA to a glucocorticoid is both steroid-sparing and enhances GRE-dependent transcription. In this simple system, neither LABA activated the GRE reporter construct but markedly potentiated glucocorticoid-induced transcription (2.6- and 2.1-fold for salmeterol and formoterol, respectively at the E_{max}). In addition, salmeterol and formoterol were glucocorticoid-sparing in this model. Thus, both fluticasone and budesonide at concentrations that evoked 90% of the maximum response produced a 12- to 15-fold induction of the luciferase gene. However, in the presence of salmeterol (100 nM) or formoterol (10 nM), which were inactive, the same degree of gene induction was achieved at a concentration of glucocorticoid that was significantly (~10-fold) lower. N.B., this measurement was made at the EC_{90} concentration of glucocorticoid (because the upper asymptote, by definition, is never reached) and so the degree to which the LABAs are steroid sparing is under-estimated.

Prior analysis of dexamethasone-inducible genes in pulmonary A549 cells identified candidate genes for subsequent analysis. Thus, dexamethasone inducibility of GILZ and TTP was confirmed in BEAS-2B cells, and this is predicted to produce anti-inflammatory benefit by reducing NF-κB and/or AP-1 activation and reducing stability of AU rich element-containing mRNAs, respectively (Mittelstadt and Ashwell, 2001; Smoak and Cidlowski, 2006; Eddleston et al., 2007). Likewise, aminopeptidase N (ANPEP, CD13), which cleaves neuropeptides (encephalins, neurokinin A), vasoactive peptides (kallidin, angiotensins), and chemoattractants (MCP-1, fMLP), was induced by dexamethasone and may therefore reduce chemotactic responses (Bauvois and Dauzonne, 2006). Likewise, plasminogen activator inhibitor (PAI)-1, a potent inhibitor of the fibrinolytic cascade, may also be important in alveolar cell repair after wounding and glucocorticoid-inducibility could promote repair after injury (Maquerlot et al., 2006). However, although potentially advantageous effects may be ascribed to GILZ, TTP, aminopeptidase N, and plasminogen activator inhibitor 1, no evidence was found for enhancement by salmeterol or forskolin. Conversely, FKBP51, a component of the hsp90-GR complex, which promotes glucocorticoid resistance (Westberry et al., 2006), may provide feedback inhibition such that lack of enhancement by β_2-adrenoceptor agonists is desirable.

Analysis of DNB5 (SLC45A1), p57KIP2, MT1X, and MKP-1 showed induction by dexamethasone and both forskolin and salmeterol further enhanced expression in BEAS-2B cells. In the case of DNB5, a gene whose function is unclear, there was no effect of either salmeterol or forskolin until 18 h. Although this was still a case of synergy, in that neither cAMP-elevating agent showed any effect alone, the response kinetic was delayed relative to the simple GRE reporter and suggested indirect mechanisms of action. In contrast, both MT1X and, in particular, p57KIP2 revealed synergy at times that coincided with reporter activation. In the case of MT1X, which is induced by cytotoxic stresses (Stennard et al., 1994), there are no data supporting a beneficial role in controlling airways inflammation. However, p57KIP2 is a cell cycle kinase inhibitor that is involved in the antiproliferative effects of dexamethasone (Samuelsson et al., 1999). Given epithelial and HASM cell hyperplasia in the remodelling that characterizes asthma, we speculate that enhanced expression of p57KIP2 may be one factor that contributes to the superior therapeutic benefit of LABA/ICS combination therapies relative to ICSs. Indeed, a functional conventional GRE is present in the p57KIP2 promoter, which suggests that LABA/ICS combinations can act synergistically in the induction of real genes, which could be important in modifying airways disease (Alheim et al., 2003). This idea is supported by the finding that p57KIP2 expression is also synergistically enhanced in primary HASM cells. Therefore, these data

provide crucial proof-of-concept by showing that real glucocorticoid-inducible genes may be synergistically induced by a LABA/ICS combination to achieve enhanced benefit and/or steroid-sparing effects in airway cells. This could therefore provide an explanation for synergy observed at the level of repression of gene expression (Edwards et al., 2006).

Lack of enhancement by salmeterol or forskolin on the remaining five glucocorticoid-inducible genes suggests that, unlike the 2×GRE reporter, glucocorticoid-dependent regulation is not primarily mediated via simple GREs. This proposal is consistent with the finding that most glucocorticoid-inducible genes are not associated with conventional GREs (So et al., 2007). For example, glucocorticoid inducibility of MKP-1 may not occur via a simple GRE mechanism because the simple GRE transactivation-defective steroid, RU24858 (Vayssiere et al., 1997), and a dimerization-defective GR mutant both induce expression (Abraham et al., 2006; Chivers et al., 2006). Furthermore, MKP-1 induced by LABAs and forskolin, and these interact with glucocorticoids in a purely additive manner at 1 h. This is of potential benefit in the resolution of inflammation and may, in part, explain the additive ability of LABAs and glucocorticoids to repress inflammatory gene expression (Pang and Knox, 2000, 2001; Korn et al., 2001).

From a mechanistic perspective, there are numerous ways to account for the augmentation of GRE-dependent transcription by β_2-adrenoceptor agonists. One possibility is that β_2-adrenoceptor agonists enhance the translocation of GR to the nucleus (Eickelberg et al., 1999; Usmani et al., 2005). Further support for this assertion is that combination therapies are associated with increased GR DNA binding (Roth et al., 2002). Certainly, this is consistent with reports of enhanced GR DNA binding after PKA overexpression (Rangarajan et al., 1992). Although such actions could contribute to the synergy seen between β_2-adrenoceptror agonists and glucocorticoids, a formal reinterrogation of such potential mechanisms is beyond the scope of the present study.

In conclusion, β_2-adrenoceptor agonists enhance glucocorticoid-induced simple GRE-dependent transcription via the cAMP-PKA pathway in human bronchial epithelial cells. This effect is class-specific effect and occurs with real effector genes. Given equivalent effects in HASM cells, our data provide evidence for a general mechanism of action, which is both steroid sparing and yields levels of glucocorticoid-dependent transcription that cannot be achieved by glucocorticoid alone. These data are consistent with the clinical literature, and we propose that this may help to explain the superior clinical efficacy of LABA/ICS combination therapies in the treatment of asthma and COPD over ICS alone.

Acknowledgments

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