

Long-acting β_2 -adrenoceptor agonists synergistically enhance glucocorticoid-dependent transcription in human airways epithelial and smooth muscle cells

Manminder Kaur, Joanna E. Chivers, Mark A. Giembycz, and Robert Newton

Airway Inflammation Group, Departments of Cell Biology and Anatomy, and Pharmacology and Therapeutics, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada

a) Running Title: β_2 -adrenoceptor agonists enhance GRE-dependent transcription

b) Corresponding Author: Dr. Robert Newton

Address: Department of Cell Biology & Anatomy, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, T2N 4N1, Canada.

Tel: 001 403 210 3938 Fax: 403 270 8928 e-mail: rnewton@ucalgary.ca

c)	Number of Text pages	36 (main text)
	Number of Figures	8 (main text), 1 supplemental
	Number of references	41
	Number of words in abstract	250 (max 250)
	Number of words in introduction	591 (max 750)
	Number of words in discussion	1499 (max 1500)

d) Abbreviations: AHR, airways hyper-responsiveness; CRE, cAMP response element; DMSO, dimethyl sulphoxide; DNB5, deleted in neuroblastoma 5; FKBP51, FK508 binding protein 51; GILZ, glucocorticoid-inducible leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid response element; HASM, human airways smooth muscle; ICS, inhaled corticosteroid; LABA, long-acting β_2 -adrenoceptor agonist; MT1X, metallothionein 1X; MKP-1, mitogen-activated protein kinase phosphatase 1; PAI-1, plasminogen activator inhibitor 1; PCR, polymerase chain reaction; PG, prostaglandin; PKA, cAMP-dependent protein kinase; SE, standard error; SFM, serum-free medium; SMART, Symbicort maintenance and reliever therapy; TTP, tristetraprolin.

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. Since 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 (CRE)-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, 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. Since such effects also occur in primary human airways 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, which is characterised by reversible airways obstruction, airways remodelling and non-specific airways hyper-responsiveness (AHR). 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 AHR. Acting via the glucocorticoid receptor (GR), glucocorticoids repress the expression of inflammatory cytokines, their receptors, 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 airways 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; O'Byrne *et al.*, 2005; Pauwels *et al.*, 1997; Shrewsbury *et al.*, 2000; Woolcock *et al.*, 1996). 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; Calverley *et al.*, 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 airways smooth muscle relaxation (Giembycz and Newton, 2006). It is well established that glucocorticoids can augment β_2 -adrenoceptor-mediated signalling 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 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, in some way, 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 airways smooth muscle (HASM) cells as model systems. These cell types were selected on the basis that they elaborate a plethora of bio-active lipids, chemokines, cytokines and pro-fibrotic mediators, and, accordingly, are primary targets for the anti-inflammatory actions of ICSs (Barnes, 2006; Schwiebert *et al.*, 1996).

Materials and Methods

Cell Culture, Drugs and Stimuli. Human bronchial epithelial BEAS-2B cells were grown to confluence in Dulbecco's modified eagle medium (DMEM)/F12 supplemented with 10% fetal calf serum (FCS). Culture of airways smooth muscle cells was established from normal human lung tissue, which was obtained via a tissue retrieval service and with local ethics approval. Briefly 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% non-essential amino acids, 20 µg/ml of 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% CO₂:95% air for 30 - 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%CO₂:95% air. A further 2 ml of medium was added after 3 – 4 days and thereafter the medium was replaced every 5 days. HASM cells between passages 3 – 8 were utilized for experiments. In all cases, the cells (BEAS-2B and HASM) were incubated for 24 h in serum-free medium (SFM) prior to treatments.

Dexamethasone (9α-Fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione) as a 2-hydroxypropyl-β-cyclodextrin complex (Sigma) was dissolved in Hank's balanced salt solution (Sigma). Forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one), salmeterol ((±) 4-hydroxy-α-1-[[[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 ((±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol hydrochloride), 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 sulphoxide (DMSO) (Sigma). Prostaglandin (PG) E₂ (Sigma) and 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cAMP) (Sigma) were dissolved in ethanol or distilled H₂O respectively. Final concentrations of DMSO 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 previously described (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 both 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 in lower case underlined) (sense strand 5'-Tca ACA GGA Tca TCT-3') were as described (Chivers *et al.*, 2004). In each case, BEAS-2B cells at ~70% confluence in T162 flasks were transfected with 8 μ g of plasmid DNA and 20 μ l of Lipofectamine 2000 (Invitrogen, Burlington, Ontario). After 24 h, G-418 selection (0.1 mg/ml) was commenced until foci of stable transfectants appeared. The 2 \times GRE reporter, pGL3.neo.TATA.2GRE, was introduced into HASM cells by electroporation. Approximately 1 x 10⁶ HASM cells, in DMEM supplemented with 10% FCS, were placed with 10 μ g of plasmid DNA on ice for 10 mins in 4 mm Gene Pulser cuvettes (BioRad, Mississauga, Ontario, Canada). Cells were electroshocked at 200 V and 950 μ F for 1 sec in a Gene Pulser II electroporator connected to a capacitance extender (both BioRad) prior to 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 is randomized. For experiments, confluent cells in 24 well plates were incubated in SFM, without G-418, for 24 h prior to treatments. Cells were harvested 6 h after treatments in 1 x reporter lysis buffer (Promega, Madison, WI) and luciferase activity was measured using a BD Monolight Luminometer (BD Biosciences, San Diego, California).

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

Microarray Analysis. Total RNA was prepared as previously described and assayed for quality using RNA LabChips[®] (Agilent Technologies, California) (Chivers *et al.*, 2006). RNA (5 μ g) was reverse transcribed to generate cDNA and subsequently transcribed *in vitro* to generate biotin labelled cRNA prior to fragmentation and hybridization with the GeneChip[®] expression arrays (Human genome U95Av2 & 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 visualise hybridized cRNA and the GeneChip[®] was scanned to quantify gene expression. Following 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, Ontario). RNA (1 μ g) was reverse transcribed as previously described (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, California) on 2.5 μ l of cDNA in 20 μ l reactions using Syber GreenER chemistry (Invitrogen, Ontario, Canada) 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; followed by 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 (ABI).

Curve Fitting and Statistical Analysis. Agonist concentration-response curves were fitted by least-squares, non-linear iterative regression to the following form of the Hill equation (equation 1) using Prism 4 (GraphPad Software Inc, San Diego, CA):

$$E = E_{min} + \frac{(E_{max} - E_{min})}{1 + 10^{(pEC_{50}) - p[A]}^n} \quad (1)$$

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 \pm standard error (SE). Multiple comparisons were analysed by ANOVA 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 *** $P < 0.001$.

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 \times CRE reporter, in the absence and presence of a concentration (1 μ M) 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 μ M), and confirms that these effects are β -adrenoceptor-mediated (Fig. 1D-F). In each case the EC₅₀ values and maximum fold induction of luciferase evoked by salmeterol, formoterol and salbutamol were the same irrespective of whether the experiments were conducted in the absence or presence of dexamethasone (Table 2; Fig. 1A-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 \times GRE reporter (pGL3.neo.TATA.2GRE), a reporter with 2 mutated GRE sites (pGL3.neo.TATA.2GREmut) and the parent construct containing just a TATA box driving luciferase expression (pGL3.neo.TATA). Following treatment with dexamethasone, the 2 \times GRE reporter was activated in a concentration-dependent manner (Fig. 2). In contrast, dexamethasone activated neither the basal TATA construct, nor the mutated 2 \times 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 \times 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 μ M) (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 EC₅₀ 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 β₂-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 β₂-adrenoceptor agonist/glucocorticoid combination therapies that are used clinically (i.e. *Advair* and *Symbicort*). Indeed, as shown in figure 2, nine different β₂-adrenoceptor agonist/glucocorticoid combinations were tested and in every case GRE-dependent transcription was augmented by approximately two-fold (see Table 3 for full details). Thus, β₂-adrenoceptor agonists synergistically enhance GRE-dependent transcription in BEAS-2B cells.

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 EC₅₀ values of 1.1 nM, 31.8 pM and 166 nM respectively (Fig. 3A). For each β₂-adrenoceptor 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 β₂-adrenoceptor agonists tested to enhance dexamethasone-induced GRE-dependent transcription was abolished in cells pre-treated with the selective β₂-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 PGE₂ (1 μM), rolipram (30 μM), forskolin (10 μM) and 8-Br-cAMP (1 mM) did not activate GRE-dependent transcription at concentrations that are near maximal for stimulating a CRE reporter construct (Meja *et al.*, 2004). In contrast, treatment of 2×GRE BEAS-2B cells with PGE₂, rolipram, forskolin or 8-Br-cAMP concurrently with dexamethasone (1 μM) enhanced GRE-dependent transcription 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 interact 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.PKI α . This construct directs over-expression of the highly selective inhibitor of PKA, PKI α , which has no effect on cGMP-dependent protein kinase (PKG) (Meja *et al.*, 2004). At a MOI of 30, PKI α is expressed in > 95 % of BEAS-2B cells and CRE-dependent transcription induced by 8-Br-cAMP, forskolin, rolipram, PGE₂ or salbutamol 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 PKI α 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). Importantly, the viral vector alone had no significant effect on activation of the 2 \times 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 classical 2 \times GRE reporter. To examine this effect in respect of 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 - 350 Affymetrix probe sets that showed greater than 2-fold inducibility by dexamethasone (data not shown). Of these, 9 genes, which were 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 μ M) in the absence or presence of forskolin (10 μ M), 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 following 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 following 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 (PAI-1) revealed inductions by dexamethasone of 20.1 to 54.0 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 (DNB) 5, the cyclin-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). Similarly, 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- to 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, vs 11.0-fold for dexamethasone alone). However, this interaction was of a purely additive nature and was lost by 2 h post-treatment.

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). Following 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. Similarly, 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 suggests 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 cells types, a stable GRE-dependent reporter cell line was generated in primary HASM cells. As described for BEAS-2B cells, the 2 \times GRE reporter revealed robust activation by dexamethasone (1 μ M), but was unaffected by salbutamol (1 μ M), salmeterol (100 nM), formoterol (10 nM), terbutaline (10 μ M), isoprenaline (1 μ M), procaterol (1 μ M) 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. Whilst 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 ($1 \mu\text{M}$), salmeterol (100 nM), forskolin ($10 \mu\text{M}$) 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 and this argues against mechanisms that combine to elicit additive effects (Howarth *et al.*, 2000; Roberts *et al.*, 1999). 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).

As 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. While glucocorticoid-dependent enhancement of β_2 -adrenoceptor agonist signalling 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 GM-CSF, IL-8, eotaxin) in cell culture systems (Korn *et al.*, 2001; Pang and Knox, 2000;

Pang and Knox, 2001). However, there is generally little evidence for enhanced effects other than simple additivity (but see (Edwards *et al.*, 2006). While potentially helpful in respect of 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 to 300% without affecting the potency of the glucocorticoid. Since β_2 -adrenoceptor agonists alone did not activate GRE-dependent transcription, this is a case of synergy and is consistent with the clinical observations. Furthermore, the effect is class-specific as 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 is shown for both the formoterol/budesonide and salmeterol/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, if physiological responses *in vivo* are regulated similarly, explain 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). Similarly, 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 adenoviral-delivered PKI α to prevent the enhancement of budesonide-induced GRE-dependent transcription by formoterol. Thus, specificity of PKI α for PKA, and not PKG in BEAS-2Bs supports a classical β_2 -adrenoceptor-cAMP-PKA effector pathway that enhances GRE-dependent transcription (Meja *et al.*, 2004).

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- κ B and/or AP-1 activation and reducing stability of AU rich element-containing mRNAs respectively (Eddleston *et al.*, 2007; Mittelstadt and Ashwell, 2001; Smoak and Cidlowski, 2006). Similarly, 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 following wounding and glucocorticoid-inducibility could promote repair following injury (Maquerlot *et al.*, 2006). However, whilst potentially advantageous effects may be ascribed to GILZ, TTP, aminopeptidase N and PAI-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. Whilst this was still a case of synergy, as neither cAMP-elevating agent showed any effect alone, the response kinetic is delayed relative to the simple GRE reporter and suggests indirect mechanisms of action. In contrast, both MT1X and, particularly, 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 airway inflammation. However, p57KIP2 is a cell cycle kinase inhibitor, which is involved in the anti-proliferative effects of dexamethasone (Samuelsson *et al.*, 1999). Given epithelial and HASM cell hyperplasia in the remodelling that characterises 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 classical GRE is present in the p57KIP2 promoter and this 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 airways 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 classical GREs (So *et al.*, 2007). For example, glucocorticoid inducibility of MKP-1 may not occur via a simple GRE mechanism since the simple GRE transactivation-defective steroid, RU24858, 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 (Korn *et al.*, 2001; Pang and Knox, 2000; Pang and Knox, 2001).

Mechanistically, 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 following PKA over-expression (Rangarajan *et al.*, 1992). Whilst such actions could contribute to the synergy seen between β_2 -adrenoceptor agonists and glucocorticoids a formal re-interrogation 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

We thank the Sanofi-Aventis Genomics Center (Bridgewater, NJ) for conducting the Affymetrix microarray analysis and Anna Miller-Larsson for critical evaluation of the manuscript.

References

- Abraham SM and Clark AR (2006) Dual-Specificity Phosphatase 1: a Critical Regulator of Innate Immune Responses. *Biochem Soc Trans* **34**: 1018-1023.
- Abraham SM, Lawrence T, Kleiman A, Warden P, Medghalchi M, Tuckermann J, Saklatvala J, and Clark AR (2006) Antiinflammatory Effects of Dexamethasone Are Partly Dependent on Induction of Dual Specificity Phosphatase 1. *J Exp Med* **203**: 1883-1889.
- Aksoy MO, Mardini IA, Yang Y, Bin W, Zhou S, and Kelsen SG (2002) Glucocorticoid Effects on the Beta-Adrenergic Receptor-Adenylyl Cyclase System of Human Airway Epithelium. *J Allergy Clin Immunol* **109**: 491-497.
- Alheim K, Corness J, Samuelsson MK, Bladh LG, Murata T, Nilsson T, and Okret S (2003) Identification of a Functional Glucocorticoid Response Element in the Promoter of the Cyclin-Dependent Kinase Inhibitor P57Kip2. *J Mol Endocrinol* **30**: 359-368.
- Barnes PJ (2002) Scientific Rationale for Inhaled Combination Therapy With Long-Acting Beta2-Agonists and Corticosteroids. *Eur Respir J* **19**: 182-191.
- Barnes PJ (2006) Corticosteroids: the Drugs to Beat. *Eur J Pharmacol* **533**: 2-14.
- Bauvois B and Dauzonne D (2006) Aminopeptidase-N/CD13 (EC 3.4.11.2) Inhibitors: Chemistry, Biological Evaluations, and Therapeutic Prospects. *Med Res Rev* **26**: 88-130.
- Calverley PM, Anderson JA, Celli B, Ferguson GT, Jenkins C, Jones PW, Yates JC, and Vestbo J (2007) Salmeterol and Fluticasone Propionate and Survival in Chronic Obstructive Pulmonary Disease. *N Engl J Med* **356**: 775-789.
- Calverley PM, Boonsawat W, Cseke Z, Zhong N, Peterson S, and Olsson H (2003) Maintenance Therapy With Budesonide and Formoterol in Chronic Obstructive Pulmonary Disease. *Eur Respir J* **22**: 912-919.

- Chivers JE, Cambridge LM, Catley MC, Mak JC, Donnelly LE, Barnes PJ, and Newton R (2004) Differential Effects of RU486 Reveal Distinct Mechanisms for Glucocorticoid Repression of Prostaglandin E Release. *Eur J Biochem* **271**: 4042-4052.
- Chivers JE, Gong W, King EM, Seybold J, Mak JC, Donnelly LE, Holden NS, and Newton R (2006) Analysis of the Dissociated Steroid, RU24858, Does Not Exclude a Role for Inducible Genes in the Anti-Inflammatory Actions of Glucocorticoids. *Mol Pharmacol* **70**: 2084-2095.
- Eddleston J, Herschbach J, Wagelie-Steffen AL, Christiansen SC, and Zuraw BL (2007) The Anti-Inflammatory Effect of Glucocorticoids Is Mediated by Glucocorticoid-Induced Leucine Zipper in Epithelial Cells. *J Allergy Clin Immunol* **119**: 115-122.
- Edwards MR, Johnson MW, and Johnston SL (2006) Combination Therapy: Synergistic Suppression of Virus-Induced Chemokines in Airway Epithelial Cells. *Am J Respir Cell Mol Biol* **34**: 616-624.
- Eickelberg O, Roth M, Lorx R, Bruce V, Rudiger J, Johnson M, and Block LH (1999) Ligand-Independent Activation of the Glucocorticoid Receptor by Beta2-Adrenergic Receptor Agonists in Primary Human Lung Fibroblasts and Vascular Smooth Muscle Cells. *J Biol Chem* **274**: 1005-1010.
- Giembycz MA and Newton R (2006) Beyond the Dogma: Novel {Beta}2-Adrenoceptor Signalling in the Airways. *Eur Respir J* **27**: 1286-1306.
- Greening AP, Ind PW, Northfield M, and Shaw G (1994) Added Salmeterol Versus Higher-Dose Corticosteroid in Asthma Patients With Symptoms on Existing Inhaled Corticosteroid. Allen & Hanburys Limited UK Study Group. *Lancet* **344**: 219-224.
- Howarth PH, Beckett P, and Dahl R (2000) The Effect of Long-Acting Beta2-Agonists on Airway Inflammation in Asthmatic Patients. *Respir Med* **94 Suppl F**: S22-S25.
- Korn SH, Jerre A, and Brattsand R (2001) Effects of Formoterol and Budesonide on GM-CSF and IL-8 Secretion by Triggered Human Bronchial Epithelial Cells. *Eur Respir J* **17**: 1070-1077.

- Maquerlot F, Galiacy S, Malo M, Guignabert C, Lawrence DA, d'Ortho MP, and Barlovatz-Meimon G (2006) Dual Role for Plasminogen Activator Inhibitor Type 1 As Soluble and As Matricellular Regulator of Epithelial Alveolar Cell Wound Healing. *Am J Pathol* **169**: 1624-1632.
- Meja KK, Catley MC, Cambridge LM, Barnes PJ, Lum H, Newton R, and Giembycz MA (2004) Adenovirus-Mediated Delivery and Expression of a CAMP-Dependent Protein Kinase Inhibitor Gene to BEAS-2B Epithelial Cells Abolishes the Anti-Inflammatory Effects of Rolipram, Salbutamol, and Prostaglandin E2: a Comparison With H-89. *J Pharmacol Exp Ther* **309**: 833-844.
- Mittelstadt PR and Ashwell JD (2001) Inhibition of AP-1 by the Glucocorticoid-Inducible Protein GILZ. *J Biol Chem* **276**: 29603-29610.
- Newton R and Holden NS (2007) Separating Transrepression and Transactivation: A Distressing Divorce for the Glucocorticoid Receptor? *Mol Pharmacol* ..
- Nordeen SK, Moyer ML, and Bona BJ (1994) The Coupling of Multiple Signal Transduction Pathways With Steroid Response Mechanisms. *Endocrinology* **134**: 1723-1732.
- O'Byrne PM (2007) Acute Asthma Intervention: Insights From the STAY Study. *J Allergy Clin Immunol* **119**: 1332-1336.
- O'Byrne PM, Bisgaard H, Godard PP, Pistolesi M, Palmqvist M, Zhu Y, Ekstrom T, and Bateman ED (2005) Budesonide/Formoterol Combination Therapy As Both Maintenance and Reliever Medication in Asthma. *Am J Respir Crit Care Med* **171**: 129-136.
- Pang L and Knox AJ (2000) Synergistic Inhibition by Beta(2)-Agonists and Corticosteroids on Tumor Necrosis Factor-Alpha-Induced Interleukin-8 Release From Cultured Human Airway Smooth-Muscle Cells. *Am J Respir Cell Mol Biol* **23**: 79-85.
- Pang L and Knox AJ (2001) Regulation of TNF-Alpha-Induced Eotaxin Release From Cultured Human Airway Smooth Muscle Cells by Beta2-Agonists and Corticosteroids. *FASEB J* **15**: 261-269.

- Pauwels RA, Lofdahl CG, Postma DS, Tattersfield AE, O'Byrne P, Barnes PJ, and Ullman A (1997) Effect of Inhaled Formoterol and Budesonide on Exacerbations of Asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group. *N Engl J Med* **337**: 1405-1411.
- Rangarajan PN, Umesono K, and Evans RM (1992) Modulation of Glucocorticoid Receptor Function by Protein Kinase A. *Mol Endocrinol* **6**: 1451-1457.
- Roberts JA, Bradding P, Britten KM, Walls AF, Wilson S, Gratziau C, Holgate ST, and Howarth PH (1999) The Long-Acting Beta2-Agonist Salmeterol Xinafoate: Effects on Airway Inflammation in Asthma. *Eur Respir J* **14**: 275-282.
- Roth M, Johnson PR, Rudiger JJ, King GG, Ge Q, Burgess JK, Anderson G, Tamm M, and Black JL (2002) Interaction Between Glucocorticoids and Beta2 Agonists on Bronchial Airway Smooth Muscle Cells Through Synchronised Cellular Signalling. *Lancet* **360**: 1293-1299.
- Samuelsson MK, Pazirandeh A, Davani B, and Okret S (1999) P57Kip2, a Glucocorticoid-Induced Inhibitor of Cell Cycle Progression in HeLa Cells. *Mol Endocrinol* **13**: 1811-1822.
- Schwiebert LM, Stellato C, and Schleimer RP (1996) The Epithelium As a Target of Glucocorticoid Action in the Treatment of Asthma. *Am J Respir Crit Care Med* **154**: S16-S19.
- Shrewsbury S, Pyke S, and Britton M (2000) Meta-Analysis of Increased Dose of Inhaled Steroid or Addition of Salmeterol in Symptomatic Asthma (MIASMA). *BMJ* **320**: 1368-1373.
- Smoak K and Cidlowski JA (2006) Glucocorticoids Regulate Tristetraprolin Synthesis and Posttranscriptionally Regulate Tumor Necrosis Factor Alpha Inflammatory Signaling. *Mol Cell Biol* **26**: 9126-9135.
- So AY, Chaivorapol C, Bolton EC, Li H, and Yamamoto KR (2007) Determinants of Cell- and Gene-Specific Transcriptional Regulation by the Glucocorticoid Receptor. *PLoS Genet* **3**: e94.
- Stennard FA, Holloway AF, Hamilton J, and West AK (1994) Characterisation of Six Additional Human Metallothionein Genes. *Biochim Biophys Acta* **1218**: 357-365.

- Theilhaber J, Bushnell S, Jackson A, and Fuchs R (2001) Bayesian Estimation of Fold-Changes in the Analysis of Gene Expression: the PFOLD Algorithm. *J Comput Biol* **8**: 585-614.
- Usmani OS, Ito K, Maneechotesuwan K, Ito M, Johnson M, Barnes PJ, and Adcock IM (2005) Glucocorticoid Receptor Nuclear Translocation in Airway Cells After Inhaled Combination Therapy. *Am J Respir Crit Care Med* **172**: 704-712.
- Westberry JM, Sadosky PW, Hubler TR, Gross KL, and Scammell JG (2006) Glucocorticoid Resistance in Squirrel Monkeys Results From a Combination of a Transcriptionally Incompetent Glucocorticoid Receptor and Overexpression of the Glucocorticoid Receptor Co-Chaperone FKBP51. *J Steroid Biochem Mol Biol* **100**: 34-41.
- Woolcock A, Lundback B, Ringdal N, and Jacques LA (1996) Comparison of Addition of Salmeterol to Inhaled Steroids With Doubling of the Dose of Inhaled Steroids. *Am J Respir Crit Care Med* **153**: 1481-1488.

Footnotes

a) Dr Newton is a Canadian Institutes of Health Research (CIHR) New Investigator and Alberta Heritage Foundation for Medical Research (AHFMR) Scholar. Dr. Giembycz is an AHFMR Senior Scholar. The current study was funded in part by a research award from AstraZeneca and a NHLI Foundation Studentship. Work in Dr Newton's and Dr. Giembycz's laboratories is also supported by CIHR, AHFMR and research awards from Altana and GlaxoSmithKline.

b) Address correspondence to: Dr. Robert Newton, Department of Cell Biology & Anatomy, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, T2N 4N1, CANADA

e-mail: rnewton@ucalgary.ca

Legends for Figures

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 \times CRE reporter cells were treated with various concentrations of **A**, salmeterol, **B** formoterol and **C**, salbutamol in the absence (\circ) and presence of dexamethasone (1 μ M) (\bullet). Alternatively, cells were pre-incubated for 30 min with 0.5 μ M propranolol (Prop) before treating with **D**, salmeterol (100 nM) (Salm), **E**, formoterol (10 nM) (Form) and **F**, salbutamol (1 μ M). Cells were harvested after 6 h for luciferase activity determination. Data (n = 6 - 7 for **A**, **B** & **C**, and n = 3 for **D**, **E** & **F**) expressed as fold induction are plotted as mean \pm SE.

Fig 2. β_2 -adrenoceptor agonists enhance GRE-dependent transcription. BEAS-2B 2 \times GRE reporter cells were treated with various concentrations of fluticasone, budesonide or dexamethasone in the absence (\circ) or presence (\bullet) of the β_2 -adrenoceptor agonists **A**, salmeterol (100 nM), **B**, formoterol (10 nM) and **C**, salbutamol (1 μ M). Cells were harvested after 6 h for luciferase activity determination. Data (n = 4 - 6) expressed as fold induction are plotted as mean \pm SE.

Fig 3. Enhancement of GRE-dependent transcription by β_2 -adrenoceptor agonists is concentration-dependent and antagonised by ICI 118,551. **A**, BEAS-2B 2 \times GRE reporter cells were stimulated with dexamethasone (1 μ M) 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 \pm SE. **B**, BEAS-2B 2 \times GRE cells were pre-treated for 30 min with ICI 118,551 (2 μ M) prior to stimulation with dexamethasone (1 μ M) in the absence and presence of salbutamol (1 μ M) (Salb), salmeterol (100 nM) (Salm), formoterol (10 nM) (Form), terbutaline (10 μ M) (Terb), procaterol (1 μ M) (Proc) and isoprenaline (1 μ M) (Iso) as indicated. The effect of ICI 118,551 on unstimulated and dexamethasone-

induced reporter activity is shown (right panel). Cells were harvested as before for luciferase activity determination. Data (left panel; n = 5, middle panel; n = 4, right panel; n = 9) expressed fold induction are plotted as mean \pm SE.

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 μ M) in the absence and presence of PGE₂ (1 μ M), 8-Br-cAMP (1 mM), forskolin (10 μ M) (Forsk) or rolipram (30 μ M) (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 \pm SE. **B**, BEAS-2B 2xGRE reporter cells were stimulated with dexamethasone (1 μ M) 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 \pm SE.

Fig 5. Effect of PKI α on the enhancement of budesonide-induced GRE-dependent transcription by formoterol. BEAS-2B 2xGRE reporter cells were either; **A**, not incubated with adenoviral expression vector (naive), **B**, incubated with empty Ad5 vector at 30 MOI (null), or **C**, incubated with Ad5.CMV.PKI α at 30 MOI (PKI α). 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 \pm SE.

Fig 6. Effect of LABAs and forskolin on the expression of glucocorticoid-inducible genes. **A**, BEAS-2B cells were treated with dexamethasone (1 μ M) (Dex) in the absence and presence of forskolin (10 μ M) (Forsk) or salmeterol (100 nM) (Salm). Cells were harvested at the times indicated and RNA was extracted. Following 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 \pm SE. See main text for abbreviations. **B**, BEAS-2B cells were treated with budesonide (1 μ M) in the absence or presence of formoterol (10 nM). Cells were harvested after 1 or 2 h as indicated and RNA was extracted. Following 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 \pm SE.

Fig. 7. Enhancement of glucocorticoid-induced transcription by β_2 -adrenoceptor agonists and forskolin in HASM cells. **A**, HASM 2 \times GRE reporter cells were pre-treated for 30 min with ICI 118,551 (2 μ M) prior to stimulation with dexamethasone (1 μ M) (Dex) in the absence and presence of salbutamol (1 μ M) (Salb), salmeterol (100 nM) (Salm), formoterol (10 nM) (Form), terbutaline (10 μ M) (Terb), isoprenaline (1 μ M) (Iso), procaterol (1 μ M) (Proc) or forskolin (10 μ M) (Fosk) as indicated. The effect of ICI 118,551 on unstimulated and dexamethasone-induced reporter activity is shown (right panel). Cells were harvested after 6 h for luciferase activity determination. Data (n = 3 or 4) expressed fold induction are plotted as mean \pm SE. **B**, Primary HASM cells were treated with dexamethasone (1 μ M) in the absence and presence of forskolin (10 μ M) (Forsk) or salmeterol (100 nM) (Salm). Cells were harvested at the times indicated and RNA was extracted. Following 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 \pm SE.

Fig. 8. LABA/ICS combinations are both steroid-sparing and enhance GRE-dependent transcription. The data in figure 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 re-drawn 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 (since the upper asymptote, by definition, is never reached) and so the degree to which the LABAs are steroid sparing is under-estimated.

Tables

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'

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 μ M) and formoterol (1 pM to 10 nM) in the absence and presence of dexamethasone (1 μ M). After 6 h cells were harvested for luciferase activity determination. pEC₅₀ 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	<i>N</i>	pEC ₅₀ (M)	CRE Activation (Fold)
Salmeterol	6	-9.88 (\pm 0.30)	12.5 (\pm 1.43)
Salmeterol + Dexamethasone	6	-9.87 (\pm 0.50)	10.7 (\pm 1.70)
Formoterol	7	-10.62 (\pm 0.14)	13.3 (\pm 2.02)
Formoterol + Dexamethasone	7	-10.16 (\pm 0.32)	12.5 (\pm 1.36)
Salbutamol	6	-7.94 (\pm 0.28)	11.9 (\pm 1.68)
Salmeterol + Dexamethasone	6	-8.18 (\pm 0.32)	11.1 (\pm 1.73)

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 μ M) in the absence and presence of fixed concentrations of salmeterol (100 nM), salbutamol (1 μ M) and formoterol (10 nM). After 6 h cells were harvested for luciferase activity determination. pEC₅₀ values were calculated for each glucocorticoid in the presence or absence of each β_2 -adrenoceptor agonist. Overall activation of the GRE reporter (Fold) and the enhancement (Fold) evoked by each β_2 -adrenoceptor over that produced by the glucocorticoid alone are shown. See Fig. 2 and text for further details.

Treatments		<i>N</i>	pEC ₅₀ (M) (\pm SE)	GRE activation (Fold)	Enhancement (Fold)
Glucocorticoid	β_2 -adrenoceptor agonist				
Fluticasone	-	18	-8.69 (\pm 0.08)	13.8	-
	+ Salmeterol	6	-8.74 (\pm 0.19)	31.8**	2.30
	+ Formoterol	6	-8.70 (\pm 0.19)	29.9*	2.16
	+ Salbutamol	6	-8.65 (\pm 0.17)	37.1**	2.68
Budesonide	-	17	-8.65 (\pm 0.05)	15.4	-
	+ Salmeterol	6	-8.56 (\pm 0.09)	30.0*	1.94
	+ Formoterol	6	-8.57 (\pm 0.05)	34.7*	2.25
	+ Salbutamol	5	-8.54 (\pm 0.05)	28.3***	1.84
Dexamethasone	-	16	-7.73 (\pm 0.07)	14.5	-
	+ Salmeterol	6	-7.75 (\pm 0.11)	34.4**	2.38
	+ Formoterol	4	-7.57 (\pm 0.10)	37.7*	2.61
	+ Salbutamol	6	-7.60 (\pm 0.15)	32.9*	2.27

TABLE 4

Effect of PKI α 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.PKI α (PKI α), an empty vector, Ad5.CMV (null) or left untreated (naive). Cells were treated with various concentrations of budesonide (30 pM to 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. pEC₅₀ values were calculated for budesonide in the presence or absence of formoterol. Overall 2 \times GRE reporter activation and the β_2 -adrenoceptor-dependent enhancement over the glucocorticoid alone was also determined.

Treatment		N	pEC ₅₀ (M) (\pm SE)	GRE activation (Fold)	Enhancement (Fold)
Virus	Drugs				
Naïve	Bud	4	-8.62 (\pm 0.12)	12.2	-
	Bud + form	4	-8.58 (\pm 0.10)	30.1*	2.4
Null	Bud	5	-8.78 (\pm 0.23)	17.1	-
	Bud + form	5	-8.82 (\pm 0.20)	39.7***	2.3
PKI α	Bud	5	-8.75 (\pm 0.23)	15.7	-
	Bud+ form	5	-8.74 (\pm 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 μ M) for 2, 4, 6 or 18 h. RNA was prepared and microarray analysis performed using recommended Affymetrix methodology. 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*). Abbreviations are found in the main text except for: SLC45A1, solute carrier family 45, member 1; DNb, deleted in neuroblastoma; TTP, tristetraprolin; TIS, TPA-inducible sequence.

Gene symbol (common name)	Accession No. (Ref Seq)	Affymetrix ID	2 h		4 h		6 h		18 h	
			Fold	<i>P</i>	Fold	<i>P</i>	Fold	<i>P</i>	Fold	<i>P</i>
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

Fig. 1

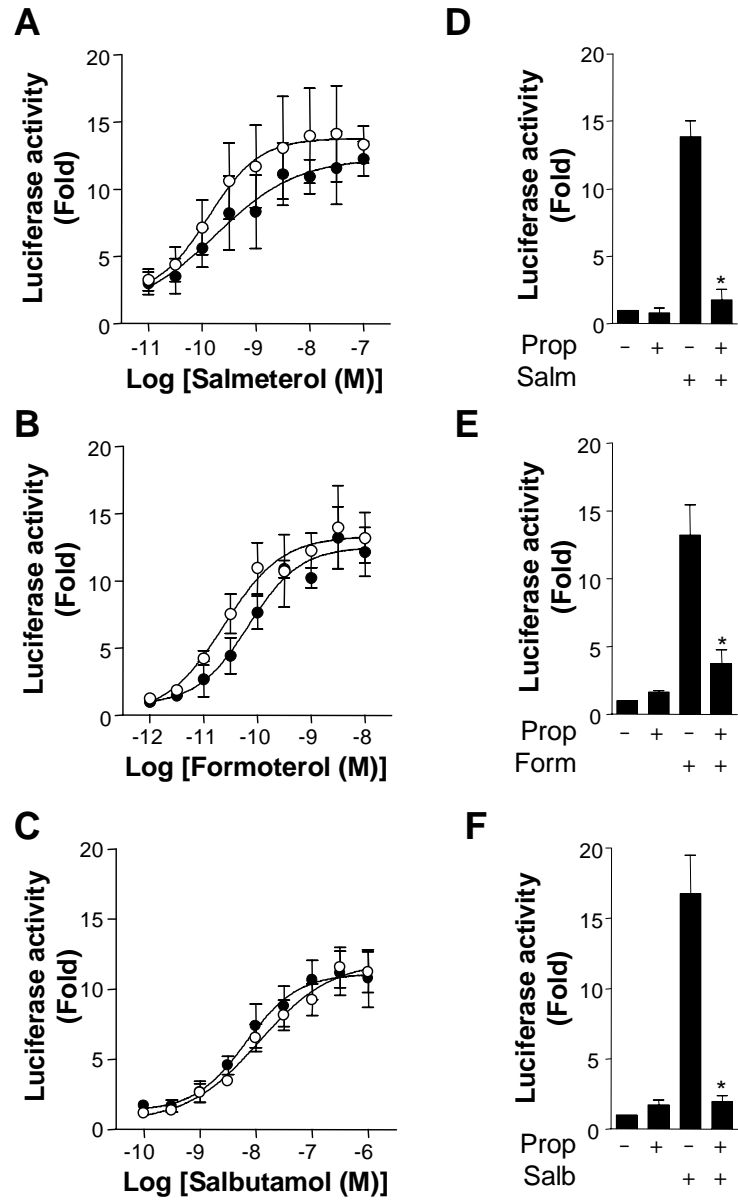


Fig. 2

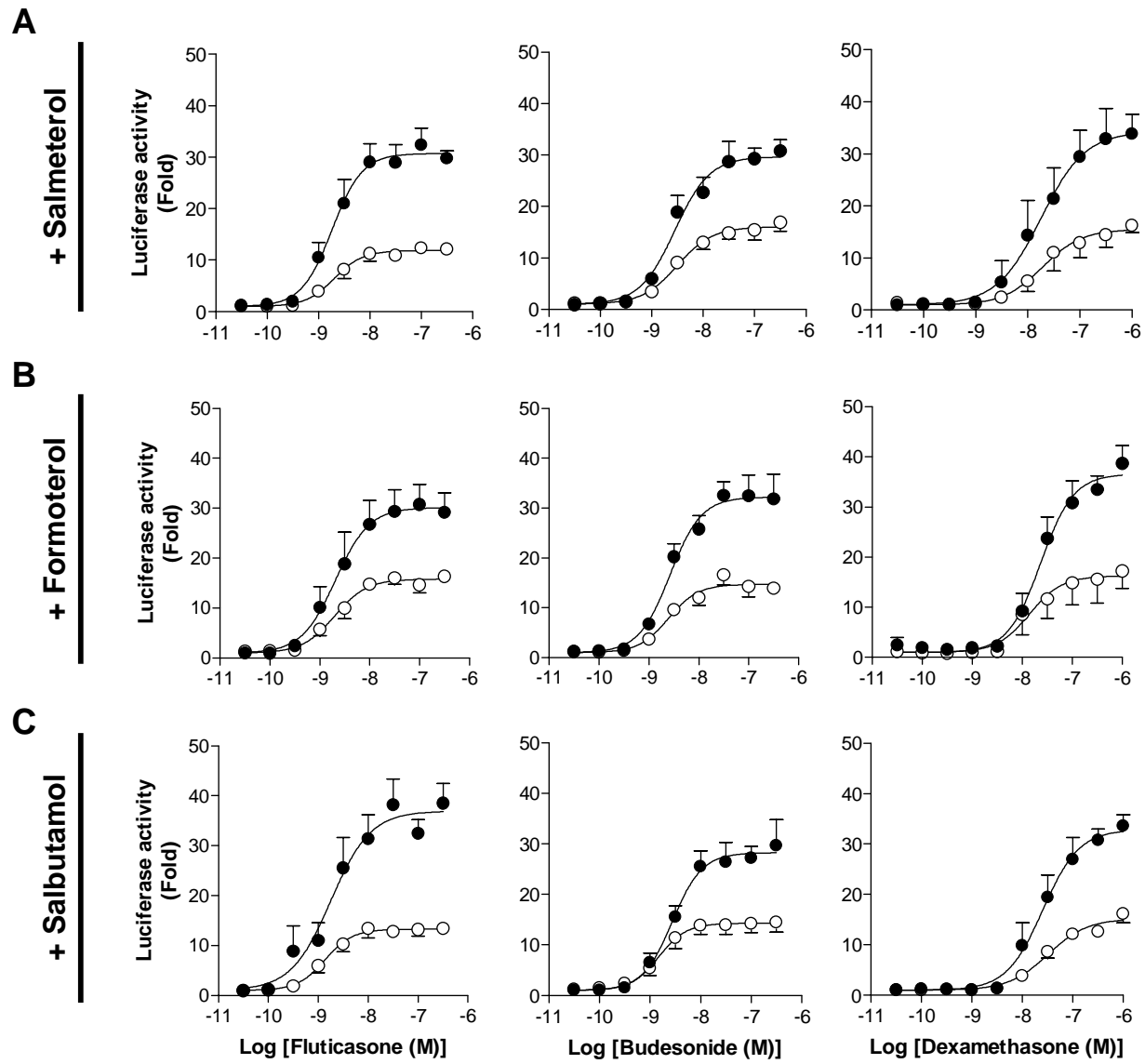


Fig. 3

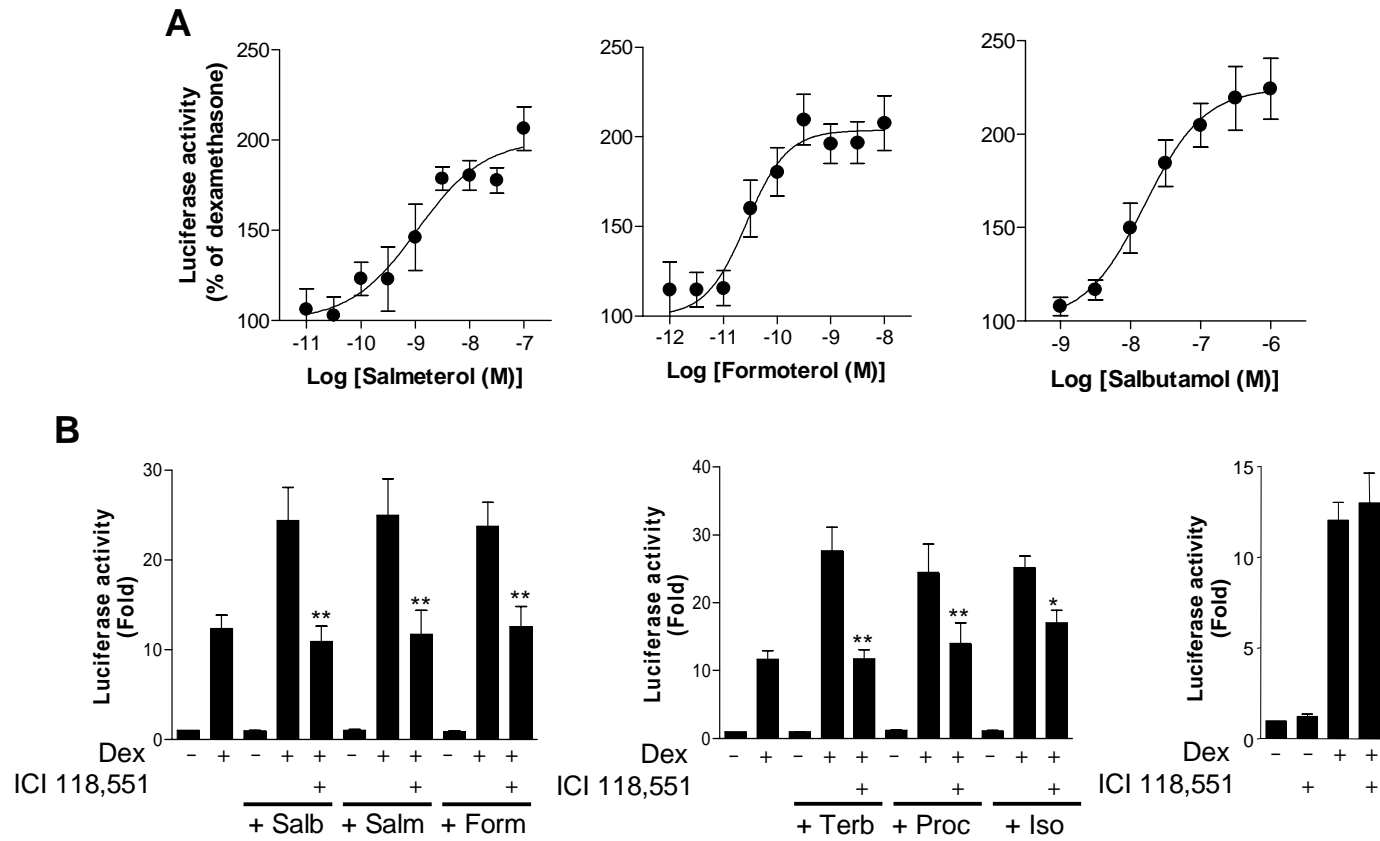


Fig. 4

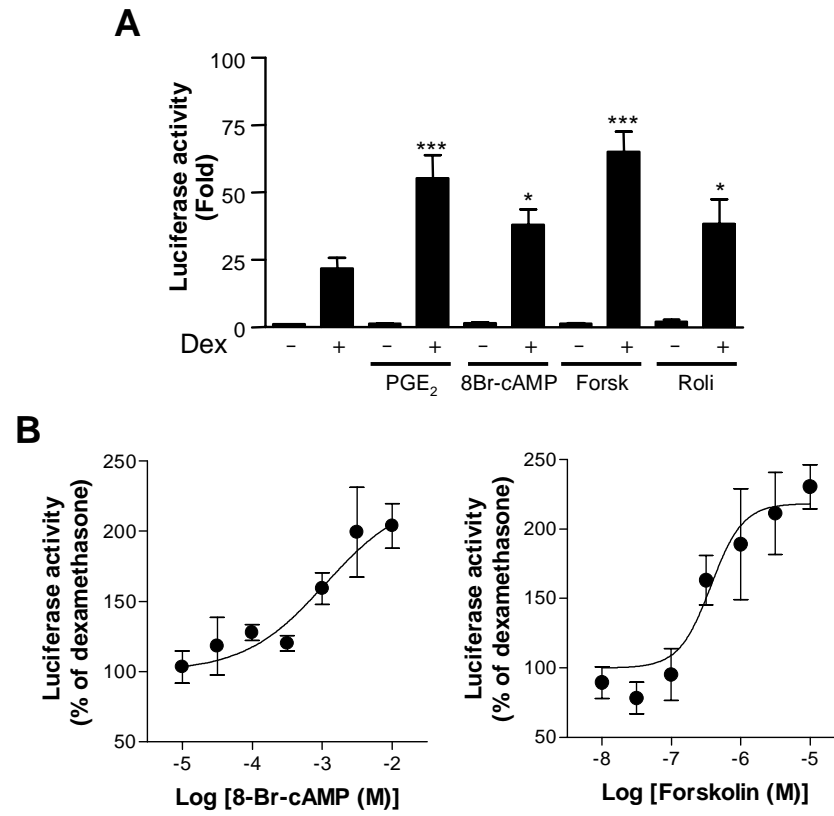


Fig. 5

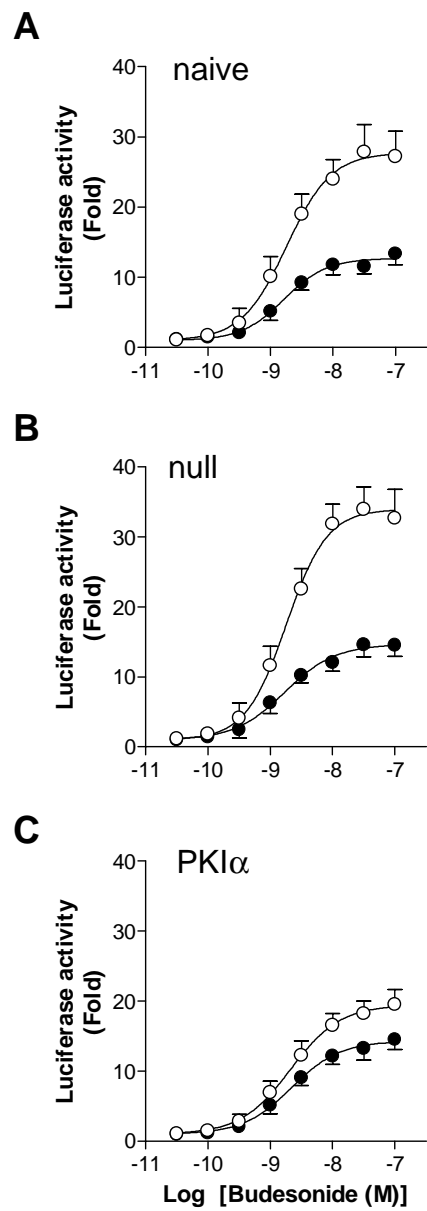


Fig. 6

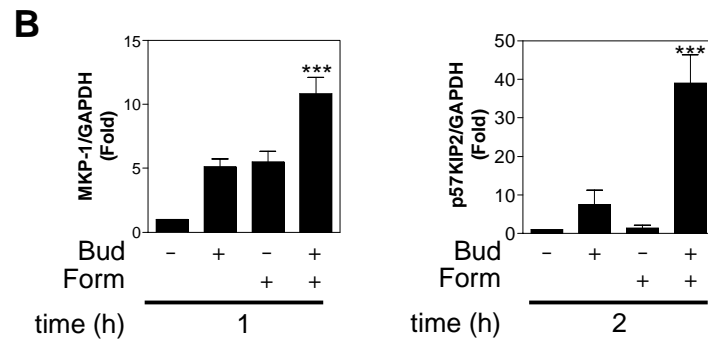
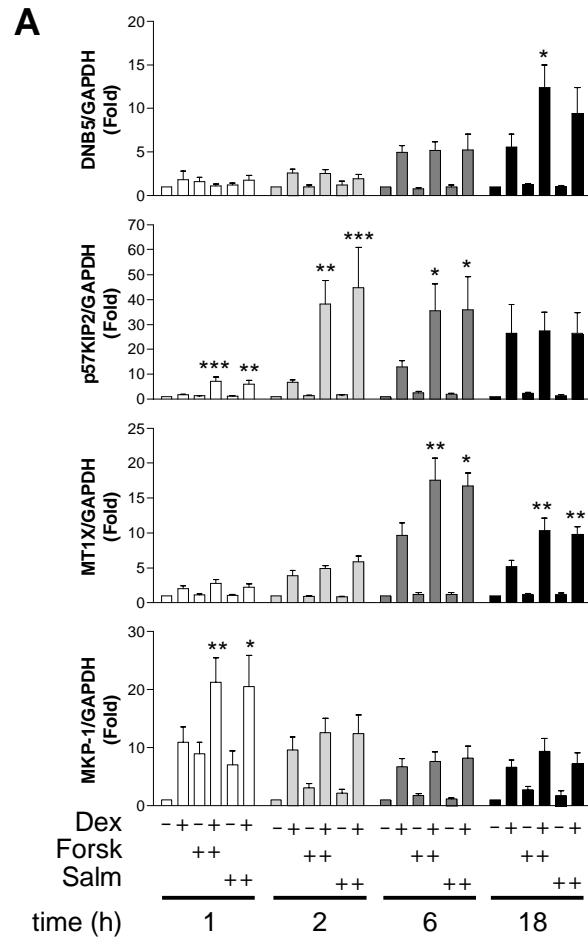


Fig. 7

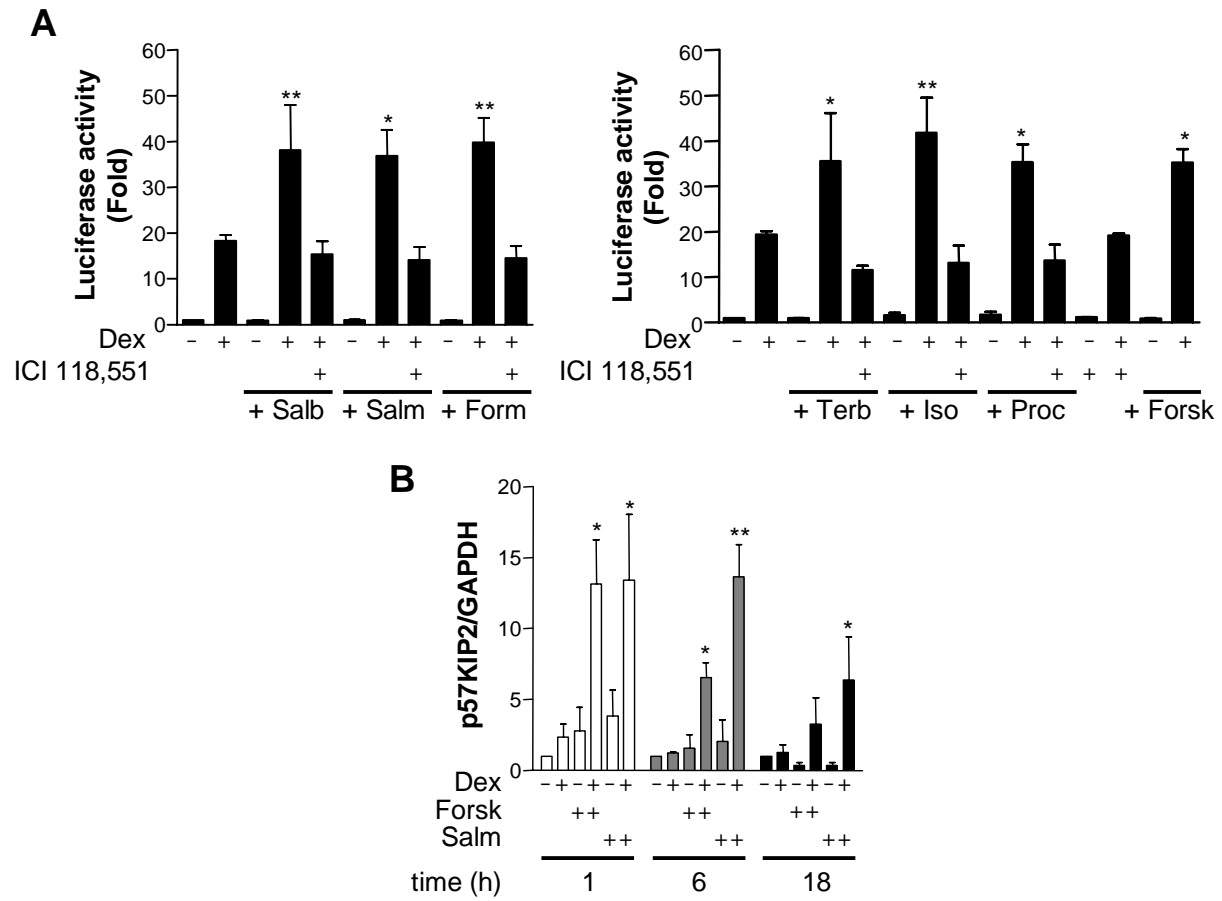
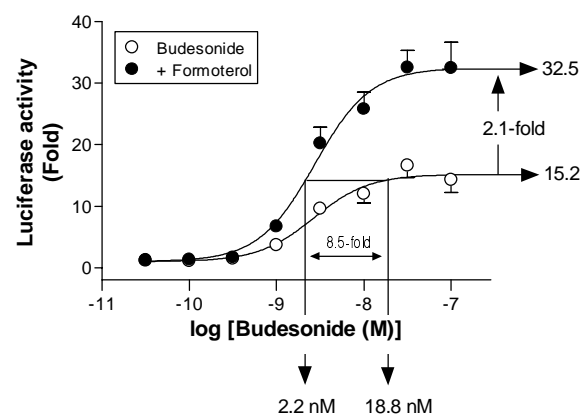


Fig. 8

A



B

