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Induction of RGS2 expression by long-acting β_2 -adrenoceptor agonists and glucocorticoids in human airway epithelial cells

Neil S. Holden¹, Tresa George, Christopher F. Rider, Ambika Chandrasekhar, Suharsh Shah, Manminder Kaur², Malcolm Johnson, David P. Siderovski, Richard Leigh, Mark A. Giembycz, Robert Newton

Airways Inflammation Research Group, Snyder Institute for Chronic Diseases, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada (N.S.H., T.G., C.F.R., A.C., S.S., M.K., R.L., M.A.G., R. N.), GlaxoSmithKline Research and Development, Uxbridge, Middlesex, UK. (M.J.), Department of Physiology and Pharmacology, School of Medicine, West Virginia University, West Virginia, USA. (D.P.S.).

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b) Corresponding author: Robert Newton, Department of Cell Biology and Anatomy, Faculty of Medicine, University of Calgary, 3330 Hospital Drive NW, Calgary, Alberta, Canada T2N 4N1. Tel: 001 403 210 3938; Fax: 001 403 210 7944; E-mail: rnewton@ucalgary.ca

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ABBREVIATIONS: 2-APB, 2-aminorthyldiphenyl borate; AP, activator protein; ASM, airway smooth muscle; COPD, chronic obstructive pulmonary disease; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; ERK, extracellular-regulated kinase; F, fluorescence; FCS, fetal calf serum; FKBP51, FK506-binding protein 51; FP, fluticasone propionate; GAP, GTPase-activating protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GILZ, glucocorticoid-induced leucine zipper; GPCR, G protein coupled receptor; HBE, human bronchial epithelial; ICS, inhaled

corticosteroid; IP₃, inositol(1,4,5)triphosphate; LABA, long-acting β_2 -adrenoceptor agonist; LT, leukotriene; MOI, multiplicity of infection; PDE, phosphodiesterase; PG, prostaglandin; PLC β , phospholipase C β ; PKA, protein kinase A; RGS, regulator of G protein signalling; MAPK, mitogen-activated protein kinase; MKP, mitogen-activated protein kinase phosphatase; NF, nuclear factor; SABA, short-acting β_2 -adrenoceptor agonist.

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ABSTRACT

In asthma and chronic obstructive pulmonary disease (COPD) multiple mediators act on $G\alpha_q$ -linked G protein coupled receptors (GPCRs) to cause bronchoconstriction. However, acting on the airway epithelium, such mediators may also elicit inflammatory responses. In human bronchial epithelial BEAS-2B cells, regulator of G protein signalling (RGS) 2 mRNA and protein was synergistically induced in response to combinations of long-acting β_2 -adrenoceptor agonist (LABA) (salmeterol, formoterol) plus glucocorticoid (dexamethasone, fluticasone propionate, budesonide). Equivalent responses occurred in primary human bronchial epithelial cells. Concentrations of glucocorticoid plus LABA required to induce RGS2 expression in BEAS-2B cells were consistent with the levels achieved therapeutically in the lungs. As RGS2 is a GTPase-activating protein that switches off $G\alpha_q$, intracellular free calcium ($[Ca^{2+}]_i$) flux was used as a surrogate of responses induced by histamine, methacholine and the thromboxane receptor agonist, U46619. This was significantly attenuated by salmeterol plus dexamethasone pre-treatment, or RGS2 over-expression, and the protective effect of salmeterol plus dexamethasone was abolished by RGS2 RNA silencing. While methacholine and U46619 induced IL-8 release and this was inhibited by RGS2 over-expression, the repression of U46619-induced IL-8 release by salmeterol plus dexamethasone was unaffected by RGS2 knockdown. Given a role for $G\alpha_q$ -mediated pathways in inducing IL-8 release, we propose that RGS2 acts redundantly with other effector processes to repress IL-8 expression. Thus, RGS2 expression is a novel effector mechanism, in the airway epithelium, that is induced by glucocorticoid/LABA combinations. This could contribute to the efficacy of glucocorticoid/LABA combinations in asthma and COPD.

Introduction

Globally, 300 million people have asthma, while smoking-related chronic obstructive pulmonary disease (COPD) will soon become the 3rd leading cause of death. Both conditions are characterized by chronic airways inflammation, in which the epithelium takes an active role via the secretion of multiple inflammatory mediators (Knight and Holgate, 2003). In both diseases, inhaled glucocorticoids, clinically referred to as inhaled corticosteroids (ICSs), form a central part of clinical therapy and target the epithelium to reduce production of inflammatory mediators (Bateman et al., 2008; Rabe et al., 2007; Knight and Holgate, 2003). While ICSs are the mainstay of asthma therapy, short-acting β_2 -adrenoceptor agonists (SABAs) provide "as-needed" relief from bronchospasm and practice guidelines recommend ICS/long-acting β_2 -adrenoceptor agonist (LABA) combination inhalers when symptoms are not controlled by ICS alone (Bateman et al., 2008). Similarly, long-acting bronchodilators are indicated in mild/moderate COPD and ICS/LABA combinations for more severe disease (Bateman et al., 2008; Rabe et al., 2007). In both asthma and COPD, large multicenter randomized controlled clinical trials confirm therapeutic benefits to ICS/LABA combinations that are not achieved by either component alone (Shrewsbury et al., 2000; Rabe et al., 2007). This phenomenon indicates synergistic interactions between these two drug classes (Giembycz et al., 2008; Newton et al., 2010b).

In terms of designing new combination therapies exploiting this effect, it is essential that a thorough mechanistic basis is established. While some studies indicate enhanced repressive effects of glucocorticoid/LABA combinations on inflammatory gene expression (Pang and Knox, 2000; Edwards et al., 2006), others show LABAs to enhance glucocorticoid-dependent transcription to a level that cannot be achieved by glucocorticoid alone (Kaur et al., 2008). In this context, glucocorticoids induce mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1/DUSP1) expression to reduce activation of key pathways, including MAPKs, nuclear factor (NF)- κ B and activator protein (AP)-1,

that are implicated in inflammatory gene expression (Clark et al., 2008; Diefenbacher et al., 2008; King et al., 2009). However, while MKP-1 expression is also increased by β_2 -adrenoceptor agonists, combination of glucocorticoid plus β_2 -adrenoceptor agonist produces an additive effect on MKP-1 expression (Kaur et al., 2008; Manetsch et al., 2012). Conversely, in human airways smooth muscle cells, expression of regulator of G-protein signalling 2 (RGS2), a $G\alpha_q$ -selective GTPase-activating protein (Heximer, 2004; Kimple et al., 2009), is synergistically enhanced by glucocorticoid plus LABA (Holden et al., 2011). Since RGS2 attenuates responses from $G\alpha_q$ -linked G protein-coupled receptors (GPCRs), such as airway smooth muscle contraction (Holden et al., 2011; Xie et al., 2012), we reasoned that relevant responses in other cells that express the β_2 -adrenoceptor, including airway epithelia, might be affected similarly.

Agonists of various GPCRs, including acetylcholine, histamine, cysteinyl-leukotrienes and certain prostanoids, activate phospholipase C β (PLC β) via $G\alpha_q$ and increase the intracellular cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) via an inositol(1,4,5)trisphosphate (IP $_3$)-dependent mechanism (Gosens et al., 2006; Penn and Benovic, 2008). It is well established that increasing $[Ca^{2+}]_i$ promotes a variety of pro-inflammatory effects (Gosens et al., 2006; Gwilt et al., 2007; Holgate et al., 2003; Gelfand, 2002). Indeed, leukotriene (LT) D $_4$ induces, and CysLT $_1$ receptor antagonism reduces, inflammatory cell infiltration, eosinophil trafficking, cytokine release and other inflammatory responses (Henderson, Jr. et al., 2002; Henderson, Jr. et al., 2006; Holgate et al., 2003). Similarly, in human airway epithelial cells, histamine acts on the $G\alpha_q$ -linked H $_1$ receptor to increase $[Ca^{2+}]_i$, NF- κ B activation and the expression of cytokines (Takizawa et al., 1995; Muller et al., 2006; Matsubara et al., 2006; Holden et al., 2007). Equally, acetylcholine activates extracellular regulated kinase (ERK) and NF- κ B to promote cytokine release from airway epithelial cells (Profita et al., 2008; Koyama et al., 1992; Koyama et al., 1998). Furthermore, muscarinic receptor antagonists, which are commonly prescribed as bronchodilators for COPD, have anti-inflammatory activities (Gwilt et al., 2007; Sales, 2010).

Likewise, thromboxane, which is elevated in the exhaled breath condensates of asthmatic subjects (Huszar et al., 2005), induces the expression of inflammatory cytokines and chemokines (Suzuki et al., 2008) through the $G\alpha_q$ -linked TP-receptor (Jones et al., 2009).

In this manuscript, we describe, using human bronchial epithelial (HBE) cells, the effect of glucocorticoids and LABAs alone and in combination on both the expression of RGS2 and agonist-induced $[Ca^{2+}]_i$ mobilisation. The central role of $[Ca^{2+}]_i$ in driving a diverse array of responses provides a reliable and well-validated marker of $G\alpha_q$ -linked GPCR activation with which to assess the functional consequences of RGS2 up-regulation. Effects on IL-8 release are also investigated.

Materials and Methods

Cell culture, drugs and stimuli

Human bronchial epithelial BEAS-2B cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Invitrogen, Burlington, ON, Canada), supplemented with 10 % fetal calf serum (FCS) (Invitrogen). Primary human HBE cells were isolated from normal human lungs and cultured as described (Newton et al., 2007). Cells were incubated overnight in serum-free, or growth factor-free, medium prior to experiments and then changed to fresh serum-free medium containing stimuli and/or drugs (all from Sigma-Aldrich Canada, Oakville, ON Canada unless otherwise stated). Formoterol fumarate (*rac*-(R,R)-*N*-[2-hydroxy-5-[1-hydroxy-2-[1-(4-methoxyphenyl)propan-2-ylamino]ethyl]phenyl]formamide) (formoterol) and budesonide (16,17-(butylidenebis(oxy))-11,21-dihydroxy-, (11- β ,16- α)-pregna-1,4-diene-3,20-dione) (both AstraZeneca, Mölndal, Sweden), salmeterol xinafoate ((*RS*)-2-(hydroxymethyl)-4-{1-hydroxy-2-[6-(4-phenylbutoxy)hexylamino]ethyl}phenol) (salmeterol), fluticasone propionate (S-(fluoromethyl)-6 α ,9-difluoro-11 β ,17-dihydroxy-16 α -methyl-3-oxoandrosta-1,4-diene-17 β -carbothioate, 17-propanoate) (FP), U46619 ((*Z*)-7-[(1*S*,4*R*,5*R*,6*S*)-5-[(*E*,3*S*)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid) (Cayman Chemical, Ann Arbor, MI), forskolin ((3*R*,4*aR*,5*S*,6*S*,6*aS*,10*S*,10*aR*,10*bS*)-6,10,10b-trihydroxy-3,4*a*,7,7,10*a*-pentamethyl-1-oxo-3-vinyldodecahydro-1*H*-benzo[*f*]chromen-5-yl acetate), prostaglandin (PG) E₂, LTB₄, LTD₄, rolipram ((*RS*)-4-(3-cyclopentyloxy-4-methoxy-phenyl)pyrrolidin-2-one), ICI 118,551 (3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy]butan-2-ol) and 2-aminoethyldiphenyl borate (2-APB), Ro 31-8220 (3-(3-(4-(1-methyl-1*H*-indol-3-yl)-2,5-dioxo-2,5-dihydro-1*H*-pyrrol-3-yl)-1*H*-indol-1-yl)propyl carbamimidothioate), G $\square$ 6976 (5,6,7,13-tetrahydro-13-methyl-5-oxo-12*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-12-propanenitrile), and GF109203X (2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide) were dissolved in dimethylsulphoxide (DMSO). Final concentrations of DMSO were <0.1 %. Dexamethasone

((8*S*,9*R*,10*S*,11*S*,13*S*,14*S*,16*R*,17*R*)-9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-3*H*-cyclopenta[*a*]phenanthren-3-one), histamine (2-(1*H*-imidazol-4-yl)ethanamine) and methacholine (-(acetyloxy)-*N,N,N*-trimethylpropan-1-aminium) were dissolved in Hanks's balanced salt solution (Sigma-Aldrich Canada).

Western blotting, ELISA, siRNA knock-down and adenoviral infections

Western blotting, siRNA transfections, adenoviral infections and ELISA were performed according to standard protocols (King et al., 2009; Holden et al., 2011). Commercially available antibodies were: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (4699-9555(ST)) from AbD Serotec (Raleigh, NC, USA); anti-HA (clone HA-7) (H3663) from Sigma-Aldrich (St. Louis, MO, USA); phospho-p44/42 MAPK (Erk1/2) (9101); phospho-SAPK/JNK (9251); phospho-p38 MAPK (9211); phospho-ATF2 (9221) from Cell Signaling Technology (Danvers, MA, USA); and MKP-1 (M-18) (sc-1102) from Santa Cruz Biotechnology (Dallas, TX, USA); and the RGS2 antibody was previously described (Holden et al., 2011). Detection was by enhanced chemiluminescence (GE Healthcare Bio-Sciences Inc) with visualization by autoradiography. Transfections for siRNA were performed using Lipofectamine™ 2000 as described (King et al., 2009; Holden et al., 2011). Targeted sequences were: RGS2 siRNA 2 (SI00045773; 5'-AAC GTG GTG TCT CAC TCT GAA-3'); RGS2 siRNA 6 (SI03036082; 5'-AAG GGT ATA CAG CTT GAT GGA-3'); Lamin siRNA (SI03650332; 5'-AAC TGG ACT TCC AGA AGA ACA-3') (all Qiagen, Mississauga, ON, Canada); green fluorescent protein siRNA (control siRNA) (P-002048-03-20; 5'-GGC AAG CTG ACC CTG AAG TTC-3') (Dharmacon, Chicago, IL, USA). BEAS-2B cells at ~ 70 % confluence were incubated for 24 h in DMEM/F12 + 10 % FCS containing the indicated multiplicity of infections (MOIs) of adenoviral (Ad5) expression vectors: Ad5-RGS2.HA, Ad5-PK1 α , Ad5-MKP-1; or either empty Ad5 vector (null), Ad5-LacZ and Ad5-GFP (QbioGene, Montreal, Quebec, Canada) were as described (King et al., 2009; Holden et al.,

2011). Cells were incubated overnight in serum-free media before experiments. IL-8 ELISA was performed using a commercial kit (R&D Systems, Hornby, ON, Canada)

RNA isolation, cDNA synthesis and SYBR green real-time PCR

Total RNA extracted by RNeasy mini kit (Qiagen) was reverse transcribed (QScript reverse transcriptase; Quanta Biosciences, Gaithersburg, MD) and subjected to real-time PCR (SYBR GreenER, Invitrogen) using ABI 7900HT and StepOne Plus instruments (Applied Biosystems Inc, Foster City, CA, USA) (King et al., 2009). Relative cDNA concentrations of target genes were obtained from a standard curve of serially diluted cDNA. Amplification primers for GAPDH were as described (King et al., 2009), and for RGS2 were: forward: 5'-CCT CAA AAG CAA GGA AAA TAT ATA CTG A-3', reverse: 5'-AGT TGT AAA GCA GCC ACT TGT AGC T-3'. Specificity of primers was determined by dissociation (melt) curve analysis.

Calcium assay

$[Ca^{2+}]_i$ release was measured using Fluo-4 NW according to the manufacturer's instructions (Invitrogen). Confluent cells were loaded for 30 min at room temperature with Fluo-4. Using 494 nm excitation, basal fluorescence (F_0) was measured at 516 nm for 1.6 seconds (FLUOstar OPTIMA plate reader, BMG Labtech, Offenburg, Germany). Agonists were injected and fluorescence measured for 50 seconds. The peak fluorescence (F), which occurred 5 - 15 seconds post-stimulation, was expressed as F/F_0 as a proxy for $[Ca^{2+}]_i$.

Data presentation and statistical analysis

Data are presented as mean $\pm$ SE. Statistical analysis between groups was performed using one-way analysis of variance (ANOVA) with a Bonferroni or Dunnett's post-test or a paired t-test as indicated. Significance between groups was assumed where; * $P < 0.05$, ** $P < 0.01$ or *** $P < 0.001$.

Results

Effect of glucocorticoids and LABAs on the expression of RGS2 in airway epithelial cells.

BEAS-2B cells, treated with a maximally effective concentration of dexamethasone (1 μ M) (Kaur et al., 2008), induced only very low and variable levels of RGS2 protein (Fig. 1A, Supplemental Fig. 1A). Conversely, the LABA, salmeterol, produced a readily detectible increase in RGS2 expression that was maximal at 2 h, but had declined by 6 h (Fig. 1A, Supplemental Fig. 1A). In combination, dexamethasone plus salmeterol resulted in a robust and prolonged elevation in RGS2 expression that was apparent by 30 min and extended for at least 12 to 15 h post treatment (Fig. 1A & B). This response was significantly greater than the effect of either treatment alone at 1, 2 and 6 h (Fig. 1A, Supplemental Fig. 1A). Equivalent effects were observed with salmeterol in the presence of FP and by combinations of formoterol plus budesonide (Fig. 1A & B, Supplemental Fig. 1). Forskolin, an activator of adenylyl cyclase, mimicked the effect of the LABAs at inducing RGS2 expression alone and in synergizing with dexamethasone (Fig. 1A, Supplemental Fig. 1A).

These changes in RGS2 protein correlated closely with the modulation of RGS2 mRNA expression (Fig. 2A & B). Dexamethasone was a weak inducer of RGS2 mRNA, while formoterol or salmeterol produced larger increases, which, when combined with dexamethasone, synergistically enhanced RGS2 mRNA expression (Fig. 2A & B). These effects were again mimicked by forskolin. In primary HBE cells, dexamethasone induced RGS2 mRNA expression and this was significantly enhanced by LABAs and forskolin (Fig. 2C). These responses were also observed in the primary HBE cells, at the level of RGS2 protein, in respect of dexamethasone combined with either salmeterol or forskolin (Fig. 2D).

Enhancement of RGS2 expression by β_2 -adrenoceptor agonists occurs via the β_2 -adrenoceptor, involves PKA and is reproduced by cAMP-elevating compounds. BEAS-2B cells were treated with dexamethasone in the presence of salbutamol, salmeterol or formoterol. Combinations of β_2 -adrenoceptor agonist plus dexamethasone robustly increased the expression of RGS2 in a manner that

was significantly prevented by prior addition of the β_2 -adrenoceptor antagonist, ICI 118,551 (Fig. 3A, Supplemental Fig. 2A). ICI 118,551 alone, or in the presence of dexamethasone, was without effect. This ability of ICI 118,551 to block, in the presence of dexamethasone, the enhancement of RGS2 by β -adrenoceptor agonists was also observed for terbutaline, procaterol and the non-selective β -agonist, isoprenaline (Supplemental Fig. 2B).

To explore the role of the cAMP-dependent kinase, protein kinase A (PKA), BEAS-2B cells were infected with an adenovirus over-expressing PKI α , a potent and selective inhibitor of PKA (Meja et al., 2004). In each case, PKI α -expressing virus, but not a null virus, significantly prevented the enhancement of RGS2 expression by salbutamol, salmeterol or formoterol in the presence of dexamethasone (Fig. 3B, Supplemental Fig. 2C).

In figure 1, we showed that forskolin mimics the effect of β_2 -adrenoceptor agonists at enhancing the expression of RGS2 in the presence of glucocorticoid. As the effect of β_2 -adrenoceptor agonists was blocked by ICI 118,551 and PKI α , a classical β_2 -adrenoceptor/adenylyl cyclase/cAMP/PKA-dependent mechanism is indicated (Giembycz and Newton, 2006). This suggests that other means of increasing cAMP may also promote RGS2 expression. Since, in BEAS-2B cells, PGE₂ activates PKA and enhances GRE-dependent transcription (Meja et al., 2004; Kaur et al., 2008), BEAS-2Bs were treated with PGE₂ in the absence and presence of dexamethasone. PGE₂, in the presence of dexamethasone, produced a robust increase in RGS2 expression (Fig. 3C). Equally, inhibition of phosphodiesterase (PDE) 4, which is highly expressed in BEAS-2B and primary HBE cells (Fuhrmann et al., 1999; Dent et al., 1998), enhances GRE-dependent transcription (Kaur et al., 2008). Therefore, cells were treated with rolipram, a PDE4 inhibitor, in the absence and presence of dexamethasone. RGS2 expression was markedly increased by dexamethasone plus rolipram (Fig. 3C).

Effect of glucocorticoid and LABA concentration on RGS2 expression and relationship with clinically used combination therapies. To explore whether such enhancements in RGS2 expression could be achieved at therapeutic concentrations of drug, BEAS-2B cells were treated with either a fixed

concentration of salmeterol (100 nM) or formoterol (10 nM) in the absence or presence of various concentrations of either FP or budesonide, respectively, prior to harvesting for western blot analysis of RGS2 and GAPDH proteins (Fig. 4A) or mRNA (Fig. 4B). Conversely, a fixed concentration of FP (300 nM) or budesonide (300 nM) was used and the concentration of salmeterol or formoterol was varied prior to harvesting for protein or RNA. Each drug alone resulted in relatively modest enhancements of RGS2 expression, but combination of glucocorticoid plus LABA resulted in synergistically increased expression (Fig. 4A & B). In the presence of maximal concentrations of LABA, FP and budesonide gave EC₅₀ values of 2.3 nM and 690 pM, respectively, at the RNA level (Fig. 4B, left panels) and these were consistent with the protein data (Fig. 4A, left panels). Likewise, in the presence of maximal concentrations of either FP or budesonide, salmeterol and formoterol produced EC₅₀ values of 43 pM and 20 pM, respectively, on RGS2 mRNA (Fig. 4B, right panels) that were again consistent with RGS2 protein expression (Fig. 4A, right panels).

In clinically used inhaled combination therapies, the LABA and ICS are in fixed ratios (Advair 250/50 & 500/50, and Symbicort 100/6 & 200/6). Therefore, the drugs were combined at starting concentrations of 200 nM FP plus 100 nM salmeterol and 1 μM FP plus 100 nM salmeterol or 175 nM budesonide plus 10 nM formoterol and 350 nM budesonide plus 10 nM formoterol to reflect the different clinical formulations. Each combination was subjected to 10 fold dilutions and applied to the cells for 6 h prior to western blot analysis. RGS2 expression was clearly induced following 1000-fold dilutions of each drug combination (Fig. 4C). This corresponds to low nanomolar and sub-nanomolar concentrations of each glucocorticoid and picomolar concentrations of the LABAs.

Effect of glucocorticoid and LABA on [Ca²⁺]_i induced by agonists of Gq-linked GPCRs. BEAS-2B cells were treated with various concentrations of histamine, the acetylcholine analog, methacholine, or the selective thromboxane receptor agonist, U46619. Each agonist produced a concentration-dependent increase in [Ca²⁺]_i, as measured by increases in Fluo-4 fluorescence (Fig. 5A), in a manner

that was prevented by the IP₃ receptor antagonist, 2-APB (Fig. 5B). These data support the involvement of a classical G α_q /PLC pathway.

To examine the functional significance of RGS2 induced by LABAs plus glucocorticoid, BEAS-2B cells were treated with salmeterol plus dexamethasone for 6 h, prior to stimulation with maximally effective concentrations of histamine, methacholine or U46619. In each case, prior treatment with either dexamethasone or salmeterol showed no effect on agonist-stimulated [Ca²⁺]_i (Fig. 5C). However, in combination, salmeterol plus dexamethasone significantly attenuated the rise in [Ca²⁺]_i that was induced by each agonist (Fig. 5C). Thus, inhibition of responses mediated by G α_q -linked GPCRs by salmeterol plus dexamethasone correlates with the expression of RGS2.

RGS2 attenuates elevations in [Ca²⁺]_i induced by agonists of G α_q -linked GPCRs. BEAS-2B cells were infected with Ad5-RGS2.HA resulting in a MOI-dependent increase in HA tagged RGS2 expression (Fig. 6A). At MOIs of 10, 30 and 100, the rise in [Ca²⁺]_i produced by histamine, methacholine and U46619, was significantly inhibited, whereas an adenovirus over-expressing LacZ was without effect (Fig. 6B). Stimulation of BEAS-2B cells with either LTB₄ or LTD₄ resulted in very small increases in [Ca²⁺]_i. While this may be due to low receptor expression and/or weak coupling efficiency, these responses were, nevertheless, prevented by RGS2 over-expression and were unaffected by the LacZ virus (Fig. 6C).

To address whether RGS2 plays a role in the repression of agonist-induced elevations in [Ca²⁺]_i, BEAS-2B cells were transfected with siRNA directed to RGS2. In each case, two RGS2 targeting, but not a control GFP siRNA, markedly decreased the expression of RGS2 that was induced by salmeterol plus dexamethasone (Fig. 6D). Therefore, naive cells, and cells transfected with RGS2 targeting and control siRNA were pretreated with salmeterol plus dexamethasone prior to stimulation with histamine, methacholine or U46619. Salmeterol plus dexamethasone attenuated agonist-enhanced [Ca²⁺]_i in the naive and GFP control cells (Fig. 6E). However, in the presence of the two RGS2 targeting siRNA, this ability of salmeterol plus dexamethasone to reduce agonist-enhanced [Ca²⁺]_i was prevented. These data

confirm that RGS2 expression induced by salmeterol plus dexamethasone is responsible for attenuating the rise in $[Ca^{2+}]_i$ that is elicited by histamine, methacholine and U46619.

A role for RGS2 in the repression of IL-8 expression by salmeterol plus dexamethasone? In BEAS-2B cells stimulated with histamine (10 μ M), MCh (30 μ M) or U46619 (1 μ M), IL-8 release was increased at 6 and 18 h in a manner that was prevented by 6 h pre-treatment with salmeterol, dexamethasone or salmeterol plus dexamethasone (Fig. 7A). MCh and U46619 produced greater responses than histamine and IL-8 release predominantly occurred within 6 h. This is consistent with the rapid rise in IL-8 mRNA that was maximal at 1 - 2 h, but had declined by 6 h (Supplemental Fig. 3). Therefore, to assess effect of RGS2 expression, BEAS-2B cells were infected with various MOIs of RGS2 expressing, or control, adenoviruses prior to stimulation with MCh or U46619 to induce IL-8 release (Fig. 7B). This was significantly reduced by increasing MOIs of the RGS2 adenovirus, but not the control virus. Over-expression of RGS2 was confirmed by western blotting (data not shown).

To examine the role of RGS2 in the repression of IL-8 expression by salmeterol plus dexamethasone, BEAS-2B cells were transfected with two RGS2-targeting siRNAs prior to treatment for 6 h with salmeterol plus dexamethasone (Fig. 7C). The expression of RGS2, induced by salmeterol plus dexamethasone, was substantially ablated by the two RGS2-targeting siRNA, but was unaffected by transfection lipid or transfection of an unrelated siRNA. Note: different gel-running conditions resulted in the broad band seen in some of the earlier blots being separated into two RGS2 bands. Parallel tissue culture wells were also treated with U46619 to induce IL-8 release (Supplemental Fig. 4 & Fig. 7D). This was unaffected by any of the siRNA treatments (Supplemental Fig. 4). While salmeterol plus dexamethasone significantly reduced the release of IL-8, this repression was unaffected by both the RGS2 targeting and the control siRNAs. Thus despite a near total loss of RGS2 expression (Fig. 7C), repression of IL-8 release by salmeterol plus dexamethasone was maintained (Fig. 7D).

Possible explanations for this result are two-fold. One, the ability of Ad5-RGS2 to repress IL-8 release represents an artifact due to over-expression and RGS2 may not in fact play any role in the

observed repression. Alternatively, RGS2 may, as shown for agonist-induced $[Ca^{2+}]_i$ (Fig. 6), reduce signalling pathways that lead to IL-8 expression, but this effect may be redundant with other effector processes that are activated by salmeterol plus dexamethasone (see main discussion for further detail). To help distinguish these alternatives, BEAS-2B cells were treated with U46619 in the presence of: *i*) 2-APB, which blocks IP_3 receptors and, as shown in Fig. 5B, prevents agonist-stimulated rises in $[Ca^{2+}]_i$; and, *ii*) Ro 31-8220, G α 6976, or GF109203X, which are all inhibitors of PKC (Fig. 7E). As before, U46619 induced IL-8 release and this was significantly reduced by both 2-APB and the PKC inhibitors. Such data argue that the activation, by U46619, of signalling pathways and secondary messengers, i.e. $[Ca^{2+}]_i$ and PKC that are downstream of $PLC\beta$, are indeed necessary for the observed increases in IL-8 release. Since salmeterol plus dexamethasone-induced RGS2 reduces agonist-induced levels of $[Ca^{2+}]_i$ (Fig. 6), the current data support functional redundancy in the repressive effect of salmeterol plus dexamethasone on IL-8 release.

Roles for MAPKs in the expression and repression of IL-8? To provide experimental support for the concept of functional redundancy, the role of MAPKs, which are inactivated by the glucocorticoid and β_2 -adrenoceptor agonist-induced gene, MKP-1 (Kaur et al., 2008; Manetsch et al., 2012), was investigated. Following treatment with histamine, MCh or U46619, rapid activation, as evidenced by increased T-x-Y phosphorylation, of the p54/p46 JNK and p38 MAPKs was apparent, with more modest increases in respect of ERK (Fig. 8A). Parallel phosphorylation of activating transcription factor (ATF) 2, an established target of multiple MAPKs that is implicated in the transcriptional regulation of IL-8 was also observed (Kyriakis and Avruch, 2001; Hisatsune et al., 2008). Furthermore, inhibitors of the ERK (PD098058, U0126), p38 (SB203580) and JNK (JNK inhibitor 8) MAPK pathways significantly reduced the expression of IL-8 when induced by U46619 (Fig. 8B).

The above data suggest that repression of MAPK pathways by salmeterol and/or dexamethasone may be predicted to reduce IL-8 expression. Indeed, expression of the MAPK phosphatase, MKP-1, was robustly, but transiently, induced by salmeterol (Fig. 8C). Similarly, dexamethasone also induced

MKP-1 expression at all times examined and in the additional presence of salmeterol, MKP-1 expression was strongly enhanced at both 1 and 2 h relative to either treatment alone (Fig. 8C).

Using western blotting of phosphorylated ERK, p38 and JNK, as well as the downstream transcription factor, ATF-2, we found that both dexamethasone and salmeterol, alone, and in combination, reduced activation of MAPKs (Fig. 8D). Note, however, that activation of ERK and p38 was, as shown in Fig. 8A, relatively modest. A similar, but generally more robust repressive effect, was also observed on the downstream transcription factor, ATF2, where the effect of the LABA/glucocorticoid combination showed the greatest repression (Fig. 8D).

Given the ability of MKP-1 to switch off MAPK signalling (Clark et al., 2008), including in airway epithelial cells (King et al., 2009), we infected BEAS-2B cells with an MKP-1 expressing adenovirus prior to stimulation with either MCh or U46619 (Fig. 8E). Western blotting confirmed over-expression of MKP-1 (data not shown) and we have previously shown this to prevent MAPK activation in A549 and BEAS-2B cells by IL-1 β and TNF α (Newton et al., 2010a; King et al., 2009). The MKP-1 expressing, but not a control adenovirus resulted in significant repression of IL-8 release induced by MCh or U46619 (Fig. 8E). Thus, the ability of salmeterol and dexamethasone to inhibit MAPKs, for example via the induction of MKP-1, is suggested to inhibit IL-8 expression independently of the effects of RGS2 expression.

Discussion

Evidence for synergy. In the treatment of asthma and COPD, combination therapies consisting of an ICS and a LABA provide superior clinical benefit compared to either monotherapy. However, for drug development programs to more successfully exploit this pharmacology, it is desirable to comprehensively understand the underlying mechanistic basis. While we previously identified *RGS2* as a gene whose expression was increased by glucocorticoids in A549 pulmonary epithelial cells (Chivers et al., 2006), *RGS2* expression is also enhanced by agonists that activate the cAMP signalling cascade (Tsingotjidou et al., 2002; Roy et al., 2006). Since inhaled therapies necessarily impact on the airway epithelium, which express both GR and β_2 -adrenoceptors, we selected human bronchial epithelial cells to explore combinatorial effects of these pathways on the expression of *RGS2*. Using BEAS-2B bronchial epithelial cells, glucocorticoids produced weak and variable increases in *RGS2* expression, whereas LABAs resulted in robust, but transient, increases in *RGS2* expression. In combination, LABAs plus glucocorticoids produced rapid increases in *RGS2* expression that extended at least 12 h post-treatment. This interaction was one of synergy since *RGS2* expression in the presence of glucocorticoid plus LABA was considerably more than the simple sum of each component alone. This effect is similar to effects observed in human airway smooth muscle, where LABA plus glucocorticoid also induced *RGS2* expression in a synergistic manner (Holden et al., 2011). Taken together, these data suggest a common response that is likely to also occur in other cells types. Given the ability of *RGS2* to attenuate signal transduction from $G\alpha_q$, such effects may therefore be relevant to multiple physiological and pathophysiological situations.

The ability to synergistically induce *RGS2* expression was a class effect that occurred with three different glucocorticoids (dexamethasone, budesonide and FP) and two LABAs (formoterol and salmeterol). In addition, SABAs plus glucocorticoid also enhanced *RGS2* expression and the effect of LABAs, SABAs and the non-selective β -adrenoceptor agonist, isoprenaline, was prevented by a

selective β_2 -adrenoceptor antagonist. Finally, over-expression of PKI α , a potent and selective inhibitor of PKA (Meja et al., 2004), prevented the enhancement of RGS2 expression by β_2 -adrenoceptor agonists. Thus, in the presence of glucocorticoids, the classical β_2 -adrenoceptor-cAMP-PKA pathway is responsible for enhancing RGS2 expression. This effect was mimicked by other cAMP-elevating agents, including the adenylyl cyclase activator, forskolin, the PDE4 inhibitor, rolipram, and PGE₂, raising the prospect of additional therapeutic agents that could be usefully combined with a glucocorticoid. This is consistent with prior findings that these cAMP-elevating agents all enhance the activation of glucocorticoid-dependent transcription and that PDE inhibitors and adenosine A_{2B} agonists may enhance glucocorticoid-induced expression of multiple, potentially protective, genes including RGS2 (Kaur et al., 2008; Moodley et al., 2013; Greer et al., 2013).

Comparison of *in vivo* and *in vitro* drug administration. While a functional role for RGS2 induced by glucocorticoid plus LABA is suggested, it is important to consider the relationship between the drug concentration needed to induce RGS2 expression and the levels that are achieved therapeutically. In the presence of maximal concentrations of each LABA, EC₅₀ values for FP and budesonide for the induction of RGS2 were 2.3 and 0.69 nM, respectively. These are consistent with the ability of these glucocorticoids to enhance simple GRE-dependent transcription (Kaur et al., 2008). Similarly, with maximal concentrations of glucocorticoid, salmeterol and formoterol induced RGS2 with EC₅₀ values of 43 and 20 pM respectively. Again these potencies are broadly consistent with their enhancement of GRE-dependent transcription (Kaur et al., 2008). Likewise, dilutions of LABA plus glucocorticoid in fixed ratios resulted in clear enhancements in RGS2 expression with FP or budesonide concentrations in the 0.1 - 1 nM range and LABA concentrations of 0.1 nM for salmeterol and 10 pM for formoterol. In the context of inhaled therapeutics, lung concentrations of ICS can readily reach 1-100 nM (Esmailpour et al., 1997; Miller-Larsson and Selroos, 2002; Todorova et al., 2006; van den Brink et al., 2008). Furthermore, in mild atopic asthmatics, inhalation of a moderate dose of budesonide (800 μ g/day) for 10 days significantly increased the expression of glucocorticoid-induced

leucine zipper (GILZ/TSC22D3) and FK506-binding protein 51 (FKBP51/FKBP5) (Kelly et al., 2012). Thus, glucocorticoid concentrations necessary to induce gene expression are achieved by ICS and glucocorticoid-dependent increases in gene expression occur with therapeutic intervention. Likewise, at the clinical dose of 50 µg, salmeterol inhalation gives low nanomolar concentrations within the lung (Johnson, 2000). Such considerations support the concept that inhaled LABA/glucocorticoid combination therapies may synergize at inducing therapeutically-relevant genes, such as RGS2, in the lung. This effect could contribute to the enhanced clinical efficacy of inhaled combination therapies over the effect of ICS alone as a monotherapy (Giembycz et al., 2008; Newton et al., 2010b). However, while we have shown clear synergy in respect of RGS2 expression in cell culture, i.e. where drug-containing media continuously bathes the cells for relatively short periods of time (typically up to 24 h), it remains to be established whether such effects occur *in vivo* in relevant patient populations. Thus the duration of treatment, the frequency of delivery, the dose, as well as the specific pharmacodynamic and pharmacokinetic properties of each drug will lead to variable and most frequently sub-maximal concentrations that are difficult to accurately model *in vitro*. Hence the current demonstration of biological synergy *in vitro* should promote clinical studies to address this effect in individuals undergoing relevant therapies.

Effects of RGS2 downstream of $G\alpha_q$. The functional significance of RGS2 induction was examined using a range of agonists, which promote Ca^{2+} mobilization via $G\alpha_q$ -linked receptors that are relevant to the pathogenesis of asthma and/or COPD. Increased $[Ca^{2+}]_i$ is a readily measurable and established proximal marker of $G\alpha_q$ -linked GPCR activation that is central to downstream responses (Gosens et al., 2006; Penn and Benovic, 2008). Thus, histamine (acting through the H_1 -receptor), methacholine (acting through the M_3 -receptor), and the TP-receptor agonist, U46619, concentration-dependently increased $[Ca^{2+}]_i$ in BEAS-2B airway epithelial cells. For each agonist, this effect was prevented by the IP_3 receptor antagonist, 2-APB, supporting a $G\alpha_q$ -linked pathway involving $PLC\beta$, IP_3 and the IP_3 -dependent release of Ca^{2+} from intracellular stores. Treatment of cells with salmeterol or

dexamethasone for 6 h had no effect on $[Ca^{2+}]_i$ induced by methacholine, histamine or U46619 and was consistent with the low levels of RGS2 expression induced by each drug alone. However, combination of dexamethasone plus salmeterol significantly attenuated agonist-induced rises in $[Ca^{2+}]_i$. This was reproduced by RGS2 over-expression and was prevented by siRNA knock-down of RGS2. As similar expression data were obtained using human primary airway epithelial cells, we suggest that up-regulation of RGS2 in airway epithelia by ICS/LABA combinations could help confer protection against pathological $G\alpha_q$ -dependent responses in asthma and COPD. This would not only complement the previously described bronchoprotective effect in airway smooth muscle (Holden et al., 2011; Xie et al., 2012), but is consistent with combination therapies reducing exacerbation rates of asthma and COPD (Pauwels et al., 1997; Calverley et al., 2007; Giembycz et al., 2008).

Functional redundancy of LABA plus glucocorticoid combination. In terms of understanding functional roles for RGS2, it is important to note that in addition to direct GR transrepression of inflammatory gene transcription, glucocorticoids induce the expression of multiple "anti-inflammatory" genes (Newton and Holden, 2007), and together these processes may repress inflammatory gene expression (King et al., 2013; Newton, 2013). For example, MKP-1 expression is increased by glucocorticoids and this switches off MAPK pathways to decrease expression of many inflammatory genes (Clark et al., 2008). Likewise, GILZ is profoundly induced by glucocorticoids and inhibits inflammatory gene expression by reducing activation of inflammatory transcription factors such as NF- κ B and AP-1 (Ayroldi and Riccardi, 2009). Equally, while GILZ did not show significant enhancement by LABAs (Kaur et al., 2008; Rider et al., 2011), the effect of glucocorticoid plus LABA on MKP-1 is additive (Kaur et al., 2008; Manetsch et al., 2012). Other genes, for example $p57^{KIP2}$ (*CDKN1C*), a cell cycle regulator that is implicated in inhibition of the c-Jun N-terminal kinase MAPK pathway (Chang et al., 2003; Yamamoto et al., 2007), show enhancement of glucocorticoid-driven transcription by LABAs (Kaur et al., 2008; Rider et al., 2011). Taken with the current data for RGS2, this illustrates how combining a LABA with an ICS could produce superior clinical benefits when compared to each

monotherapy. However, this discussion also illustrates the difficulties in assigning specific anti-inflammatory properties to any one individual effector gene. If these genes are all induced by glucocorticoids, particularly in the presence of LABA, then considerable redundancy of action is anticipated (Newton and Holden, 2007; Newton et al., 2010b). Thus, over-expression of RGS2 significantly repressed MCh- or U46619-induced IL-8 release, yet there was no effect of knocking down RGS2 expression in the context of repression by salmeterol plus dexamethasone. One explanation for such data is that the effect of RGS2 over-expression was an artifact and there is no role in the repression of IL-8 release by dexamethasone plus salmeterol. However, the induction of both $[Ca^{2+}]_i$ and IL-8 by U46619 was inhibited by IP₃R antagonist, 2-APB. Likewise, multiple PKC inhibitors reduced IL-8 release and together these data support the activation of a $G\alpha_q$, PLC β , $[Ca^{2+}]_i$ /PKC dependent pathway leading to IL-8 release. As RGS2 is responsible for the salmeterol plus dexamethasone-dependent repression of $[Ca^{2+}]_i$ (Fig. 6), these data strongly support a redundant effect of RGS2 in mediating repression of IL-8.

To provide experimental support for this contention, we show that histamine, MCh and U46619 activate MAPKs pathways and that inhibitors of each MAPK pathway (ERK, p38 and JNK) prevent U46619-induced IL-8 release. Furthermore, pre-treatment with dexamethasone and/or salmeterol inhibits MAPK phosphorylation, an effect that is most obvious for dexamethasone and dexamethasone plus salmeterol treatments. MKP-1 expression is induced by these same treatments and over-expression represses IL-8 release induced by both MCh and U46619. This supports a role for MKP-1 in repressing IL-8 expression and provides experimental evidence of functional redundancy between RGS2 and MKP-1. Since conventional transrepression and gene products, including GILZ and p57KIP2, may reduce IL-8 expression, the issue of widespread redundancy is clear. This point is further illustrated by the fact that both salmeterol and dexamethasone independently reduce IL-8 expression, whereas both in combination are needed to induce RGS2 expression at 6 h. Thus non-RGS2-dependent mechanisms of repression clearly exist and that in the combination, RGS2 is one of many factors, or mechanisms, that

collectively act to ensure repression. However, given the non-redundant role of RGS2 in repressing agonist-induced $[Ca^{2+}]_i$, we predict the existence of functional outputs, which if exclusively dependent on $[Ca^{2+}]_i$, may show RGS2-dependent repression. Physiologically, we believe these data make sense, as the ability to induce the expression of multiple effector processes enables the repression of signalling and gene expression events that are the result of a wide variety of different inflammatory stimuli.

Conclusions. We show that the combination of LABA plus glucocorticoid synergistically induce RGS2 expression in human bronchial epithelial BEAS-2B cells and primary HBE cells. This effect was reproduced with other cAMP-elevating agents suggesting that enhanced effects could also be realized by additional combination therapies involving ICS. Functionally, the expression of RGS2 induced by dexamethasone plus salmeterol attenuated $[Ca^{2+}]_i$ elevations elicited by agonists of $G\alpha_q$ -linked GPCRs that are implicated in the pathology of asthma and COPD. While this pathway is implicated in the expression of inflammatory genes, our data suggest that the repressive effect of RGS2 on IL-8 expression is redundant with other effector processes that are also induced by glucocorticoids and LABAs. However, assuming that non-redundant roles for RGS2 do exist (Holden et al., 2011), the induction of RGS2 expression in patients taking ICS/LABA combination therapies could represent a mechanism for the superior clinical efficacy in asthma and COPD.

Authorship Contributions

Participated in research design and/or obtained funding: Holden, Johnson, Leigh, Giembycz, Newton

Conducted experiments: Holden, George, Rider, Chandrasekhar, Shah, Kaur

Contributed new reagents or analytical tools: Siderovski

Performed data analysis: Holden, George, Rider, Kaur, Newton

Wrote or contributed to the writing of the manuscript: Holden, George, Rider, Chandrasekhar, Shah, Kaur, Johnson, Siderovski, Leigh, Giembycz, Newton.

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Footnotes

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¹ Current address: AstraZeneca R&D Mölndal, Pepparedsleden 1, Mölndal, SE-43183, Sweden.

² Current address: University of Manchester, NIHR Translational Research Facility, University Hospital of South Manchester, UK.

Figure Legends

Fig 1. Glucocorticoid plus LABA combinations synergistically enhance RGS2 protein expression. A, BEAS-2B cells were treated with dexamethasone (Dex) (1 μ M) in the absence or presence of salmeterol (Salm) (100 nM) or forskolin (Forsk) (10 μ M) (upper panels). Alternatively, BEAS-2B cells were treated with combinations of salmeterol (100 nM) and fluticasone propionate (FP) (300 nM) or formoterol (Form) (10 nM) and budesonide (Bud) (300 nM) (lower panels). Total proteins were harvested at the indicated times for western blot analysis of RGS2 and GAPDH. All data are representative of at least 3 experiments. Blots from the Dex/Salm/Forsk experiments (upper panels) were subjected to densitometric analysis and data (n = 4) are presented in Supplemental Figure 1A. B, BEAS-2B cells were treated with either salmeterol (100 nM) plus fluticasone propionate (300 nM), salmeterol (100 nM) plus dexamethasone (1 μ M) or formoterol (10 nM) plus budesonide (300 nM) prior to harvesting at the indicated times for western blot analysis of RGS2 and GAPDH. Blots representative of 3 experiments are shown and densitometric data is presented in Supplemental Figure 1B.

Fig 2. Glucocorticoid plus LABA combinations synergistically enhance RGS2 mRNA expression. A, BEAS-2B cells were treated, as indicated, with formoterol (10 nM) and dexamethasone (1 μ M) prior to harvesting for RNA and real-time PCR analysis of RGS2 and GAPDH mRNAs. Data (n = 5), normalized as RGS2/GAPDH and expressed as fold induction, are plotted as means $\pm$ SE. B, BEAS-2B cells were treated, as indicated, for 6 h, with dexamethasone (1 μ M), salmeterol (100 nM) and forskolin (10 μ M) prior to harvesting for RNA and real-time PCR analysis. Data (n = 7, left panel; 6, right panel) normalized as RGS2/GAPDH and expressed as fold induction, are plotted as means $\pm$ SE. C, Human primary bronchial epithelial cells were treated with the indicated drugs as described in A, above. At the indicated times, the cells were harvested for RNA and real-time PCR analysis of RGS2

and GAPDH. Data ($n = 6 - 7$), normalized as RGS2/GAPDH and expressed as fold induction, are plotted as means $\pm$ SE. D, Human primary bronchial epithelial cells were treated as in C and harvested after 2 h for western blot analysis of RGS2 and GAPDH. Data representative of 2 experiments is shown. In panels A, B and C, statistical analysis was performed by ANOVA with a Bonferroni post-test. All treatments were tested relative to untreated control plus the shown paired analyses. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 3. Role of the β_2 -adrenoceptor-PKA pathway in the enhancement of RGS2 expression by LABA/glucocorticoid combinations. A, BEAS-2B cells were pre-treated with ICI 118,551 (2 μ M) for 30 min prior to treatment with dexamethasone (Dex) (1 μ M) in the absence or presence of salbutamol (Salb) (1 μ M), salmeterol (100 nM) or formoterol (10 nM). After 6 h, the cells were harvested for western blot analysis of RGS2 and GAPDH. Blots representative of 3 experiments are shown. Densitometric analysis is presented in Supplemental Figure 2A. B, BEAS-2B cells were infected with either PKI α -expressing or empty (Null) adenovirus at a MOI of 30. Cells were subsequently treated with dexamethasone (1 μ M) in the absence or presence of salbutamol (1 μ M), salmeterol (100 nM) or formoterol (10 nM). After 6 h, the cells were harvested for western blot analysis of RGS2 and GAPDH. Blots representative of 3 experiments are shown. Densitometric analysis is presented in Supplemental Figure 2B. C, BEAS-2B cells were treated with dexamethasone (1 μ M) in the absence and presence of PGE₂ (1 μ M) or rolipram (30 μ M). Blots representative of 6 experiments are shown.

Fig. 4. Effect of concentration on the enhancement of RGS2 expression by glucocorticoid plus LABA combinations. A, BEAS-2B cells were variously treated with salmeterol (100 nM) plus increasing concentrations of fluticasone propionate (FP) or fluticasone propionate (300 nM) in the presence of increasing concentrations of salmeterol (upper panels). Alternatively, the cells were treated with

formoterol (Form) (10 nM) in the presence of increasing concentrations of budesonide (Bud) or budesonide (300 nM) in the presence of increasing concentrations of formoterol. After 6 h, cells were harvested for western blot analysis of RGS2 and GAPDH. Blots representative of 3 - 4 such experiments are shown. B, BEAS-2B cells were treated, as in A, with the indicated concentrations of salmeterol, fluticasone, formoterol and budesonide. Drug alone were: salmeterol (100 nM), formoterol (10 nM), fluticasone propionate 1 μ M (left panel) and 300 nM (right panel), budesonide (1 μ M) (left panel) and 300 nM (right panel). After 2 h, the cells were harvested for RNA and real-time PCR analysis of RGS2 and GAPDH mRNA. Data (n = 5), normalized as RGS2/GAPDH and expressed as a percentage of the LABA alone, were plotted as means $\pm$ SE. C, Salmeterol plus fluticasone propionate or formoterol plus budesonide were combined at the indicated concentrations (log dilution = 0). Each combination was subject to serial 10-fold dilution. BEAS-2B cells were either not treated or treated with undiluted and diluted drug combinations. After 6 h, the cells were harvested for western blot analysis of RGS2 and GAPDH. Following densitometric analysis, data (n = 3), normalized as RGS2/GAPDH are plotted as means $\pm$ SE.

Fig. 5. Effect of dexamethasone plus salmeterol on agonist-stimulated $[Ca^{2+}]_i$. A, BEAS-2B cells loaded with Fluo-4 were stimulated with various concentrations of; histamine, methacholine (MCh) or U46619. Peak fluorescence at 516 nm divided by the fluorescence at time 0 (F/F_0) from 5 or more such experiments are plotted as means $\pm$ SE. B, Cells were treated as in A, except that 2-APB (10 μ M) was added 30 min before addition of histamine (10 μ M), methacholine (30 μ M) or U46619 (1 μ M). Data (n = 3 - 7) expressed as F/F_0 are plotted as means $\pm$ SE. Significance was tested by paired t-test. C, Cells were treated as in A, except that salmeterol (Salm) (100 nM) and/or dexamethasone (Dex) (1 μ M) were added 6 h before the histamine (10 μ M), methacholine (30 μ M) or U46619 (1 μ M). Data (n = 3 - 8) expressed as peak F/F_0 are plotted as means $\pm$ SE. Significance was tested relative to untreated control

and the indicated treatments by ANOVA with a Bonferroni post-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 6. Attenuation of agonist-stimulated $[Ca^{2+}]_i$ by RGS2. A, BEAS-2B cells were infected with the indicated MOIs of Ad5.RGS2.HA or AD5.LacZ and incubated for 24 h. Following overnight incubation in serum-free medium, cells were harvested for western blot analysis of the HA epitope tag and GAPDH. Blots are representative of 4 experiments. Note: there is a faint non-specific (n.s.) band that appears just below the correct RGS2.HA band. B, Following loading with Fluo-4, cells were stimulated with histamine (10 μ M), methacholine (MCh) (30 μ M) or U46619 (1 μ M). Peak fluorescence at 516 nm divided by the fluorescence at time 0 (F/F_0) from 4 - 6 such experiments are plotted as means $\pm$ SE. Significance between RGS2 and LacZ infected samples was by t test. C, As in A and B, cells were infected with Ad5 viruses, loaded with Fluo4 and then stimulated with either LTB₄ (100 nM), or LTD₄ (30 nM), prior to measurement of F/F_0 . Data ($n = 4$) are plotted as means $\pm$ SE. Significance between RGS2 and LacZ infected samples was by t test. D, BEAS-2B cells were transfected with the indicated siRNA oligonucleotides, or transfection lipid without siRNA (Lipid) before overnight incubation in serum-free medium and treatment with salmeterol (100 nM) plus dexamethasone (1 μ M) (Salm + Dex). After 6 h, cells were harvested for western blot analysis of RGS2 and GAPDH. Blots representative of 5 experiments are shown. E, Following loading with Fluo-4, cells were treated with histamine (10 μ M), methacholine (30 μ M) or U46619 (1 μ M). Peak fluorescence at 516 nm divided by the fluorescence at time 0 (F/F_0) was measured. Data ($n = 4 - 9$) are plotted as means $\pm$ SE. For each test group (naive, GFP control siRNA, RGS2-O2 siRNA, RGS2-O6 siRNA), significance was tested between untreated and Salm + Dex treated by t test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 7. The role of RGS2 in the repression of IL-8 release by salmeterol and dexamethasone. A, BEAS-2B cells were pre-treated, as indicated, with salmeterol (100 nM) (Salm) and dexamethasone (1 μ M) (Dex) for 6 h. Cells were then stimulated with histamine (10 μ M), methacholine (30 μ M) (MCh), or U46619 (1 μ M). Supernatants were harvested at 6 and 18 h for analysis of IL-8 release. Data (n = 6) are plotted as means $\pm$ SE. Significance, between untreated and stimulated samples and then between stimulated samples and those with Salm and/or Dex was tested by ANOVA with a Bonferroni post test. B, BEAS-2B cells were infected with the indicated MOIs of Ad5-RGS2.HA or Ad5-GFP and incubated for 24 h. Following overnight incubation in serum free medium, cells were either not stimulated (NS) or stimulated with MCh (10 μ M) or U46619 (1 μ M). Supernatants were harvested at 6 h for analysis of IL-8 release and data (n = 4) are plotted as means $\pm$ SE. Significance between Ad5-RGS2 and Ad5-GFP infected samples was by t-test. C, BEAS-2B cells were transfected with the indicated oligonucleotides, or transfection lipid without siRNA (Lipid) before over-night incubation in serum free media. Cells were treated with salmeterol (100 nM) and dexamethasone (1 μ M) (Salm+Dex) for 6 h prior to harvesting for western blot analysis of RGS2 and GAPDH. Blots are representative of 4 such experiments. D, Cells treated alongside those in C, were stimulated with U46619 (1 μ M) and supernatants harvested after 6 h for analysis of IL-8 release. Data (n = 4) are plotted as means $\pm$ SE and significance was tested as in A. E, BEAS-2B cells were pre-treated with 2-APB (10 μ M), Ro 31-8220 (10 μ M), G α 6976 (1 μ M) or GF109203X (1 μ M) for 30 min prior to treatment with U46619 (1 μ M). After 6 h, supernatants were harvested for analysis of IL-8 release. Data (n = 6) are plotted as means $\pm$ SE and significance was tested as in A. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Fig. 8. MAPK activation and their role in the induction and repression of IL-8 expression. BEAS-2B cells were treated with histamine (10 μ M) (Hist), methacholine (30 μ M) (MCh) or U46619 (1 μ M) for the indicated times. Cells were harvested for western blot analysis of phospho-ERK (P-ERK), phospho-JNK (P-p54, P-p46), phospho-p38 MAPK (P-p38), phospho-ATF2 (P-ATF2) and GAPDH. Blots

representative of 4 such experiments are shown. B, BEAS-2B cells were pretreated for 30 min with PD098059 (10 μ M), U0126 (1 μ M), SB203580 (1 μ M) or JNK inhibitor 8 (1 μ M) (JNK8) prior to stimulation with U46619 (1 μ M). After 6 h, supernatants were harvested for analysis of IL-8 and data are plotted as means $\pm$ SE. Significance, between untreated and stimulated (Hist, MCh, U46619) samples and then between stimulated samples and those with Salm and/or Dex was tested by ANOVA with a Bonferroni post test. C, BEAS-2B cells were treated with salmeterol (100 nM) (Salm) and dexamethasone (1 μ M) (Dex) and harvested at the indicated times for western blot analysis of MKP-1 and GAPDH. Blots representative of 4 such experiments are shown. D, BEAS-2B cells were pretreated with salmeterol (100 nM) and dexamethasone (1 μ M) for 6 h prior to treatment with U46619 (1 μ M). After 30 min, cells were harvested for western blot analysis of phosphorylated MAPKs and ATF2. blots are representative of 6 such experiments. E, BEAS-2B cells were infected with the indicated MOI of Ad5.MKP-1 or Ad5.GFP and incubated for 24 h. Following overnight incubation in serum free medium, cells were either not stimulated (NS) or stimulated with MCh (10 μ M) or U46619 (1 μ M). Supernatants were harvested at 6 h for analysis of IL-8 release and data (n = 4) are plotted as means $\pm$ SE. Significance between Ad5.MKP-1 and Ad5.GFP infected samples was by t-test.

Fig. 1

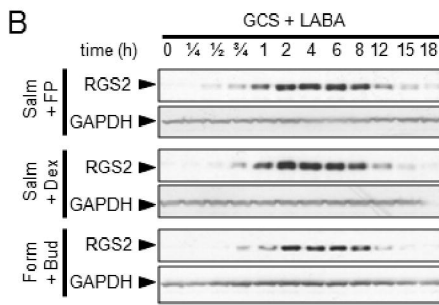
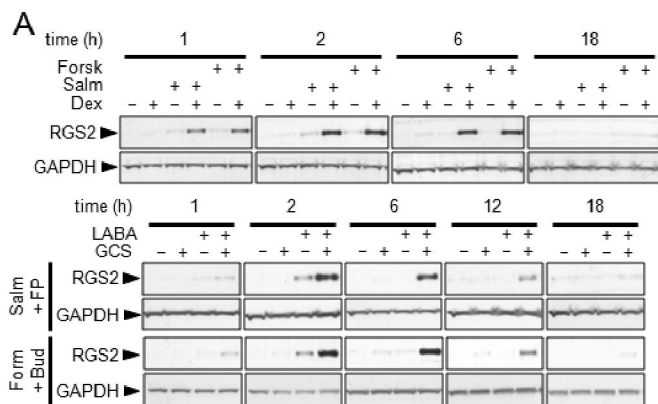


Fig. 2

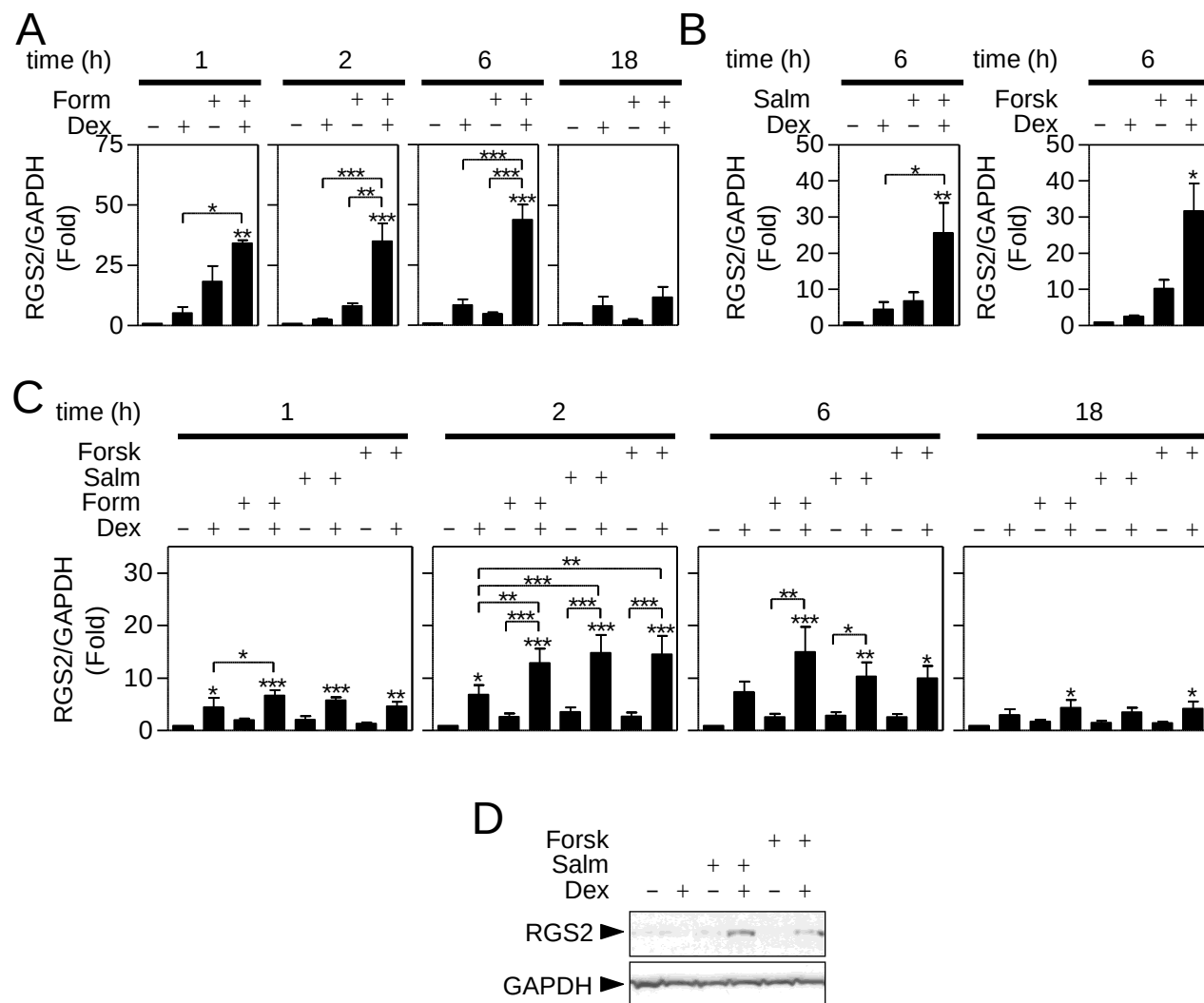


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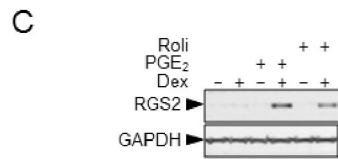
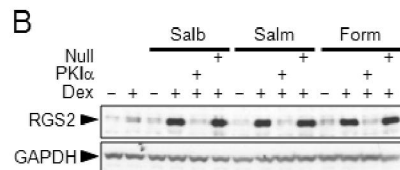
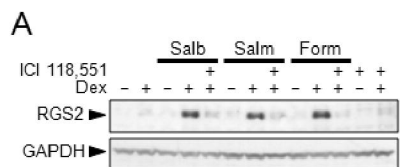


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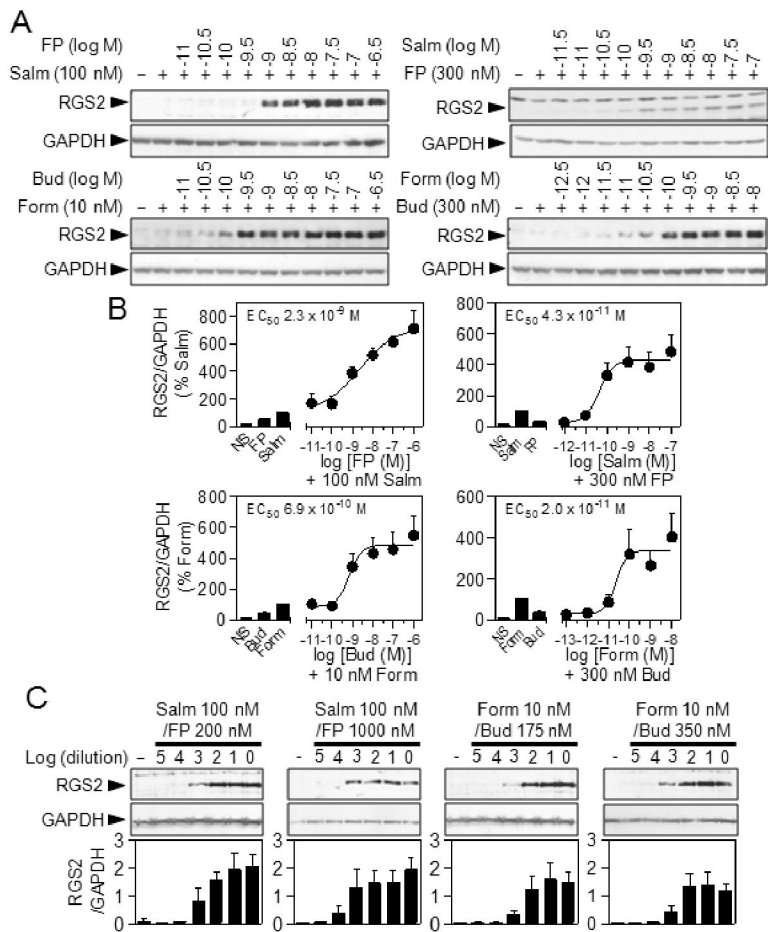


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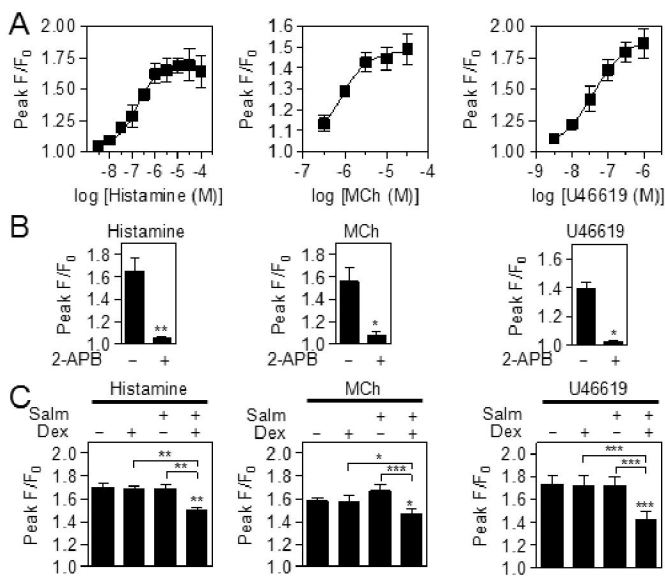


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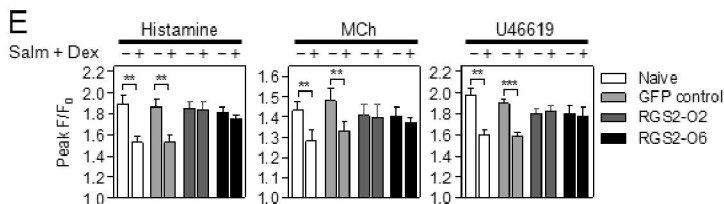
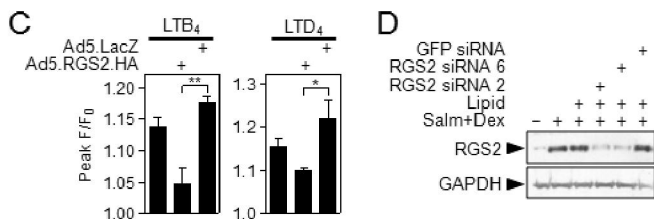
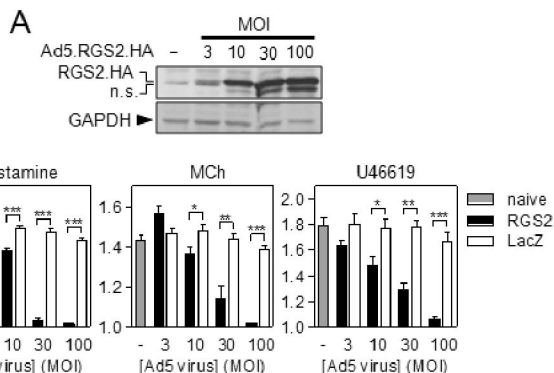


Fig. 7

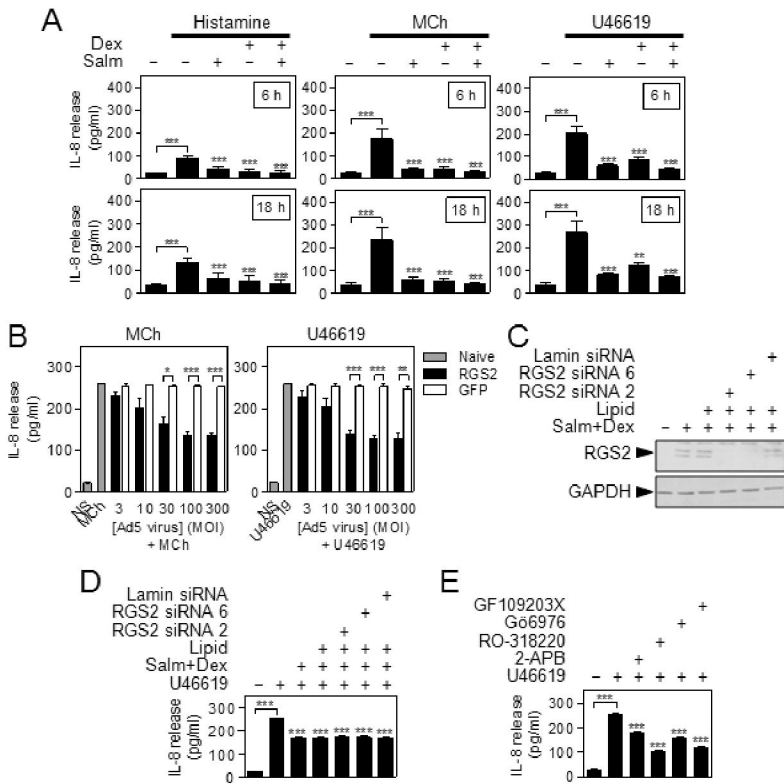
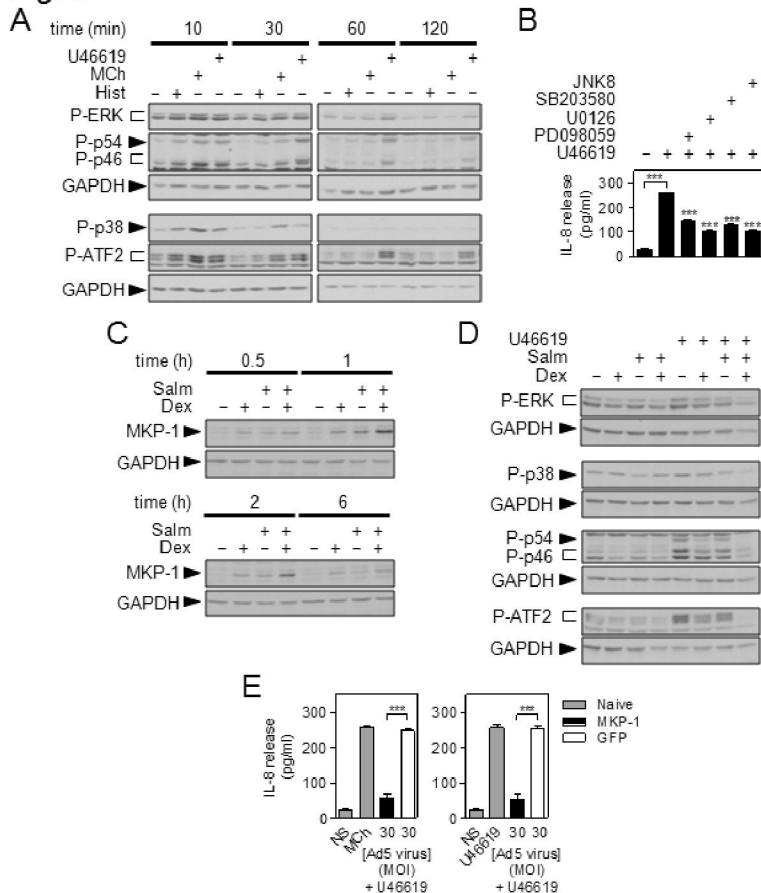


Fig. 8

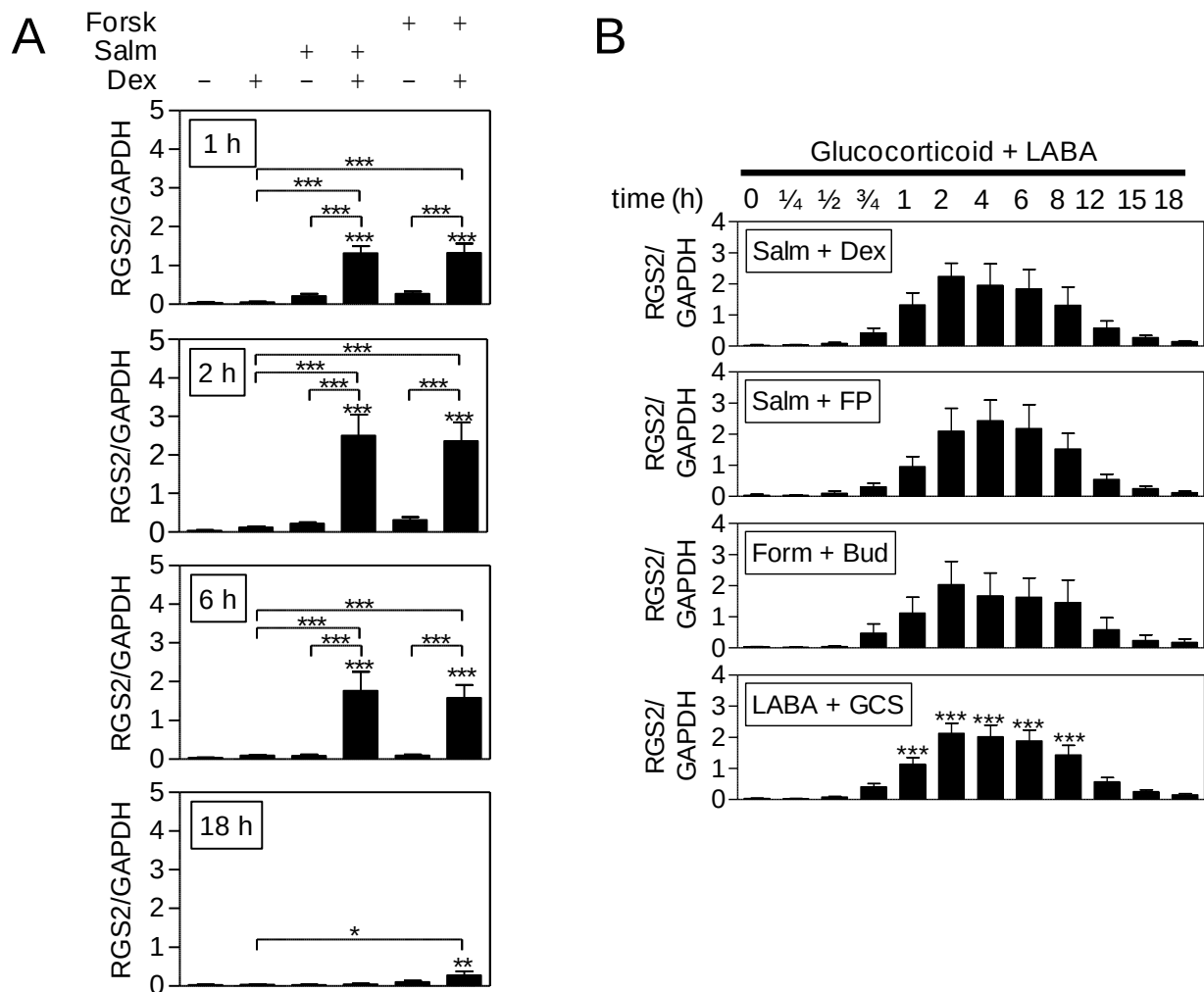


Supplemental data

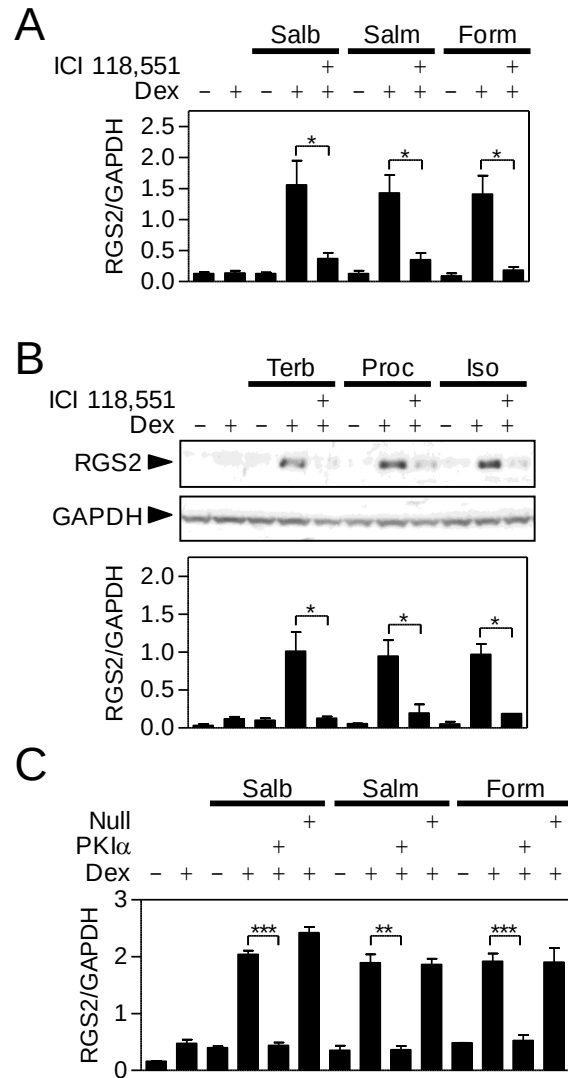
Induction of RGS2 expression by long-acting β_2 -adrenoceptor agonists and glucocorticoids in human airway epithelial cells

Neil S. Holden¹, Tresa George, Christopher F. Rider, Ambika Chandrasekhar, Suharsh Shah, Manminder Kaur², Malcolm Johnson, David P. Siderovski, Richard Leigh, Mark A. Giembycz, Robert Newton

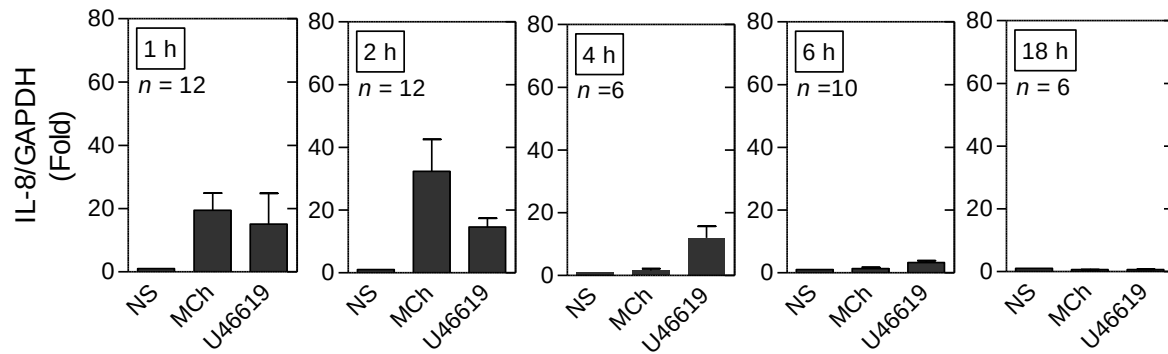
Airways Inflammation Research Group, Snyder Institute for Chronic Diseases, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada (N.S.H., T.G., C.F.R., A.C., S.S., M.K., R.L., M.A.G., R. N.), GlaxoSmithKline Research and Development, Uxbridge, Middlesex, UK. (M.J.), Department of Physiology and Pharmacology, School of Medicine, West Virginia University, West Virginia, USA. (D.P.S.).



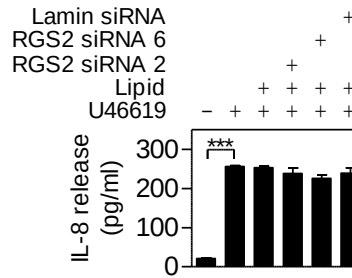
Supplemental Fig. 1. Expression of RGS2 in BEAS-2B cells. A, As described in Figure 1 of the main manuscript, BEAS-2B cells were treated with dexamethasone (Dex) (1 μ M) in the absence or presence of salmeterol (Salm) (100 nM) or forskolin (Forsk) (10 μ M). Total proteins were harvested at the indicated times for western blot analysis of RGS2 and GAPDH. Following densitometric analysis, data (n = 4), normalized as RGS2/GAPDH and expressed as fold induction, are plotted as means $\pm$ SE. Statistical analysis was performed by ANOVA with a Bonferroni post-test. Treatments for each time point were tested relative to untreated control plus the shown comparisons. B, BEAS-2B cells were treated with either salmeterol (100 nM) plus fluticasone propionate (300 nM), salmeterol (100 nM) plus dexamethasone (1 μ M) or formoterol (10 nM) plus budesonide (300 nM) prior to harvesting at the indicated times for western blot analysis of RGS2 and GAPDH. Following densitometric analysis, data (n = 3 for each treatment, upper 3 panels), normalized as RGS2/GAPDH and expressed as fold, are plotted as means $\pm$ SE. Given equivalent data in respect of each glucocorticoid + LABA combination, data were combined in the lower panel to provide an overall summation for these treatments. Statistical analyses, relative to t = 0, were performed by ANOVA with a Dunnett's post-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



Supplemental Fig. 2. Role of the β_2 -adrenoceptor-PKA pathway in the enhancement of RGS2 expression by LABA/glucocorticoid combinations. A, BEAS-2B cells were pre-treated with ICI 118,551 (2 μ M) for 30 min prior to treatment with dexamethasone (Dex) (1 μ M) in the absence or presence of salbutamol (Salb) (1 μ M), salmeterol (100 nM) or formoterol (10 nM). After 6 h, the cells were harvested for western blot analysis of RGS2 and GAPDH. Blots representative of 3 experiments are shown in Figure 3A of the main manuscript. Following densitometric analysis, data, normalized as RGS2/GAPDH, are presented as means $\pm$ SE. Paired t-test was performed between treatments in the absence and presence of ICI 118,551. B, BEAS-2B cells were pre-treated with ICI 118,551 (2 μ M) for 30 min prior to treatment with dexamethasone (Dex) (1 μ M) in the absence or presence of terbutaline (Terb) (10 μ M), procaterol (1 μ M) or isoprenaline (1 μ M). After 6 h, the cells were harvested for western blot analysis of RGS2 and GAPDH. Blots representative of 3 experiments are shown. Following densitometric analysis, data, normalized as RGS2/GAPDH, are presented as means $\pm$ SE. Paired t-tests were performed between treatments in the absence and presence of ICI 118,551. C, BEAS-2B cells were infected with either PKI α -expressing or empty (Null) adenovirus at a MOI of 30. Cells were subsequently treated with dexamethasone (1 μ M) in the absence or presence of salbutamol (1 μ M), salmeterol (100 nM) or formoterol (10 nM). After 6 h, the cells were harvested for western blot analysis of RGS2 and GAPDH. Blots representative of 3 experiments are shown in Figure 3B of the main manuscript. Following densitometric analysis, data, normalized as RGS2/GAPDH, are plotted as means $\pm$ SE. Paired t-tests were performed between treatments in the absence and presence of Ad5-PKI α . * $P < 0.05$.



Supplemental Fig 3. Effect of methacholine and U46619 on IL-8 mRNA expression. BEAS-2B cells were either not stimulated (NS) or stimulated with methacholine (30 μ M) (MCh) or U46619 (1 μ M) for the indicated times. RNA was extracted for real-time PCR analysis of IL-8 and GAPDH as previously described (King et al. 2013).



Supplemental Fig 4. BEAS-2B cells were transfected with the indicated oligonucleotides, or transfection lipid without siRNA (Lipid) before over-night incubation in serum free media. Cells were treated with U46619 (1 μ M) for 6 h and the supernatants collected for analysis of IL-8 release. Full experimental details are as described for Fig. 7D of the main manuscript. Data (n = 4) are plotted as means $\pm$ SE and significance was tested by ANOVA with a Bonferroni post test. *** $P < 0.001$.