Induction of Regulator of G-Protein Signaling 2 Expression by Long-Acting β₂-Adrenoceptor Agonists and Glucocorticoids in Human Airway Epithelial Cells


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ABSTRACT

In asthma and chronic obstructive pulmonary disease (COPD) multiple mediators act on Gαq-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 (bronchial epithelium + adenovirus 12-SV40 hybrid), regulator of G-protein signaling (RGS) 2 mRNA and protein were synergistically induced in response to combinations of long-acting β₂-adrenoceptor agonist (LABA) (salmeterol, for- meterol) 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 Gαq, intracellular free calcium ([Ca²⁺]i) flux was used as a surrogate of responses induced by histamine, methacholine, and the thromboxane receptor agonist U46619 [(Z)-7-[(15,4R,5R,6S)-5-[(E,3S)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid]. This was significantly attenuated by salmeterol plus dexamethasone pretreatment, or RGS2 overexpression, and the protective effect of salmeterol plus dexamethasone was abolished by RGS2 RNA silencing. Although methacholine and U46619 induced interleukin-8 (IL-8) release and this was inhibited by RGS2 overexpression, the repression of U46619-induced IL-8 release by salmeterol plus dexamethasone was unaffected by RGS2 knockdown. Given a role for Gαq-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 third 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 (Knight and Holgate, 2003; Rabe et al., 2007; Bateman et al., 2008). Although ICSs are the mainstay of asthma therapy, short-acting β₂-adrenoceptor agonists (SABAs) provide “as-needed” relief from bronchoconstriction, and practice guidelines recommend ICS/long-acting β₂-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 (Rabe et al., 2007; Bateman et al., 2008). 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 (Shrout et al., 2006; 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. Although 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 glucocorticoids 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 κB (NF-κB), and activator protein 1 (AP-1), that are implicated in inflammatory gene expression (Clark et al., 2008; Diefenbacher et al., 2008; King et al., 2009). However, although MKP-1 expression is also increased by β2-adrenoceptor agonists, the combination of glucocorticoid plus β2-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, the expression of regulator of G-protein signaling 2 (RGS2), a G protein-coupled receptor (GPCR), 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 β2-adrenoceptor, including airway epithelia, might be affected similarly.

Agonists of various GPCRs, including acetylcholine, histamine, cysteinyl-leukotrienes and certain prostanoids, activate phospholipase Cβ (PLCβ) via Gαq and increase the intracellular cytosolic free Ca2+ concentration ([Ca2+]i) via an inositol (1,4,5)trisphosphate (IP3)-dependent mechanism (Gosens et al., 2006; Penn and Benovic, 2008). It is well established that increasing [Ca2+]i promotes a variety of proinflammatory effects (Gelfand, 2002; Holgate et al., 2003; Gosens et al., 2006; Gwilt et al., 2007). Indeed, leukotriene (LT) D4 induces, and CysLT1 receptor antagonism reduces, inflammatory cell infiltration, eosinophil trafficking, cytokine release, and other inflammatory responses (Henderson et al., 2002, 2006; Holgate et al., 2003). Similarly, in human airway epithelial cells, histamine acts on the Gαq-linked H1 receptor to increase [Ca2+]i, NF-κB 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-κB to promote cytokine release from airway epithelial cells (Koyama et al., 1992, 1998; Profita et al., 2008). 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 Gαq-linked thromboxane A2 (TP) receptor (Jones et al., 2009). 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 [Ca2+]i mobilization. The central role of [Ca2+]i, in driving a diverse array of responses provides a reliable and well-validated marker of Gαq-linked GPCR activation with which to assess the functional consequences of RGS2 up-regulation. The effect on IL-8 release is 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 F-12 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 elsewhere (Newton et al., 2007). Cells were incubated overnight in serum-free or growth factor-free medium before 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-[(1R,4S)-4-methoxyphenyl]propan-2-yl-malonato][ethyl] phenyl]formamidine (formoterol) and budesonide [16-(butylenedioxy)-11,12-dihydroxy-((11β,16α)-pregna-1,4-diene-3,20-dione) (both AstraZeneca, Mölndal, Sweden), salmeterol xinafoate [[[(3S)-fluoromethyl]-6,9-difluoro-11β, 17-dihydroxy-16α-methyl-3-oxoandrosta-1,4-diene-17β-carbothioate, 17-propanoate] (FP), U46619 [(Z)-7-[[15(S),4R,5R,8S]-5,6,7,13-tetrahydro-13-methyl-5-oxoanandrosta-1,4-diene-3-yl]oxy]-3-hydroxyoct-1-ene-1-yl]oxy]-3-oxabicyclo[2.2.1]hept-6-yl]hepta-6-ene-5-anoic acid] (Cyman Chemical, Ann Arbor, MI), forskolin [(3,4a,7,7,10a-pentamethyl-1-oxo-3-vinyldodecahydro-1H-benzo[c]chromen-5-yl)acetate], prostaglandin (PG) E2, LTB4, LTD4, rolipram [(3,4,5-trihydroxy-4-methyl-6-[3-(isopropylamino)-1-[(7-methyl-4-indanyl)oxy] butan-2-ol], and 2-aminothyldiphenyl borate; ANOVA, analysis of variance; AP, activator protein; ASM, airway smooth muscle; ATP2, activating transcription factor 2; BEAS-2B, bronchial epithelium + adenovirus 12-SV40 hybrid cell line; COPD, chronic obstructive pulmonary disease; DMEM, Dulbecco's Modified Eagle's medium; DMSO, dimethylsulfoxide; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular-regulated kinase; F, fluorescence; FCS, fetal calf serum; FFBS, binding protein 51; FP, fluticasone propionate; GAP, GTPase-activating protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GF109203X [2-[1-(3-dimethylamino propyl)indol-3-yl]-3-(indol-3-yl) maleimide]; GILZ, glucocorticoid-induced leucine zipper; G06876, 5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile; GPCR, G-protein-coupled receptor; GRE, glucocorticoid response element; HBE, human bronchial epithelial; ICI, 0118551, 2-(3-[(4S)-2-(trans-pent-4-enyloxy)butan-2-yl)-1H-indol-3-yl]-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile; IL, interleukin; IP3, inositol (1,4,5)trisphosphate; LABA, long-acting β2-adrenoceptor agonist; LAF, leukotriene; MAPK, mitogen-activated protein kinase; MCH, methacholine; MKP, mitogen-activated protein kinase phosphatase; MOI, multiplicity of infection; NF, nuclear factor; PCR, polymerase chain reaction; PDE, phosphodiesterase; PG, prostaglandin; PGE2, prostaglandin E2; PKA, protein kinase A; PLCβ, phospholipase Cβ; RGS, regulator of G-protein signaling; Ro 31-8220, 3-(3-[(4-R,1H)-1H-indol-3-yl]-2,5-dioxo-2,5-dihydro-1H-pyrryl-3-y1]-1H-indol-1-yl)propyl carbamimidothioate; SAB, short-acting β2-adrenoceptor agonist; sRNA, small interfering RNA; TP, thromboxane A2; U46619, (Z)-7-[[15(S),4R,5R,8S]-5,6,7,13-tetrahydro-13-methyl-5-oxoanandrosta-1,4-diene-3-yl]oxy]-3-hydroxyoct-1-ene-1-yl]oxy]-3-oxabicyclo[2.2.1]hept-6-yl]hepta-6-ene-5-anoic acid.
17-dodecahydro-3H-cyclopenta[6]phenanthren-3-one], histamine [2-(1H-imidazol-4-yl)ethanamine], and methacholine [2-acetyloxy]-N,N,N-trimethylpropan-1-aminium] were dissolved in Hanks’ balanced salt solution (Sigma-Aldrich Canada).

**Western Blotting, Enzyme-Linked Immunosorbent Assay, Small Interfering RNA Knockdown, and Adenoviral Infections.** Western blotting, small interfering RNA (siRNA) transfections, adenoviral infections, and enzyme-linked immunosorbent assay (ELISA) were performed according to standard protocols (King et al., 2009; Holden et al., 2011). Commerically available antibodies were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (4699-9555/ST) from AbD Serotec (Raleigh, NC); anti-HA (clone HA-7) (H3663) from Sigma-Aldrich (St. Louis, MO); phospho-p44/42 MAPK (Erk1/2) (9101); phospho-SAPK/JNK (9251); phospho-p38 MAPK (9211); phospho-ATF2 (9221) from Cell Signaling Technology (Danvers, MA); and MKP-1 (M-18) (sc-1102) from Santa Cruz Biotechnology (Dallas, TX); and the RGS2 antibody was previously described elsewhere (Holden et al., 2011). Detection was by enhanced chemiluminescence (GE Healthcare Bio-Sciences, Pittsburgh, PA) with visualization by autoradiography. Transfections for siRNA were performed using Lipofectamine 2000 as described elsewhere (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'-AAG GGT ATA CAG CTT GAT GGA-3'), and MKP-1 siRNA (SI03650332; 5'-AAG GGT ATA CAG CTT GAT GGA-3'). These changes in RGS2 protein correlated closely with the modulation of RGS2 mRNA expression (Fig. 2, A and B). Dexamethasone was a weak inducer of RGS2 mRNA, while formoterol or salmeterol produced larger increases, which, when combined with dexamethasone, synergistically enhanced (1 µM) (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 detectable increase in RGS2 expression that was maximal at 2 hours, but had declined by 6 hours (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 minutes and extended for at least 12–15 hours after the treatment (Fig. 1, A and B). This response was significantly greater than the effect of either treatment alone at 1, 2, and 6 hours (Fig. 1A; Supplemental Fig. 1A). Equivalent effects were observed with salmeterol in the presence of FP and by combinations of formoterol plus budesonide (Fig. 1, A and B; Supplemental Fig. 1). Forskolin, an activator of adenylyl cyclase, mimicked the effect of the LABAs in 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. 2, A and B). Dexamethasone was a weak inducer of RGS2 mRNA, while formoterol or salmeterol produced larger increases, which, when combined with dexamethasone, synergistically enhanced

**Fig. 1.** Glucocorticoid plus LABA combinations synergistically enhance RGS2 protein expression. (A) BEAS-2B cells were treated with dexamethasone (Dex) (1 µM) in the absence or presence of salmeterol (Salm) (100 nM) or forskolin (Forsk) (10 µM) (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 three experiments. Blots from the Dex/Salm/Forsk experiments (upper panels) were subjected to densitometric analysis and data (n = 4) are presented in Supplemental Fig. 1A. (B) BEAS-2B cells were treated with either salmeterol (100 nM) plus fluticasone propionate (300 nM), salmeterol (100 nM) plus dexamethasone (1 µM) or formoterol (10 µM) plus budesonide (300 nM) before harvesting at the indicated times for Western blot analysis of RGS2 and GAPDH. Blots representative of three experiments are shown, and densitometric data are presented in Supplemental Fig. 1B.

**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...
RGS2 mRNA expression (Fig. 2, A and 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 $\beta_2$-Adrenoceptor Agonists Occurs via the $\beta_2$-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 $\beta_2$-adrenoceptor agonist plus dexamethasone robustly increased the expression of RGS2 in a manner that was significantly prevented by prior addition of the $\beta_2$-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 $\beta_2$-adrenoceptor agonists was also observed for terbutaline, procaterol, and the nonselective $\beta$-agonist isoprenaline (Supplemental Fig. 2B).

To explore the role of protein kinase A (PKA), a cAMP-dependent kinase, BEAS-2B cells were infected with an adenovirus overexpressing PKI$\alpha$, a potent and selective inhibitor of PKA (Meja et al., 2004). In each case, the PKI$\alpha$-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 Fig. 1, we showed that forskolin mimics the effect of $\beta_2$-adrenoceptor agonists at enhancing the expression of RGS2 in the presence of glucocorticoid. The effect of $\beta_2$-adrenoceptor agonists was blocked by ICI 118,551 and PKI$\alpha$, so a classic $\beta_2$-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. Because, in BEAS-2B cells, PGE$_2$ activates PKA and enhances glucocorticoid response element (GRE)-dependent transcription (Meja et al., 2004; Kaur et al., 2008), BEAS-2B cells were treated with PGE$_2$ in the absence and presence of dexamethasone. In the presence of dexamethasone, PGE$_2$, produced a robust increase in RGS2 expression (Fig. 3C). Equally, inhibition of phosphodiesterase 4 (PDE4), 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, before harvesting for Western blot analysis of RGS2 and GAPDH. Densitometric analysis is presented in Supplemental Fig. 2A. (B) BEAS-2B cells were infected with either PKI expressing or empty (Null) adenovirus at a MOI of 30. Cells were subsequently treated with dexamethasone (1 μM) in the absence or presence of salbutamol (1 μM), salmeterol (100 nM), or formoterol (10 nM). After 6 hours, the cells were harvested for Western blot analysis of RGS2 and GAPDH. Blots representative of three experiments are shown. (C) BEAS-2B cells were treated with dexamethasone (1 μM) in the absence or presence of PGE2 (1 μM) or rolipram (30 μM). Blots representative of six experiments are shown.

![Fig. 3. Role of the β2-adrenoceptor-PKA pathway in the enhancement of RGS2 expression by LABA/glucocorticoid combinations.](image)

**Combination Therapies.**

RGS2 Attenuates Elevations in [Ca^{2+}]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 [Ca^{2+}]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 classic Gq/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 hours, before 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 [Ca^{2+}]i (Fig. 5C). However, in combination, salmeterol plus dexamethasone significantly attenuated the rise in [Ca^{2+}]i that was induced by each agonist (Fig. 5C). Thus, inhibition of responses mediated by Gq-linked GPCRs by salmeterol plus dexamethasone correlates with the expression of RGS2.

**Agonists of Gq-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 [Ca^{2+}]i produced by histamine, methacholine and U46619 was significantly inhibited, whereas an adenovirus overexpressing LacZ was without effect (Fig. 6B). Stimulation of BEAS-2B cells with either LTB4 or LTD4 resulted in very small increases in [Ca^{2+}]i. Although this may be due to low receptor expression and/or weak coupling efficiency, these responses were nevertheless prevented by RGS2 overexpression and were unaffected by the LacZ virus (Fig. 6C).

To address whether RGS2 plays a role in the repression of agonist-induced elevations in [Ca^{2+}]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, naïve cells, and cells transfected with RGS2 targeting and control siRNA were pretreated with salmeterol plus dexamethasone before stimulation with histamine, methacholine, or U46619. Salmeterol plus dexamethasone attenuated agonist-enhanced [Ca^{2+}]i in the naïve and GFP control cells (Fig. 6E). However, formoterol produced EC_{50} values of 43 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 and 500/50, and Symbicort 100/6 and 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 hours before Western blot analysis. RGS2 expression was clearly induced after 1000-fold dilutions of each drug combination (Fig. 4C). This corresponds to low nanomolar and subnanomolar concentrations of each glucocorticoid and picomolar concentrations of the LABAs.

**Effect of Glucocorticoid and LABA on [Ca^{2+}]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 [Ca^{2+}]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 classic Gq/PLC pathway.
in the presence of the two RGS2 targeting siRNA, this ability of salmeterol plus dexamethasone to reduce agonist-enhanced \([\text{Ca}^{2+}]_i\) was prevented. These data confirm that RGS2 expression induced by salmeterol plus dexamethasone is responsible for attenuating the rise in \([\text{Ca}^{2+}]_i\) 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 \(\mu M\)), methacholine (MCh) (30 \(\mu M\)), or U46619 (1 \(\mu M\)), IL-8 release was increased at 6 and 18 hours in a manner that was prevented by 6 hours of pretreatment 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 hours. This is consistent with the rapid rise in IL-8 mRNA that was maximal at 1 to 2 hours, but had declined by 6 hours (Supplemental Fig. 3). Therefore, to assess effect of RGS2 expression, BEAS-2B cells were infected with various MOIs of RGS2 expressing, or control, adenoviruses before 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. Overexpression 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 before treatment of 6 hours 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: the 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 (Fig. 7D; Supplemental Fig. 4). This was unaffected by any of the siRNA treatments (Supplemental Fig. 4). Although 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 2-fold. The ability of Ad5-RGS2 to repress IL-8 release represents an artifact due...
to overexpression, and RGS2 may not in fact play any role in the observed repression. Alternatively, RGS2 may, as shown for agonist-induced [Ca\(^{2+}\)], (Fig. 6), reduce signaling 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 the main discussion for further detail). To help distinguish these alternatives, BEAS-2B cells were treated with U46619 in the presence of 1) 2-APB, which blocks IP\(_3\) receptors and, as shown in Fig. 5B, prevents agonist-stimulated rises in [Ca\(^{2+}\)]; and 2) Ro 31-8220, O66976, 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 (Fig. 7E). Parallel phosphorylation of activating transcription factor 2 (ATF2), 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 (PD980598, 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 hours relative to either treatment alone (Fig. 8C).

Using Western blotting of phosphorylated ERK, p38 and JNK, as well as the downstream transcription factor ATF2, 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 signaling (Clark et al., 2008), including in airway epithelial cells (King et al., 2009), we infected BEAS-2B cells with an MKP-1 expressing adenovirus before stimulation with either MCh or U46619 (Fig. 8E). Western blotting confirmed the overexpression of MKP-1 (data not shown), and we have previously shown this to prevent MAPK activation in A549 and BEAS-2B cells by IL-1β and tumor-necrosis factor α (TNFα) (King et al., 2009; Newton et al., 2010a). 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 with either monotherapy. However, for drug development programs to more successfully exploit this pharmacology, it is desirable to comprehensively understand the underlying mechanistic basis. We had previously identified RGS2 as a gene whose expression was increased by glucocorticoids in A549 pulmonary epithelial cells (Chivers et al., 2006), but RGS2 expression is also enhanced by agonists that activate the cAMP signaling cascade (Tsingotjidou et al., 2002; Roy et al., 2006). Because inhaled therapies necessarily impact 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. When we used BEAS-2B bronchial epithelial cells, the glucocorticoids

**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. After 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 2 (ATF2), 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 (PD980598, U0126), p38 (SB203580), and JNK (JNK inhibitor 8) MAPK pathways significantly reduced the expression of IL-8 when induced by U46619 (Fig. 8B).
isoprenaline was prevented by a selective LABAs, SABAs, and the nonselective (formoterol and salmeterol). In addition, SABAs plus glucocorticoids (dexamethasone, budesonide, and FP) and two LABAs to multiple physiologic and pathophysiologic 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 nonselective β2-adrenoceptor agonist isoprenaline was prevented by a selective β2-adrenoceptor antagonist. Finally, overexpression of PKIα, a potent and selective inhibitor of PKA (Meja et al., 2004), prevented the enhancement of RGS2 expression by β2-adrenoceptor agonists. Thus, in the presence of glucocorticoids, the classic β2-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 useful in combination with a glucocorticoid. This is consistent with prior findings that these cAMP-elevating agonists may enhance glucocorticoid-induced expression of multiple, potentially protective, genes including RGS2 (Kaur et al., 2008; Greer et al., 2013; Moodley et al., 2013).

**Comparison of In Vivo and In Vitro Drug Administration.** Although 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 and that PDE inhibitors and adenosine A2B agonists may enhance glucocorticoid-induced expression of multiple, potentially protective, genes including RGS2 (Kaur et al., 2008; Greer et al., 2013; Moodley et al., 2013).
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 0.1–1.0 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 μg per 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).

Fig. 7. The role of RGS2 in the repression of IL-8 release by salmeterol and dexamethasone. (A) BEAS-2B cells were pretreated, as indicated, with salmeterol (100 nM) (Salm) and dexamethasone (1 μM) (Dex) for 6 hours. Cells were then stimulated with histamine (10 μM), methacholine (30 μM) (MCh), or U46619 (1 μM). Supernatants were harvested at 6 and 18 hours for analysis of IL-8 release. Data (n = 6) are plotted as mean ± S.E. 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 hours. After overnight incubation in serum-free medium, cells were either not stimulated (NS) or stimulated with MCh (10 μM) or U46619 (1 μM). Supernatants were harvested at 6 hours for analysis of IL-8 release, and the data (n = 4) are plotted as mean ± S.E. Significance between Ad5-RGS2 and Ad5-GFP infected samples was by t test. (C) BEAS-2B cells were transfected with the indicated oligonucleotides or with transfection lipid without siRNA (Lipid) before overnight incubation in serum-free media. Cells were treated with salmeterol (100 nM) and dexamethasone (1 μM) (Salm+Dex) for 6 hours before harvesting for Western blot analysis of RGS2 and GAPDH. Blots are representative of four such experiments. (D) Cells treated alongside those in C were stimulated with U46619 (1 μM), and the supernatants were harvested after 6 hours for analysis of IL-8 release. Data (n = 4) are plotted as mean ± S.E., and significance was tested as in A. (E) BEAS-2B cells were pretreated with 2-APB (10 μM), Ro 31-8220 (10 μM), Gӧ6976 (1 μM), or GF109203X (1 μM) for 30 minutes before treatment with U46619 (1 μM). After 6 hours, supernatants were harvested for analysis of IL-8 release. Data (n = 6) are plotted as mean ± S.E., and significance was tested as in A.
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, although we have shown clear synergy in respect of RGS2 expression in cell culture—that is, where drug-containing media continuously bathe the cells for relatively short periods of time (typically up to 24 hours)—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 dosage, and the specific pharmacodynamic and pharmacokinetic properties of each drug will lead to variable and most frequently submaximal concentrations that are difficult to accurately model in vitro. Hence, the current demonstration of biologic synergy in vitro should promote clinical studies to address this effect in individuals undergoing relevant therapies.

Effects of RGS2 Downstream of Goq. The functional significance of RGS2 induction was examined using a range of agonists that promote Ca2+ mobilization via Goq-linked receptors that are relevant to the pathogenesis of asthma and/or COPD. Increased [Ca2+]i is a readily measurable and established proximal marker of Goq-linked GPCR activation, which is central to downstream responses (Gosens et al., 2006; Penn and Benovic, 2008). Thus, histamine (acting through the H1-receptor), methacholine (acting through the M3-receptor), and the thromboxane A2 (TP) receptor agonist U46619 concentration-dependently increased [Ca2+]i, in BEAS-2B airway epithelial cells. For each agonist, this effect was prevented by the IP3 receptor antagonist 2-APB, supporting a Gq-linked pathway. RGS2 induction and repression of IL-8 expression.

Fig. 8. MAPK activation and their role in the induction and repression of IL-8 expression. (A) BEAS-2B cells were treated with histamine (10 μM) (Hist), methacholine (30 μM) (MCh), or U46619 (1 μM) for the indicated times. Cells were harvested for Western blot analysis of phospho-ERK (P-ERK), phospho-JNK (P-p54), phospho-p38 MAPK (P-p38), phospho-activating transcription factor 2 (P-ATF2), and GAPDH. Blots representative of four such experiments are shown. (B) BEAS-2B cells were pretreated for 30 minutes with PD098059 (10 μM), U0126 (1 μM), SB203580 (1 μM) or JNK inhibitor 8 (1 μM) (JNK8) before stimulation with U46619 (1 μM). After 6 hours, the supernatants were harvested for analysis of IL-8, and the data are plotted as mean ± S.E. 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 μM) (Dex) and harvested at the indicated times for Western blot analysis of MKP-1 and GAPDH. Blots representative of four such experiments are shown. (D) BEAS-2B cells were pretreated with salmeterol (100 nM) and dexamethasone (1 μM) for 6 hours before treatment with U46619 (1 μM). After 30 minutes, cells were harvested for Western blot analysis of phosphorylated MAPKs and ATF2. Blots are representative of six such experiments. (E) BEAS-2B cells were infected with the indicated MOI of Ad5.MKP-1 or Ad5.GFP and incubated for 24 hours. After overnight incubation in serum-free medium, cells were either not stimulated (NS) or stimulated with MCh (10 μM) or U46619 (1 μM). Supernatants were harvested at 6 hours for analysis of IL-8 release and data (n = 4) are plotted as mean ± S.E. Significance between Ad5.MKP-1 and Ad5.GFP infected samples was by t test.
previously described bronchoprotective effect in airway smooth muscle (Holdsen 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; Gienbycz 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-κB 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 p57kip2 (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 with 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, overexpression 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 are that the effect of RGS2 overexpression 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\textsuperscript{2+}]\textsubscript{i} and IL-8 by U46619 was inhibited by the IP\textsubscript{3}R antagonist 2-APB. Likewise, multiple PKC inhibitors reduced IL-8 release, and together these data support the activation of a Gr\textsubscript{q}, PLC\beta, [Ca\textsuperscript{2+}]\textsubscript{i}/PKC dependent pathway leading to IL-8 release. As RGS2 is responsible for the salmeterol plus dexamethasone-dependent repression of [Ca\textsuperscript{2+}]\textsubscript{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, pretreatment 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 overexpression 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. Because 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 hours. 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 nonredundant role of RGS2 in repressing agonist-induced [Ca\textsuperscript{2+}]\textsubscript{i}, we predict the existence of functional outputs, which if exclusively dependent on [Ca\textsuperscript{2+}]\textsubscript{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 signaling 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\textsuperscript{2+}]\textsubscript{i} elevations elicited by agonists of Gr\textsubscript{q}-linked GPCRs that are implicated in the pathology of asthma and COPD. This pathway is implicated in the expression of inflammatory genes, but 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 nonredundant 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:** Holden, Johnson, Leigh, Giembycz, Newton.

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

** Contributed new reagents or analytic tools:** Siderovsky.

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

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

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