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Comments

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Budesonide enhances agonist-induced bronchodilation in human small airways by increasing cAMP production in airway smooth muscle

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Abstract

The non-genomic mechanisms by which glucocorticoids modulate β_2 agonist-induced bronchodilation remain elusive. Our studies aimed to elucidate mechanisms mediating the beneficial effects of glucocorticoids on agonist-induced bronchodilation. Utilizing human precision cut lung slices (hPCLS), we measured bronchodilation to formoterol, prostaglandin E₂ (PGE₂), cholera toxin (CTX) or forskolin in the presence and absence of budesonide. Using cultured human airway smooth muscle (HASM), intracellular cAMP was measured in live cells following exposure to formoterol, PGE₂, or forskolin in the presence or absence of budesonide. We showed that simultaneous budesonide administration amplified formoterol-induced bronchodilation and attenuated agonist-induced phosphorylation of myosin light chain, a necessary signaling event mediating force generation. In parallel studies, cAMP levels were augmented by simultaneous exposure of HASM cells to formoterol and budesonide. Budesonide, fluticasone and prednisone alone rapidly increased cAMP levels, but steroids alone had little effect on bronchodilation in hPCLS. Bronchodilation induced by PGE₂, CTX or forskolin was also augmented by simultaneous exposure to budesonide in hPCLS. Furthermore, HASM cells expressed membrane-bound glucocorticoid receptors that failed to translocate with glucocorticoid stimulation, and that potentially mediated the rapid effects of steroids on β_2 agonist-induced bronchodilation. Knockdown of glucocorticoid receptor α had little effect on budesonide-induced and steroid-dependent augmentation of formoterol-induced cAMP generation in HASM. Collectively, these studies suggest that glucocorticoids amplify cAMP-dependent bronchodilation by directly increasing cAMP levels. These studies identify a molecular mechanism by which the combination of glucocorticoids and β_2 agonists may augment bronchodilation in diseases such as asthma or chronic obstructive pulmonary disease.

Introduction

Combination therapy including an inhaled glucocorticoid and a long-acting β_2 agonist represents a cornerstone in the management of airways disease including asthma and chronic obstructive pulmonary disease. Conceptually, combined use of an anti-inflammatory agent with a bronchodilator improves medication adherence as compared to using each drug separately (1-3). Use of an inhaled corticosteroid (ICS) with a long-acting β_2 agonist (LABA) as rescue and maintenance therapy appears more effective than using an ICS or SABA alone or using an ICS/LABA for maintenance (4-10). Evidence now suggests, apart from enhanced adherence, combination therapy augments efficacy of either drug alone (11). We posit that this effect is mediated by a rapid, non-genomic effect of glucocorticoids. To date, the molecular mechanisms by which non-genomic effects of glucocorticoids modulate bronchodilation remain unknown. (11).

Glucocorticoids primarily mediate their effects by activating the glucocorticoid receptor (GR). In this canonical signaling, the GR resides in the cytoplasm in its inactive state and, upon ligand activation, translocates to the cell nucleus to interact with glucocorticoid response elements (GREs) and produce genomic effects altering protein expression. Recent evidence has emerged to suggest that glucocorticoids manifest rapid non-genomic actions on several signaling processes (11). These non-canonical, non-genomic effects of glucocorticoids appear to involve non-specific interactions with the cell membrane and/or specific interactions with cytosolic GRs (cGR) or membrane-bound GRs (mGR). The rapid non-genomic effects appear to, at least in part, be mediated through a putative mGR (11).

Several studies have reported an interaction of mGR with other membrane receptors, particularly G protein-coupled receptors (GPCR) (12). Involvement of mGR and GPCR-dependent mechanisms in the rapid effect (~ 1 min) of corticosterone on NMDA-evoked currents in hippocampal neurons was demonstrated (12), suggesting that mGR may couple to multiple G proteins, including $G_{\alpha s}$ and $G_{\alpha q/11}$. Other studies suggest that mGR directly activates downstream intracellular signaling pathways. Corticosterone acted via mGR to rapidly elicit PKC-dependent activation of ERK1/2 MAPK pathway in PC12 cells (13). Interestingly, proteomic analysis of CCRF-CEM cells identified 128 proteins that were differentially regulated by activation of mGR using BSA-conjugated

cortisol briefly (5 and 15 min) (14). These actions were unique to mGR, as no activation of cGR target genes (e.g. GILZ) were observed. Signal pathway analysis now provides evidence that mGR is involved in numerous pathways that are also regulated by glucocorticoids through cGR, suggesting that mGRs trigger early priming events, ultimately facilitating slower genomic activation by glucocorticoids (14).

Our study identifies a molecular mechanism by which glucocorticoids acutely amplify β_2 agonist-induced bronchodilation. By rapidly stimulating cAMP production, glucocorticoids augment the primary second messenger signal of β_2 agonists and other bronchodilators. Ultimately, identification of plasma membrane components through which glucocorticoids impact cAMP signaling and bronchodilation may provide novel therapeutic targets for airways diseases, an area that has seen little innovation in the past 45 years.

Methods

Reagents

Reagents were purchased from the following vendors: formoterol, forskolin, carbachol, and TNF α (Sigma Aldrich, St. Louis, MO); budesonide (AstraZeneca); cell culture media and components (ThermoFisher, Waltham, MA); fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA); GR antibody (Santa Cruz, Dallas, TX); β -actin antibody (Sigma Aldrich, St. Louis, MO); phosphorylated GR α (Cell Signaling Technology, Danvers, MA); rabbit and mouse secondary antibodies for immunoblotting (LiCor, Lincoln, NE); GR α targeted siRNA (Ambion, Austin, TX); non-targeting siRNA (Dharmacon, Lafayette, CO); HiPerFect transfection reagent (Qiagen, Germantown, MD); single analyte ELISA kits for IL-6, RANTES, and IL-8 (R&D Systems, Minneapolis, MN).

hPCLS generation and bronchodilation assays

Human precision cut lung slices (hPCLS) were prepared as previously described (15). Briefly, whole human lungs from non-asthma donors were dissected and inflated using 2% (wt/vol) low melting point agarose. Once the agarose set, the lobe was sectioned, and cores of 8-mm diameter were made. Cores containing small airways by

visual inspection were sliced at a thickness of 350 μm (Precisionary Instruments VF300 Vibratome, Greenville, NC) and collected in wells containing supplemented Ham's F-12 medium. To study bronchodilation, small airways contained within hPCLS were contracted with carbachol (CCh, 10^{-5} M), and then bronchodilated to formoterol (10^{-12} – 10^{-6} M), budesonide (10^{-12} – 10^{-5} M), forskolin (10^{-12} – 10^{-5} M), PGE₂ (10^{-10} – 10^{-5} M), or cholera toxin (0.01-100 $\mu\text{g/ml}$) \pm budesonide (10^{-5} M, simultaneous administration). Human lung tissue samples were commercially obtained from anonymous donors (National Disease Research Interchange, Philadelphia, PA or the International Institute for the Advancement of Medicine, Edison, NJ), and are therefore exempt from IRB (Institutional Review Board) approval. Although these samples have demographic information, there is no information linking the subject's identification to the tissue. Integrated area under the curve (AUC - % dilation/bronchodilator dose) was calculated from the dose response curves and was plotted along with maximal bronchodilation achieved over the entire dose response curve.

Human airway smooth muscle (HASM) cells

HASM cells were derived from tracheas obtained from non-asthma donor lungs that hPCLS were derived from. HASM cell culture was performed as described previously (16). Briefly, the cells were cultured in Ham's F-12 medium supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 mg/ml amphotericin B, and this medium was replaced every 72 hr. HASM cells in subculture during passages 1–5 were used, because these cells retain expression of native contractile proteins, as demonstrated by immunocytochemical staining for smooth muscle actin and myosin (17).

cAMP assays

For kinetic measurement of cAMP production in live cells, subconfluent HASM cells were plated in black-walled, clear flat bottom 96-well plates with HASM media, BacMam virus expressing the green cAMP difference detector *in situ* (cADDis) cAMP sensor (Montana Molecular, Bozeman, MT), and 1 μM trichostatin-A (Sigma Aldrich, St. Louis, MO) per well and grown overnight. Media was aspirated and replaced with PBS without calcium or magnesium, then the plate was covered and incubated at room

temperature. Cell fluorescence was read from the plate bottom using excitation/emission wavelengths of 494 nm and 522 nm, respectively, using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). A kinetic read (5 min) on unstimulated cells was performed to determine variability in each well's fluorescence ($\leq 5\%$). Cells were stimulated with agonist and fluorescence changes were read at 30 second intervals for 30 min.

Cell surface biotinylation

Cell surface protein biotinylation was carried out according to manufacturer's protocols (Pierce Biotechnology, Rockford, IL). Cells were grown to 90-95% confluence, incubated with a biotin solution, then scraped and lysed. Lysates were incubated with NeutrAvidin beads, then bound proteins eluted off. Eluates were assessed for glucocorticoid receptor, using epidermal growth factor receptor (EGFR; Cell Signaling Technology, Danvers, MA) as a positive control for cell surface biotinylation. Remaining cell lysates were examined for expression of GAPDH (Millipore, Burlington, MA) and COXIV (Cell Signaling Technology, Danvers, MA) as measures of intracellular/cytosolic proteins.

Immunohistochemistry

HASM cells were grown in chamber slides until confluent, then serum starved for 18 hr. Cells were then fixed with 1% paraformaldehyde, washed, then blocked with 1% BSA/PBS solution containing 10% FcR block (Miltenyi Biotec, Auburn, CA). Cells were stained with a GR antibody (rabbit, Santa Cruz Biotechnology, Dallas, TX) in 1% BSA/PBS solution overnight. The slides were washed, stained with biotin-coated donkey anti-rabbit antibody (Jackson Immunolabs, Bar Harbor, ME) in 1% BSA/PBS, washed, then incubated with a streptavidin-Alexa Fluor 488 conjugated antibody (Jackson Immunolabs). The slides were washed, then the cells were permeabilized with 0.01% Triton X-100 and stained with DAPI. Slides were cover-slipped and imaged.

Immunoblotting

HASM cells were treated with carbachol (25 μ M Cch, 10 min) then with formoterol (100 pM, 5 min) \pm simultaneous budesonide stimulation (1 μ M, 5 min). Cells were then treated with 500 μ M perchloric acid, plates scraped, and cells pelleted. Pellets were solubilized in RIPA and sonicated prior to being subjected to SDS PAGE and transferred to nitrocellulose membranes, as previously described (18) then assessed for phosphorylation of MLC and total MLC. Total GR α and phospho-GR α were assessed in cell lysates following siRNA transfection of HASM with non-targeting and GR α -targeted siRNA.

Single analyte ELISAs

HASM transfected with non-targeting or GR α -targeted siRNA were treated with budesonide (100 nM, 1 hr) prior to stimulation with TNF α (10 ng/ml, 24 hr). Single analyte ELISAs were utilized to assess release of IL-6, RANTES, and IL-8 into the media. Each condition represents duplicate samples from a single donor, each run in triplicate.

Statistical analyses

Standard curves for cAMP generation were fitted and unknown values extrapolated using GraphPad Prism 6.0h (GraphPad Software Inc., San Diego, CA). Data are presented as the mean \pm SEM. Statistical comparisons (t-tests and one-way analysis of variance) were performed and graphics were generated using GraphPad Prism 6.0h (GraphPad Software Inc.). Unpaired non-parametric analyses were used for hPCLS data that was not normally distributed to compare conditions. Paired parametric analysis was used for HASM experiments (ELISA, western blots, and cAMP generation).

Results

Budesonide enhances formoterol-induced bronchodilation

To examine whether a glucocorticoid and a β_2 agonist can additively promote bronchodilation, human small airways in hPCLS were precontracted to carbachol and then dilated to formoterol in the absence and presence of budesonide, with the budesonide being added simultaneously with the formoterol. Budesonide treatment

augmented formoterol-induced bronchodilation (Figure 1), increasing maximal levels of bronchodilation. Similarly, the integrated bronchodilator response as represented by Area Under the Curve (AUC) significantly increased. Budesonide alone had little effect on luminal diameter dilation despite being administered at similar concentrations as formoterol (data not shown). These data show that simultaneous administration of budesonide augments β_2 agonist-induced dilation of human small airways.

Budesonide amplifies PGE₂-, cholera toxin- and forskolin-mediated bronchodilation

Given that budesonide augments airway dilation to a β_2 agonist, we next examined whether budesonide enhances dilation mediated by activation of other GPCRs coupled to G_{αs} or via direct activation of G_{αs} or adenylyl cyclase. We showed that simultaneous administration of budesonide enhances PGE₂-induced bronchodilation of airways (Figure 2), with increases in maximal bronchodilation and AUC to PGE₂. To assess whether the effect of budesonide was due to activation of G_{αs}, a G protein shared between the β_2 AR and EP_{2/4} receptors, we utilized cholera toxin (CTX) to induce bronchodilation in hPCLS. Simultaneous administration of budesonide with CTX induced greater maximal bronchodilation overall responses to CTX compared to CTX alone (Figure 3). To assess whether budesonide enhanced direct adenylyl cyclase (AC)-induced bronchodilation, hPCLS were exposed to forskolin (FSK) in the presence or absence of budesonide. Simultaneous budesonide administration enhanced FSK-induced bronchodilation (Figure 4), significantly increasing the AUC to FSK compared to control.

Budesonide enhances formoterol-stimulated cAMP production

To dissect the molecular pathways by which budesonide augments bronchodilation of human airway smooth muscle (HASM), HASM were infected with a recombinant BacMam expressing a fluorescent cAMP sensor, cADDiS, and cAMP levels were measured after exposure to varying concentrations of formoterol. The cADDiS sensor decreases fluorescence upon binding cAMP, providing real-time assessment of intracellular cAMP levels without inclusion of phosphodiesterase (PDE) inhibitors. Formoterol decreased cADDiS fluorescence within minutes that typically stabilized within 15-20 min (Figure 5A, inset). To account for the rate and maximal levels of cAMP

production produced by formoterol, the product of the decay rate (K) and the level at steady state (plateau) were plotted for each drug concentration. Using this analysis, formoterol increased cAMP levels (Figure 5A). In parallel, formoterol-stimulated cAMP was measured in HASM treated with vehicle or 10^{-6} M budesonide given simultaneously. Budesonide shifted the formoterol concentration-response curve 3.9-fold leftward (Figure 5B). Additionally, budesonide at 1 or 10 μ M augmented formoterol-induced cAMP production, but that effect was not realized at 100 nM budesonide. The effect of budesonide on formoterol-stimulated cAMP production in HASM cells therefore mimicked the effect of budesonide on agonist-induced bronchodilation observed in hPCLS.

Budesonide enhances PGE₂- and forskolin-stimulated cAMP production

To determine whether budesonide also enhanced cAMP signaling stimulated by PGE₂, PGE₂-stimulated cAMP production was detected by cADDis in a concentration-dependent manner (Figure 5C). Inclusion of budesonide (1 μ M) shifted the PGE₂ curve leftward 8.4-fold. We next examined cAMP responses to forskolin (FSK), finding that FSK-stimulated cAMP production was shifted leftward 8.2-fold by inclusion of 1 μ M budesonide as compared to vehicle (Figure 5D). These data are consistent with the notion that budesonide enhances cAMP production initiated by multiple GPCRs in different signaling compartments in HASM cells, and that budesonide-mediated augmentation of these responses is receptor-independent.

Budesonide, fluticasone and prednisone enhance directly stimulated cAMP production

Because budesonide enhanced agonist-induced cAMP signaling, we investigated whether budesonide alone stimulated cAMP. Significant decreases in cADDis fluorescence was observed within seconds of addition of 1 or 10 μ M budesonide (data not shown). 10 μ M budesonide stimulated cAMP levels that were statistically different than vehicle within 2 minutes, while 1 μ M induced significant changes within 5 minutes. The higher concentration of budesonide stimulated changes in cADDis fluorescence that were equivalent to maximal concentrations of forskolin (10 μ M) or formoterol (not shown). However, cADDis is a readily saturated sensor so high levels of cAMP may not be distinguishable (19). We also examined cAMP production by other corticosteroids, finding

that fluticasone and prednisone (data not shown) stimulated cAMP production. Fluticasone was equi-effective as budesonide although greater inter-experimental variability was observed while prednisone was less efficacious and less potent.

A membrane-bound form of GR (mGR) is present in HASM cells

Since budesonide effects on cAMP generation and bronchodilation were rapid, we posited that budesonide activates a membrane-associated receptor. Other laboratories have identified a mGR that can evoke immediate steroid effects in other cell types (20-22). Using cell-surface biotin labeling and immunohistochemistry (Figure 6), we showed that HASM express mGRs that fail to translocate to the nucleus from the membrane despite stimulation with a glucocorticoid (dexamethasone). As expected, cytosolic GR translocated to the nucleus as shown in Figure 6.

Budesonide augments formoterol-induced dephosphorylation of myosin light chain

Contractile agonists activate GPCRs that evoke HASM shortening by promoting phosphorylation of myosin light chain (pMLC). To determine whether formoterol in the absence and presence of budesonide modulates agonist-induced excitation-contraction signaling, we examined attenuation of carbachol-induced pMLC by formoterol ± budesonide. Formoterol alone (10 pM – 10 nM) induced significant reversal of carbachol-induced pMLC, and simultaneous administration of budesonide with formoterol (at 100 pM) further augments formoterol-induced pMLC dephosphorylation (Figure 7). These data suggest that glucocorticoids can also augment formoterol effects on pro-contractile pathways in HASM cells.

GR α knockdown in HASM cells has little effect on cAMP generation in response to budesonide or budesonide + formoterol

To more directly assess the role of the GR in mediating rapid cAMP responses to glucocorticoids, we used siRNA to knock down GR α expression in HASM. Transfection of validated siRNA sequences into HASM led to a nearly complete loss of GR α immunoreactivity (detected as a doublet of approximately 94 kDa) as compared to cells transfected with a scrambled siRNA (Figure

8A). GR α mRNA expression in control HASM was readily detected by quantitative RT-PCR but was undetectable in cells transfected with GR α siRNA (not shown). We then assessed cAMP levels in HASM following siRNA transfection. Forskolin-stimulated cAMP responses were unaltered by GR α knockdown, implying that loss of GR α did not alter AC expression or function (data not shown). Budesonide (1 μ M) or formoterol (0.1 nM) each elicited equivalent cAMP responses in both GR α knockdown and control cells (Figure 8B and 8C). cAMP responses to simultaneous addition of budesonide and formoterol were greater than either agent alone, but were similar in both control and GR α knockdown conditions (Figure 8D). Knockdown of GR α was confirmed by immunoblot, and showed reversal of budesonide attenuation of TNF α -induced inflammatory mediator release (data not shown). These results suggest that GR α expression may not be required for rapid, non-genomic signaling by GC.

Discussion

In airway smooth muscle, contractile agonists stimulate Ca²⁺-dependent signaling evoking cell shortening regulated in part through increased [Ca²⁺]_i transients, inhibition of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), and pMLC inducing actin-myosin cross-bridge cycling and force generation. Agents that mobilize cAMP and activate protein kinase A (PKA) protect against or reverse agonist-induced bronchoconstriction. In epithelial cells and neurons, glucocorticoids modulate signaling pathways that promote relaxation of HASM. Dexamethasone stimulation of bronchial cells attenuated [Ca²⁺]_i currents, which was reversed by SERCA inhibitors, PKA, and activated adenylyl cyclase (AC) within minutes (20). Further, corticosterone treatment reversed ATP-induced [Ca²⁺]_i transients in mouse HT4 neuroblastoma cells (23). In primary HASM cells, budesonide stimulation alone increased cAMP levels (data not shown). Interestingly, we show that simultaneous budesonide and formoterol significantly reduced carbachol-induced pMLC (Figure 7), but little effect of budesonide alone. Others noted existence of a mGR that may mediate non-genomic, rapid effects of glucocorticoids. We demonstrate presence of mGR in HASM (Figure 6) that does not translocate upon glucocorticoid stimulation. Our

work suggests that expression of GR α is not necessary to elicit cAMP production in response to budesonide or budesonide + formoterol (Figure 8). There is a possibility that residual GR protein whose expression was not attenuated by siRNA transfection may mediate the effect, but the existence of a modified GR that may not be subject to knockdown of the protein may mediate the effects of steroids on cAMP production/augmentation of bronchodilation. Overall, these data suggest that despite the effect of glucocorticoids on attenuation of Ca²⁺-dependent pathways, glucocorticoids alone had little effect on agonist-induced bronchoconstriction of small airways (data not shown) in hPCLS but rather, amplified β_2 agonist-induced bronchodilation.

Although glucocorticoids alone induced cAMP production (data not shown), these steroids had little effect on bronchodilation of small airways in hPCLS (data not shown). Robust and sensitive assessment of cAMP pools is achieved using the cADDis reporter (19). Although the assay shows increases in cAMP production to budesonide alone, this increase in cAMP levels may be insufficient for promoting bronchodilation due to an inadequate magnitude or localization of the cAMP signal despite utilizing the same concentrations of budesonide as formoterol in the hPCLS bronchodilation assays (10^{-12} – 10^{-5} M, data not shown). Glucocorticoids may therefore be increasing cAMP levels via direct activation of G_{as}, activation of an unidentified GPCR, or inhibition of phosphodiesterases. Others suggest that increases in intracellular cAMP elicited by glucocorticoids may be due to inhibition of ABCC4, an ATP binding cassette transporter that pumps cAMP into the extracellular space. In differentiated airway epithelial cells, dexamethasone, but not budesonide, augmented activity of GRE-luciferase when given with forskolin treatment. Additionally, blockade of ABCC4 potentiated activity of the GRE-luciferase in the presence of either dexamethasone or budesonide treatment (24). Unlike airway epithelial cells, β_2 agonist or forskolin treatment had little effect on extracellular levels of cAMP in HASM (data not shown). However, we reported that ABCC4 is expressed in HASM cells, and its expression is augmented by budesonide treatment (25). In the aforementioned studies, glucocorticoid treatment was for 6-12 hours, conceivably the mechanisms modulating rapid glucocorticoid stimulation may differ. Future studies will address whether non-genomic effects of glucocorticoids are mediated by activation of ABCC4 proteins.

Others noted acute glucocorticoid stimulation activates signaling pathway components in non-muscle cells that are associated with bronchodilatory responses in smooth muscle. In bronchial epithelial cells dexamethasone inhibited agonist-induced $[Ca^{2+}]_i$, with the inhibition sensitive to AC and PKA inhibition (20). Other show a non-genomic glucocorticoid effect that was PKA-dependent in a neural cell line (23). Accordingly, to assess mechanisms by which steroid may be augmenting agonist-induced bronchodilation we examined the effect of glucocorticoids on: (1) selectivity of responses we observed to the β_2 AR; (2) direct activation of $G_{\alpha s}$; and (3) whether the response observed was receptor-dependent. We found that PGE₂-, CTX-, and FSK-stimulated bronchodilation of small airways (Figures 2, 3 and 4, respectively) was augmented by co-administration of budesonide. Similarly, PGE₂-, and FSK-induced cAMP production in HASM (Figure 5C and D) was augmented by budesonide co-administration. Despite the cADDis characterizing budesonide's effects on CTX-induced cAMP production, CTX induced such saturating levels of cAMP production in HASM cells making us unable to detect a synergism above CTX stimulation alone (data not shown).

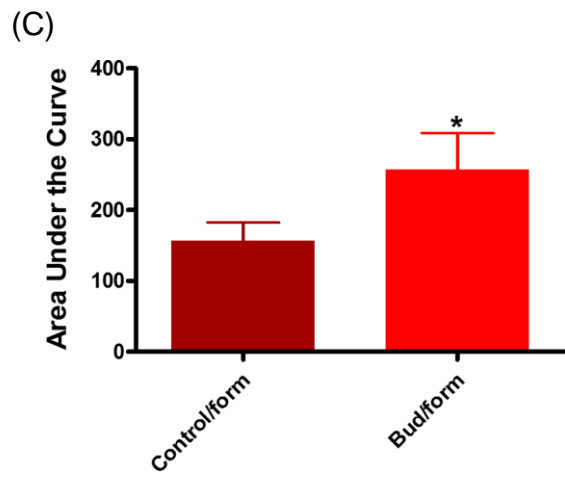
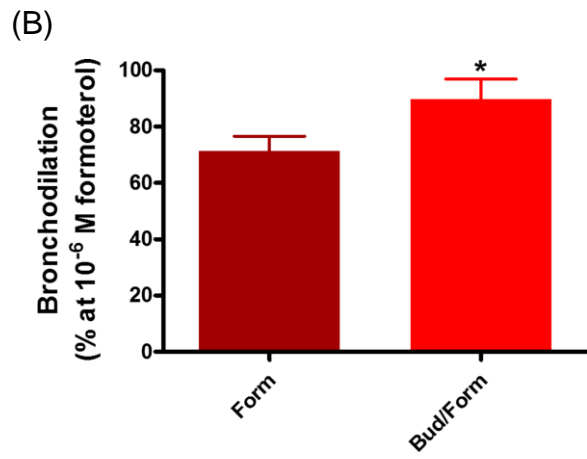
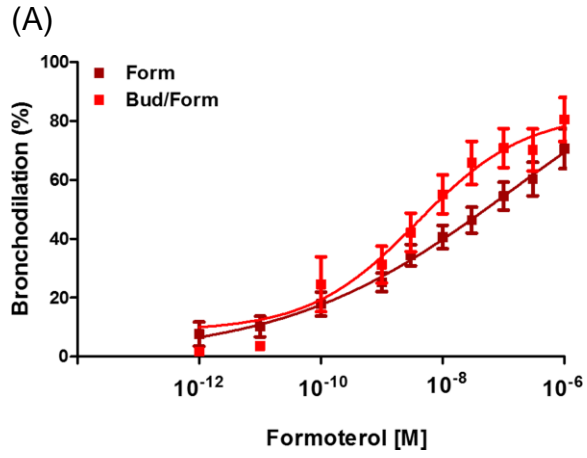
Interestingly, synergism between glucocorticoids and β_2 agonists has been examined previously. A recent study noted that beclomethasone augmented formoterol-induced reversal of pre-contracted airways (26), to about ~30% maximally (27). Although there was synergism between the two therapeutics, the beclomethasone dose was given overnight, with effects likely genomic. This is in direct contrast to what our studies show, where despite budesonide inducing cAMP production in HASM, it alone was unable to induce bronchodilation even at a concentration of 100 μ M. Additionally, the 2016 study utilized non-selective inhibitors of PKA and $G_{\alpha s}$ to establish roles for those protein in beclomethasone-induced bronchodilation in lung slices. Our studies have the advantage of observing cAMP production in live primary HASM in real time, a system in which we can genetically manipulate components of signaling pathways to more rigorously ascertain the necessity and sufficiency specific molecules in the responses we observe.

Our studies identify mechanisms by which budesonide, a potent glucocorticoid, rapidly activates cAMP production and augments agonist-mediated bronchodilation. This is the first direct demonstration that glucocorticoids stimulate cAMP production in airway smooth muscle. These mechanisms may explain, in part, how combination therapy of β_2

agonists and ICS offer greater efficacy in comparison to either drug alone. Interestingly, these beneficial effects occur separate and distinct from any anti-inflammatory effects (11). Combination therapy represents a cornerstone for maintenance therapy in asthma and COPD. Evidence also suggests that this combination can serve as an anti-inflammatory reliever therapy (when added to maintenance) that decreases exacerbation rates, improves asthma symptoms, and enhances lung function as compared to higher doses of inhaled corticosteroids (ICS) alone (4-6). Our data identify molecular mechanisms by which steroids and long-acting β_2 agonists together improve bronchodilation.

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(D)

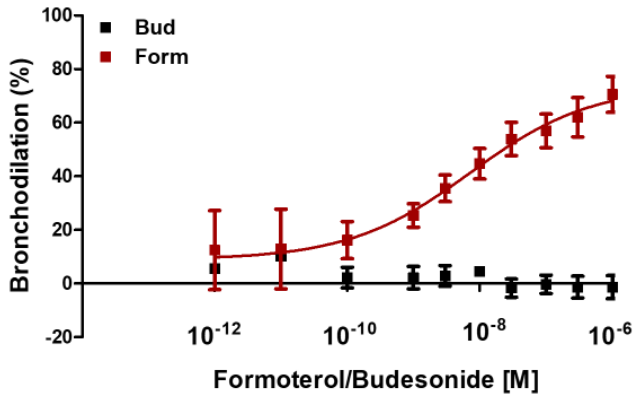
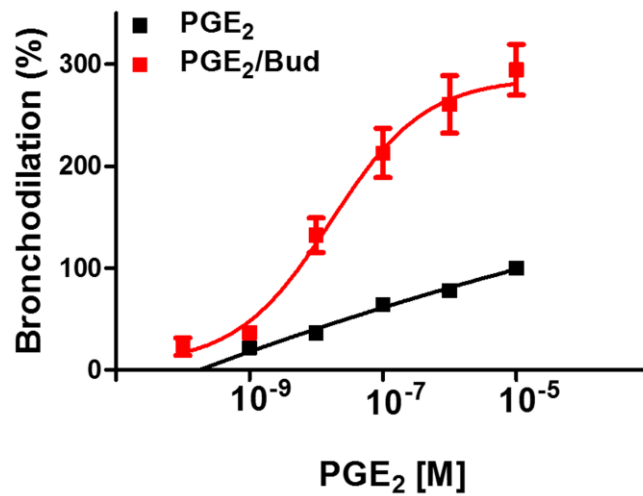
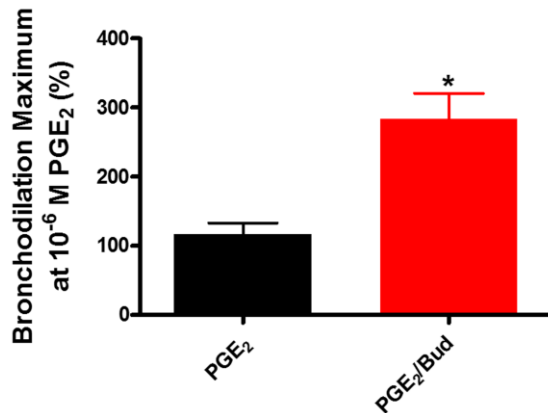


Figure 1 – Acute budesonide stimulation augments formoterol-induced bronchodilation, but does not induce bronchodilation alone. (A) Simultaneous stimulation of hPCLS with budesonide (10^{-5} M) with formoterol (10^{-12} – 10^{-6} M) augments (B) maximal bronchodilation (formoterol vs. budesonide at 10^{-6} M formoterol, $71.4 \pm 5\%$ vs. $89.7 \pm 7.3\%$), and increases the integrated (C) area under the curve vs. formoterol alone (formoterol vs. formoterol + budesonide, 156.8 ± 25.9 vs. 257.1 ± 51.6). Data represents $n=15-25$ donors, $p<0.05$ vs formoterol alone. (D) Budesonide alone does not induce bronchodilation in hPCLS. Data represents 2 (budesonide alone) - 15 (formoterol) donors, 6-36 slices/condition.

(A)



(B)



(C)

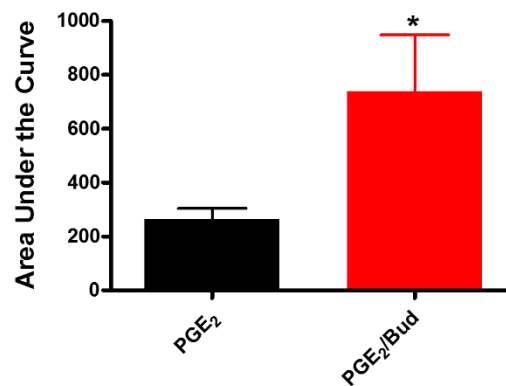
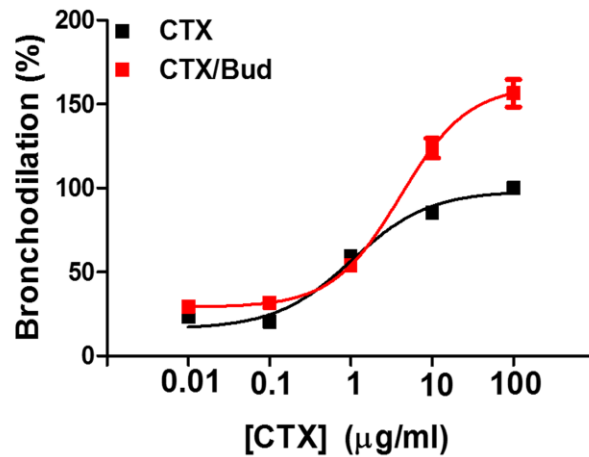
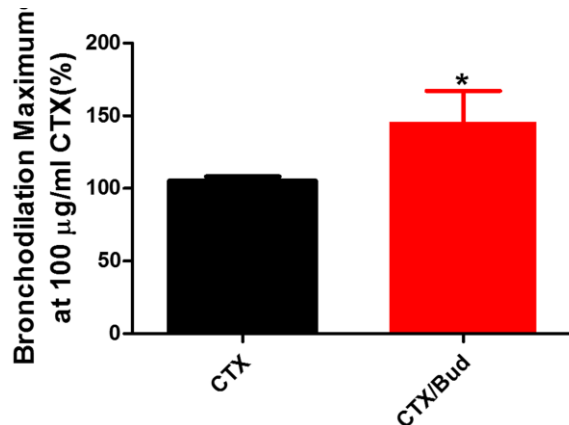


Figure 2 – Simultaneous budesonide treatment augments PGE₂-induced bronchodilation of human small airways. Budesonide (10 μM) was given simultaneously with PGE₂ (10⁻¹⁰ – 10⁻⁵ M) and bronchodilation was assessed. Concentration-response curves (A) are represented as % dilation for the combination compared to PGE₂-induced dilation alone. Maximal bronchodilation at 10⁻⁵ M PGE₂ (B, PGE₂ vs. PGE₂ + budesonide at a maximum of 10⁻⁵ M PGE₂, 116.6 ± 16.6% vs. 283.3 ± 37.3%) and integrated area under the curve (C, PGE₂ vs. PGE₂ + budesonide, 264.5 ± 40 vs. 736.2 ± 209.6) of PGE₂-induced bronchodilation were significantly increased. Data represents n=3 donors, *p<0.05 compared to PGE₂ stimulation alone.

(A)



(B)



(C)

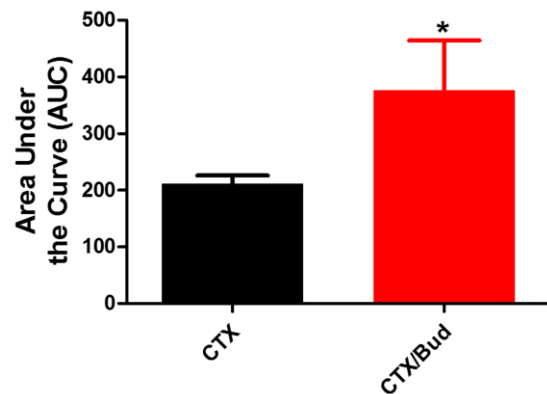
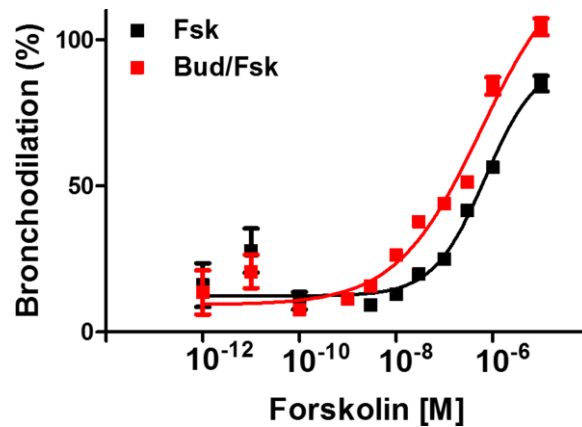
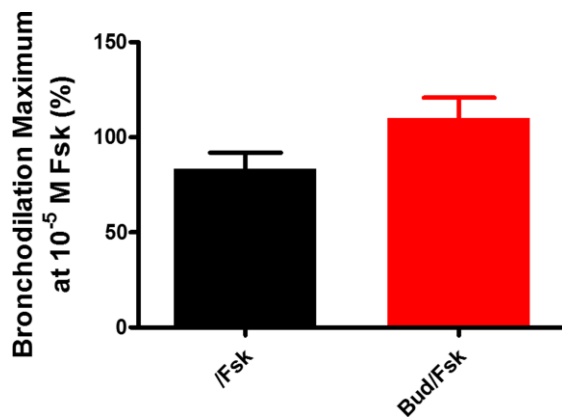


Figure 3 – Budesonide significantly augments cholera toxin-induced bronchodilation of human small airways. Budesonide (10 μM) was given simultaneously with cholera toxin (CTX, 0.01-100 $\mu\text{g/ml}$) and bronchodilation was assessed. Concentration-response curves (A) were normalized to CTX stimulation alone set to 100%. Maximum bronchodilation at 100 $\mu\text{g/ml}$ (B, CTX vs. CTX + budesonide at a maximum of 100 $\mu\text{g/ml}$ CTX, 105.5 \pm 3.9% vs. 128.4 \pm 17.5%) and area under the curve (C, (CTX vs. CTX + budesonide, 200.8 \pm 13.8 vs. 361.5 \pm 112.6) were significantly increased with budesonide stimulation. Data are representative of n=5 donors, 11-13 slices/condition, * p <0.05 compared to CTX stimulation alone.

(A)



(B)



(C)

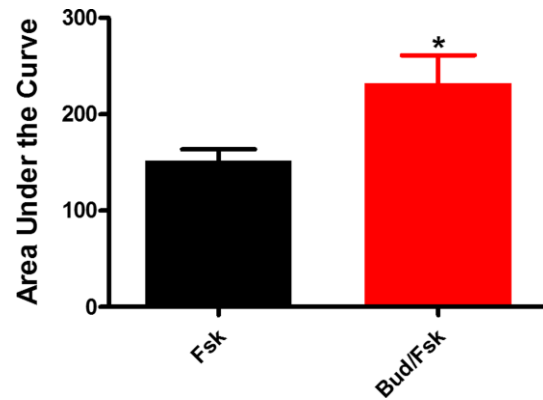
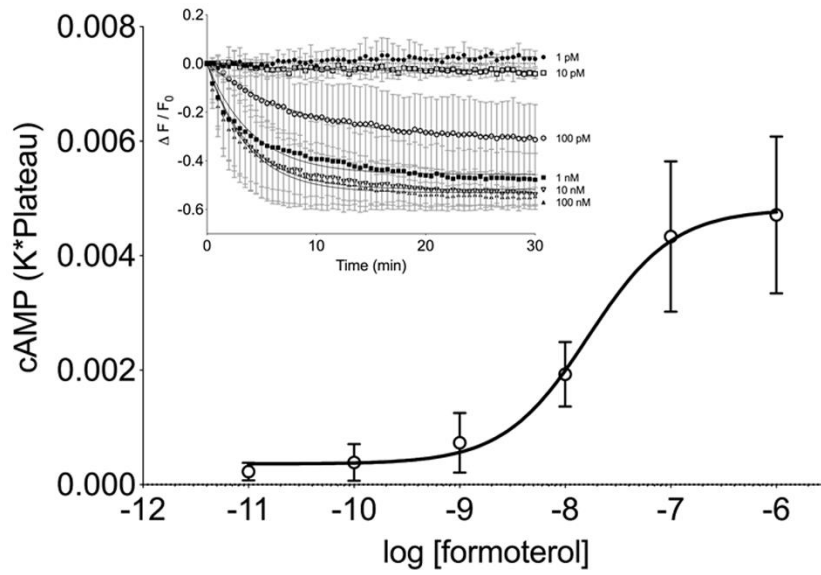
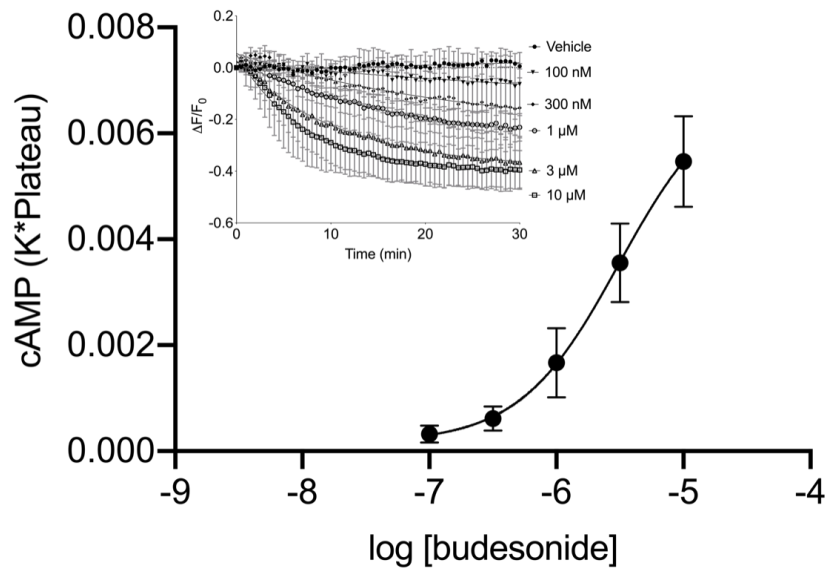


Figure 4 – Budesonide augments forskolin-induced bronchodilation of human small airways. Budesonide (10 μ M) was given simultaneously with forskolin (Fsk, 10^{-12} – 10^{-5} M) and bronchodilation was assessed. Concentration-response curves to Fsk (A) were plotted. Maximal bronchodilation at 10^{-5} M FSK (B, FSK vs. FSK + budesonide at maximum of 10^{-5} M FSK, $83.7 \pm 8.5\%$ vs. $110.1 \pm 10.6\%$) and area under the curve (C, FSK vs. FSK + budesonide, 151.9 ± 11.8 vs. 232.3 ± 29.1) were significantly increased in the presence of budesonide. Data are representative of $n=5$ donors, * $p < 0.05$ compared to control/Fsk.

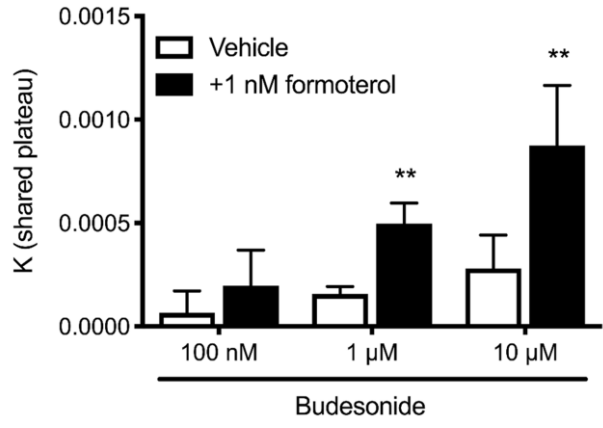
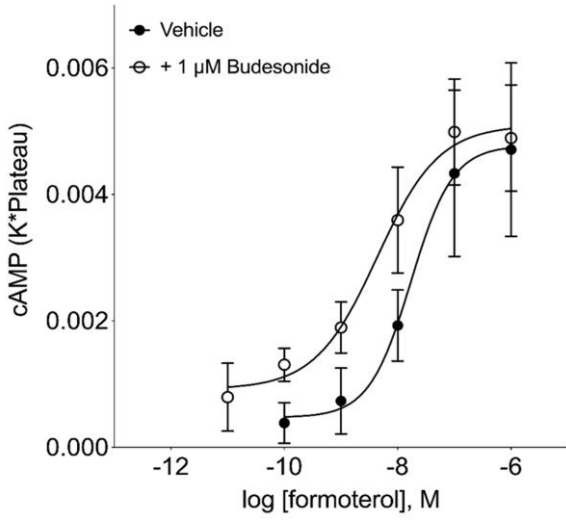
(A)



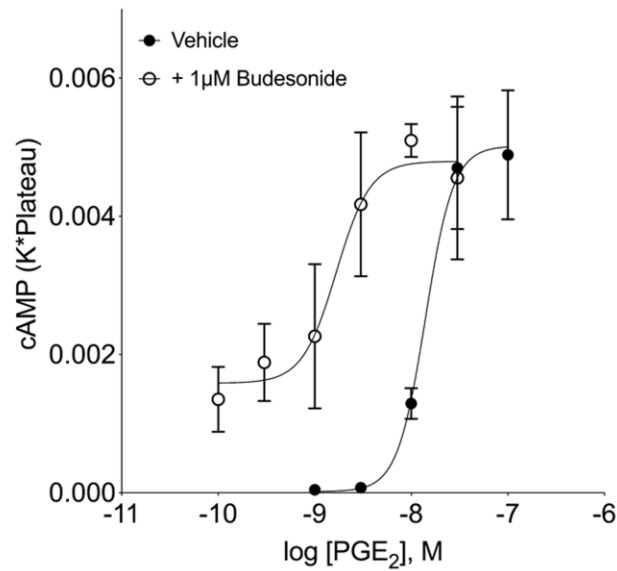
(B)



(C)



(D)



(E)

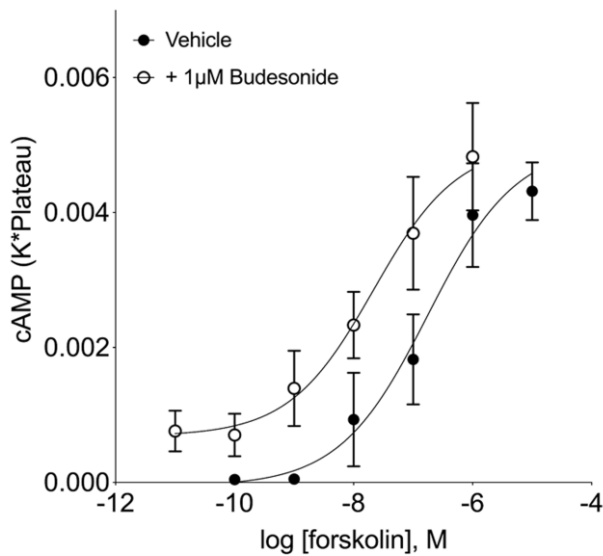
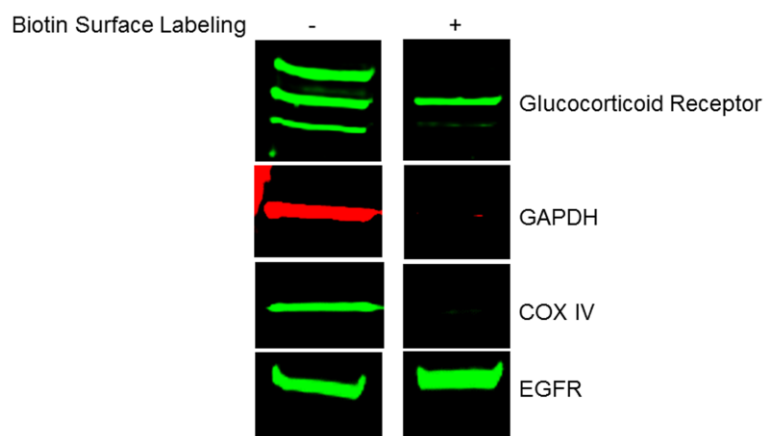


Figure 5 – Budesonide alone induces cAMP production in HASM cells, and enhances formoterol, PGE₂, and forskolin-induced cAMP production. HASM cells were incubated with recombinant BacMam virus expressing the cADDis cAMP sensor for 24 hr. After establishing baseline, fluorescence decay was monitored for 30 min after addition of drug. (A) cADDis sensor fluorescent decay curves elicited by various concentrations of formoterol were fit by one-phase decay non-linear regression analysis (inset, 10⁻¹² – 10⁻⁶ M formoterol, log EC₅₀ of -7.78 ± 0.185). The rate (K) was multiplied by the steady state change in fluorescence (plateau) for each concentration of formoterol.

Each point represents the mean \pm SEM of n=5. (B) Budesonide alone (1 or 10 μ M) elicits cAMP production in cells, and is compared to forskolin (10 μ M) stimulation. Each point represents the mean \pm SEM of n=4-6 cell lines and lines represent the fit by one-phase decay non-linear regression analysis. * denotes $p < 0.05$, ** denotes $p < 0.01$ of each time point compared to vehicle using multiple t tests and the Holm-Sidak method for correction of multiple comparisons. (C) Formoterol concentration-responses curves in the presence of vehicle or 1 μ M budesonide. Each point represents the mean \pm SEM of n=5 cell lines (log EC₅₀ formoterol vs. formoterol + budesonide, -7.76 ± 0.205 vs. -8.36 ± 0.189 , $p=0.021$). (D) PGE₂ concentration-responses curves in the presence of vehicle or 1 μ M budesonide. Each point represents the mean \pm SEM of n=4 cell lines (log EC₅₀ PGE₂ vs. PGE₂ + budesonide, -7.85 ± 0.062 vs. -8.78 ± 0.129 , $p=0.046$). (E) Forskolin concentration-responses curves in the presence of vehicle or 1 μ M budesonide. Each point represents the mean \pm SEM of n=7 cell lines (log EC₅₀ FSK vs. FSK + budesonide, -6.75 ± 0.168 vs. -7.67 ± 0.190 , $p=0.005$).

(A)



(B)

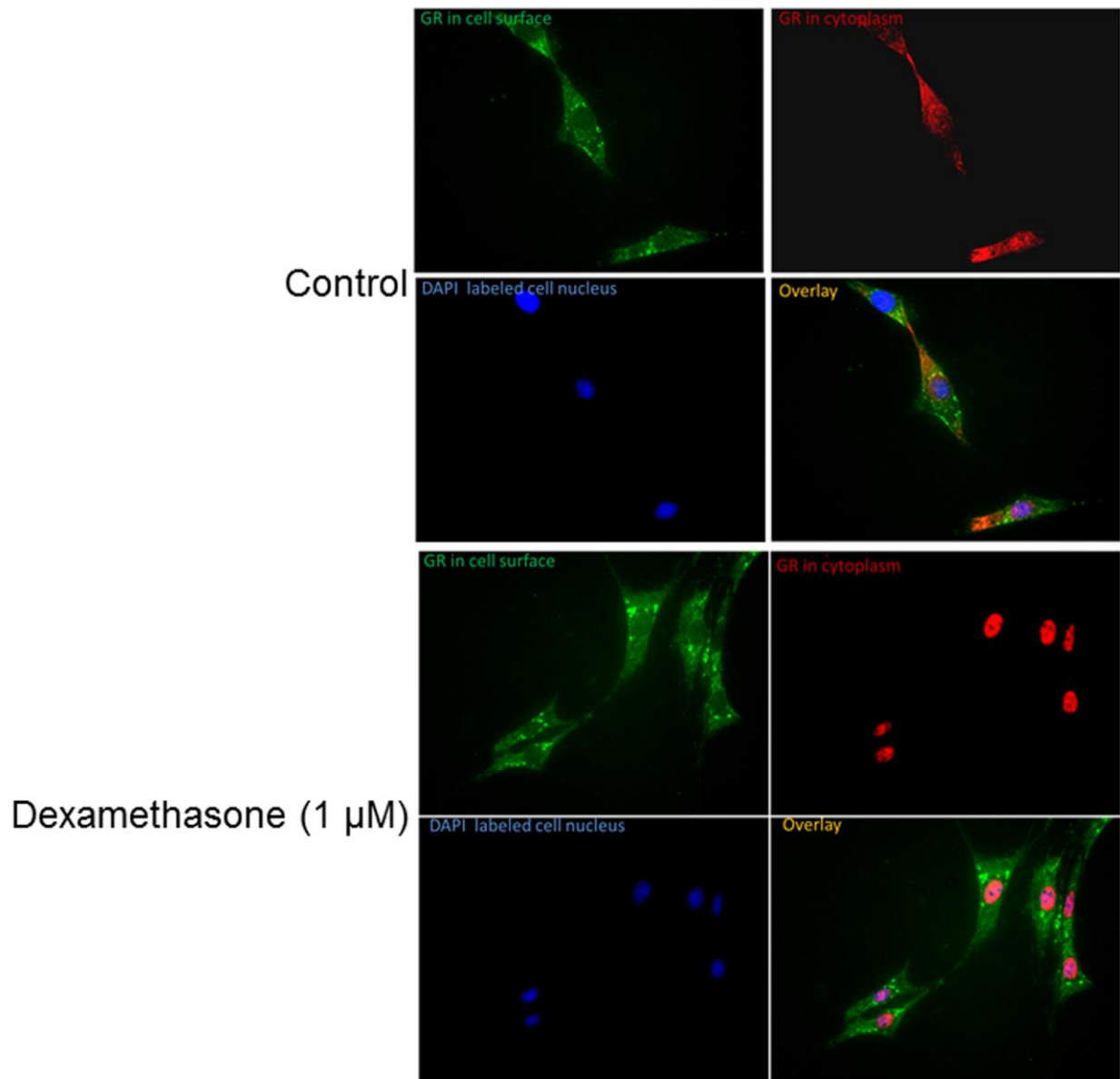
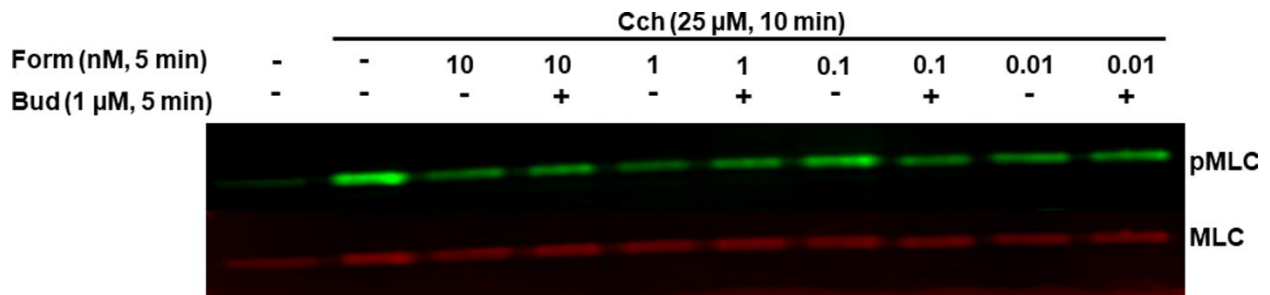


Figure 6 - mGR exists in HASM. (A) HASM from non-diseased lung donors was biotinylated and purification of a membrane fraction determined the existence of a membrane bound form of the glucocorticoid receptor. Presence of the epidermal growth factor receptor (EGFR) and absence of GAPDH and COX IV (cytosolic proteins) were used as controls for purity of membrane protein isolation. (B) HASM cells were fixed with acetone following stimulation for 30 min with dexamethasone (1 μ M). Fixed cells were then probed for glucocorticoid receptor and DAPI. Data are representative of 3 separate non-asthma donors for both (A) and (B).

(A)



(B)

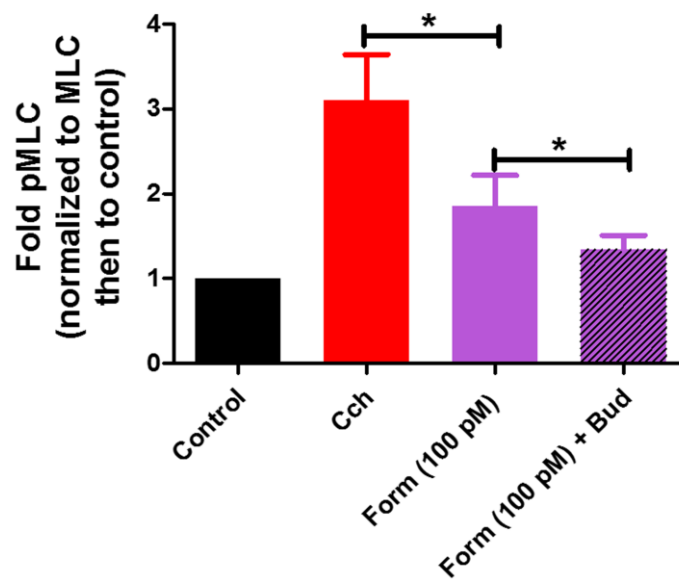


Figure 7 – Budesonide augments formoterol-mediated attenuation of carbachol (Cch)-induced phosphorylation of myosin light chain. HASM cells were treated with Cch (25 μ M, 15 min), then treated simultaneously with formoterol (10 pM – 10 nM, 5 min) and budesonide (1 μ M, 5 min). Phosphorylation of myosin light chain was assessed by immunoblot, using total myosin light chain as a loading control (A). Data are expressed as fold compared to control (B), and are representative of 5 separate HASM donors (* $p < 0.05$).

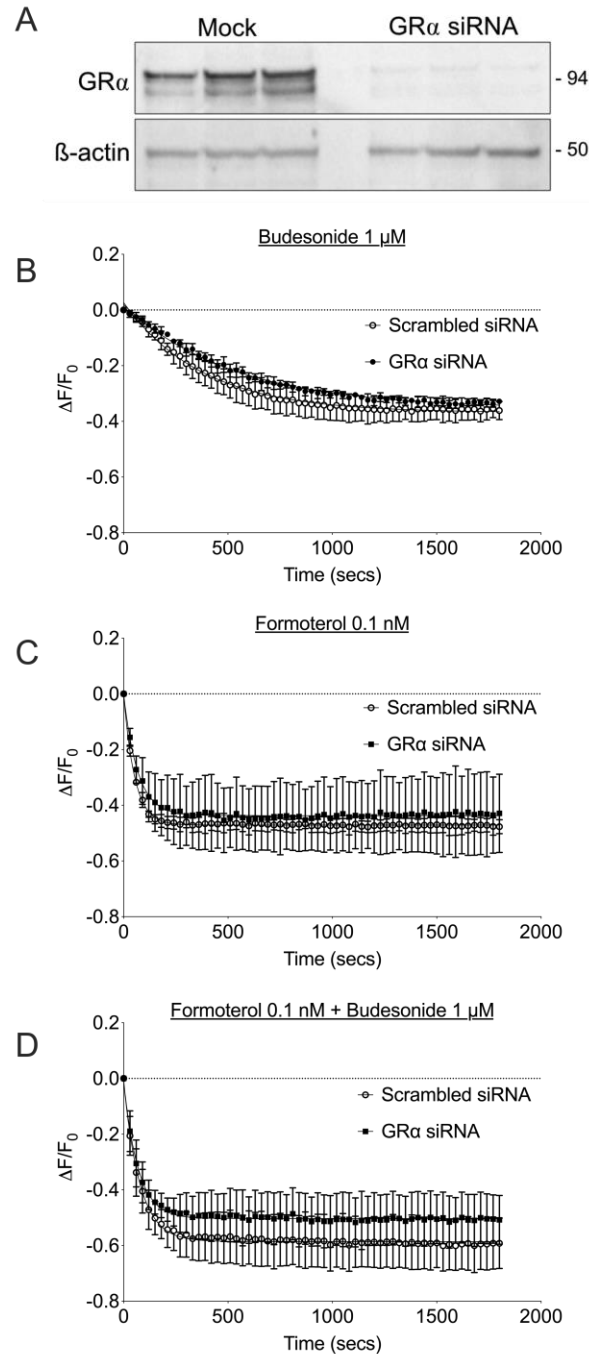


Figure 8 – Knockdown of GR α had little effect on budesonide or budesonide + formoterol-induced cAMP production in HASM. HASM were transfected with siRNA specific for GR α or scrambled control (mock) for 72 hr. A: Lysates were collected and analyzed by immunoblotting using antibodies specific for GR α or β -actin. Each lane shows one of 3 separate experiments on individual cell lines. B, C and D: cADDis sensor was expressed in HASM with a recombinant BacMam virus then cAMP responses to

budesonide (1 μ M, B), formoterol (0.1 nM, C) or both budesonide and formoterol (1 μ M and 0.1 nM, respectively, D) were measured in HASM were transfected with non-targeting siRNA or siRNA specific for GR α . Each point represents the mean \pm SEM of n=4-5 cell lines.

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