

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

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4 Cynthia Koziol-White¹, Timothy B. Johnstone², Maia L. Corpuz², Gaoyuan Cao¹, Sarah
5 Orfanos¹, Vishal Parikh¹, Brian Deeney¹, Omar Tliba³, Rennolds S. Ostrom², Ian
6 Dainty⁴, and Reynold A. Panettieri, Jr¹

7

8 ¹Rutgers Institute for Translational Medicine and Science, Rutgers University, New
9 Brunswick, NJ 08901

10

11 ²Department of Biomedical and Pharmaceutical Sciences, Chapman University School
12 of Pharmacy, Irvine, CA 92618

13

14 ³Department of Biomedical Sciences, College of Veterinary Medicine, Long Island
15 University, Brookville, NY 11548-1300

16

17 ⁴Bioscience, Research and Early Development, Respiratory, Inflammation and
18 Autoimmune (RIA), BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden

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28 ¹Address of Correspondence to Reynold A. Panettieri; rp856@rbhs.rutgers.edu

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31

32

33 Author Contributions:

34 C. Koziol-White: study design, data acquisition, data analysis and interpretation, drafting
35 and editing of the manuscript, final approval of publication

36 T. Johnstone: study design, data acquisition, data analysis and interpretation, editing of
37 the manuscript, final approval of publication

38 M. Corpuz: data acquisition, data analysis and interpretation, editing of the manuscript,
39 final approval of publication

40 G. Cao: study design, data acquisition, data analysis and interpretation, editing of the
41 manuscript, final approval of publication

42 S. Orfanos: study design, data acquisition, data analysis and interpretation, editing of
43 the manuscript, final approval of publication

44 V. Parikh: study design, data acquisition, data analysis and interpretation, editing of the
45 manuscript, final approval of publication

46 B. Deeney: data acquisition, data analysis and interpretation, editing of the manuscript,
47 final approval of publication

48 O. Tliba: study design, drafting and editing of the manuscript, final approval of
49 publication

50 R. Ostrom: study design, data acquisition, data interpretation, drafting and editing of the
51 manuscript, final approval of publication

52 I. Dainty: study design, data interpretation, editing of the manuscript, final approval of
53 publication

54 R.A. Panettieri Jr.: study design, drafting and editing of the manuscript, final approval of
55 publication

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57

58 **Abstract**

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

83

84 **Introduction**

85 Combination therapy including an inhaled glucocorticoid and a long-acting β_2
86 agonist represents a cornerstone in the management of airways disease including
87 asthma and chronic obstructive pulmonary disease. Conceptually, combined use of an
88 anti-inflammatory agent with a bronchodilator improves medication adherence as
89 compared to using each drug separately (6, 19, 26). Use of an inhaled corticosteroid
90 (ICS) with a long-acting β_2 agonist (LABA) as rescue and maintenance therapy appears
91 more effective than using an ICS or SABA alone or using an ICS/LABA for maintenance
92 (2, 3, 12, 14, 15, 21, 22). Evidence now suggests, apart from enhanced adherence,
93 combination therapy augments efficacy of either drug alone (18). We posit that this
94 effect is mediated by a rapid, non-genomic effect of glucocorticoids. To date, the
95 molecular mechanisms by which non-genomic effects of glucocorticoids modulate
96 bronchodilation remain unknown. (18).

97 Glucocorticoids primarily mediate their effects by activating the glucocorticoid
98 receptor (GR). In this canonical signaling, the GR resides in the cytoplasm in its inactive
99 state and, upon ligand activation, translocates to the cell nucleus to interact with
100 glucocorticoid response elements (GREs) and produce genomic effects altering protein
101 expression. Recent evidence has emerged to suggest that glucocorticoids manifest
102 rapid non-genomic actions on several signaling processes (18). These non-canonical,
103 non-genomic effects of glucocorticoids appear to involve non-specific interactions with
104 the cell membrane and/or specific interactions with cytosolic GRs (cGR) or membrane-
105 bound GRs (mGR). The rapid non-genomic effects appear to, at least in part, be
106 mediated through a putative mGR (18).

107 Several studies have reported an interaction of mGR with other membrane
108 receptors, particularly G protein-coupled receptors (GPCR) (27). Involvement of mGR
109 and GPCR-dependent mechanisms in the rapid effect (~ 1 min) of corticosterone on
110 NMDA-evoked currents in hippocampal neurons was demonstrated (27), suggesting
111 that mGR may couple to multiple G proteins, including G_{α_s} and $G_{\alpha_q/11}$. Other studies
112 suggest that mGR directly activates downstream intracellular signaling pathways.
113 Corticosterone acted via mGR to rapidly elicit PKC-dependent activation of ERK1/2
114 MAPK pathway in PC12 cells (20). Interestingly, proteomic analysis of CCRF-CEM cells

115 identified 128 proteins that were differentially regulated by activation of mGR using
116 BSA-conjugated cortisol briefly (5 and 15 min) (25). These actions were unique to mGR,
117 as no activation of cGR target genes (e.g. GILZ) were observed. Signal pathway
118 analysis now provides evidence that mGR is involved in numerous pathways that are
119 also regulated by glucocorticoids through cGR, suggesting that mGRs trigger early
120 priming events, ultimately facilitating slower genomic activation by glucocorticoids (25).

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

128

129 **Methods**

130

131 *Reagents*

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

142

143 *hPCLS generation and bronchodilation assays*

144 Human precision cut lung slices (hPCLS) were prepared as previously described
145 (11). Briefly, whole human lungs from non-asthma donors were dissected and inflated

146 using 2% (wt/vol) low melting point agarose. Once the agarose set, the lobe was
147 sectioned, and cores of 8-mm diameter were made. Cores containing small airways by
148 visual inspection were sliced at a thickness of 350 μm (Precisionary Instruments VF300
149 Vibratome, Greenville, NC) and collected in wells containing supplemented Ham's F-12
150 medium. To study bronchodilation, small airways contained within hPCLS were
151 contracted with carbachol (CCh, 10^{-5} M), and then bronchodilated to formoterol (10^{-12} –
152 10^{-6} M), budesonide (10^{-12} – 10^{-5} M), forskolin (10^{-12} – 10^{-5} M), PGE₂ (10^{-10} – 10^{-5} M), or
153 cholera toxin (0.01-100 $\mu\text{g/ml}$) \pm budesonide (10^{-5} M, simultaneous administration).
154 Human lung tissue samples were commercially obtained from anonymous donors
155 (National Disease Research Interchange, Philadelphia, PA or the International Institute
156 for the Advancement of Medicine, Edison, NJ), and are therefore exempt from IRB
157 (Institutional Review Board) approval. Although these samples have demographic
158 information, there is no information linking the subject's identification to the tissue.
159 Integrated area under the curve (AUC - % dilation/bronchodilator dose) was calculated
160 from the dose response curves and was plotted along with maximal bronchodilation
161 achieved over the entire dose response curve.

162

163 *Human airway smooth muscle (HASM) cells*

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

172

173 *cAMP assays*

174 For kinetic measurement of cAMP production in live cells, subconfluent HASM
175 cells were plated in black-walled, clear flat bottom 96-well plates with HASM media,
176 BacMam virus expressing the green cAMP difference detector *in situ* (cADDiS) cAMP

177 sensor (Montana Molecular, Bozeman, MT), and 1 μ M trichostatin-A (Sigma Aldrich, St.
178 Louis, MO) per well and grown overnight. Media was aspirated and replaced with PBS
179 without calcium or magnesium, then the plate was covered and incubated at room
180 temperature. Cell fluorescence was read from the plate bottom using
181 excitation/emission wavelengths of 494 nm and 522 nm, respectively, using a
182 SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). A kinetic read (5 min)
183 on unstimulated cells was performed to determine variability in each well's fluorescence
184 ($\leq 5\%$). Cells were stimulated with agonist and fluorescence changes were read at 30
185 second intervals for 30 min.

186

187 *Cell surface biotinylation*

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

197

198 *Immunohistochemistry*

199 HASM cells were grown in chamber slides until confluent, then serum starved for
200 18 hr. Cells were then fixed with 1% paraformaldehyde, washed, then blocked with 1%
201 BSA/PBS solution containing 10% FcR block (Miltenyi Biotec, Auburn, CA). Cells were
202 stained with a GR antibody (rabbit, Santa Cruz Biotechnology, Dallas, TX) in 1%
203 BSA/PBS solution overnight. The slides were washed, stained with biotin-coated
204 donkey anti-rabbit antibody (Jackson Immunolabs, Bar Harbor, ME) in 1% BSA/PBS,
205 washed, then incubated with a streptavidin-Alexa Fluor 488 conjugated antibody
206 (Jackson Immunolabs). The slides were washed, then the cells were permeabilized with
207 0.01% Triton X-100 and stained with DAPI. Slides were cover-slipped and imaged.

208

209 *Immunoblotting*

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

218

219 *Single analyte ELISAs*

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

225

226

227 *Statistical analyses*

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

236

237 **Results**

238

239 *Budesonide enhances formoterol-induced bronchodilation*

240 To examine whether a glucocorticoid and a β_2 agonist can additively promote
241 bronchodilation, human small airways in hPCLS were precontracted to carbachol and
242 then dilated to formoterol in the absence and presence of budesonide, with the
243 budesonide being added simultaneously with the formoterol. Budesonide treatment
244 augmented formoterol-induced bronchodilation (Figure 1), increasing maximal levels of
245 bronchodilation. Similarly, the integrated bronchodilator response as represented by
246 Area Under the Curve (AUC) significantly increased. Budesonide alone had little effect
247 on luminal diameter dilation despite being administered at similar concentrations as
248 formoterol (data not shown). These data show that simultaneous administration of
249 budesonide augments β_2 agonist-induced dilation of human small airways.

250

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

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

266

267 *Budesonide enhances formoterol-stimulated cAMP production*

268 To dissect the molecular pathways by which budesonide augments
269 bronchodilation of human airway smooth muscle (HASM), HASM were infected with a
270 recombinant BacMam expressing a fluorescent cAMP sensor, cADDis, and cAMP levels

271 were measured after exposure to varying concentrations of formoterol. The cADDiS
272 sensor decreases fluorescence upon binding cAMP, providing real-time assessment of
273 intracellular cAMP levels without inclusion of phosphodiesterase (PDE) inhibitors.
274 Formoterol decreased cADDiS fluorescence within minutes that typically stabilized
275 within 15-20 min (Figure 5A, inset). To account for the rate and maximal levels of cAMP
276 production produced by formoterol, the product of the decay rate (K) and the level at
277 steady state (plateau) were plotted for each drug concentration. Using this analysis,
278 formoterol increased cAMP levels (Figure 5A). In parallel, formoterol-stimulated cAMP
279 was measured in HASM treated with vehicle or 10^{-6} M budesonide given
280 simultaneously. Budesonide shifted the formoterol concentration-response curve 3.9-
281 fold leftward (Figure 5B). Additionally, budesonide at 1 or 10 μ M augmented formoterol-
282 induced cAMP production, but that effect was not realized at 100 nM budesonide. The
283 effect of budesonide on formoterol-stimulated cAMP production in HASM cells therefore
284 mimicked the effect of budesonide on agonist-induced bronchodilation observed in
285 hPCLS.

286

287 *Budesonide enhances PGE₂- and forskolin-stimulated cAMP production*

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

297

298 *Budesonide, fluticasone and prednisone enhance directly stimulated cAMP production*

299 Because budesonide enhanced agonist-induced cAMP signaling, we investigated
300 whether budesonide alone stimulated cAMP. Significant decreases in cADDiS
301 fluorescence was observed within seconds of addition of 1 or 10 μ M budesonide (data

302 not shown). 10 μ M budesonide stimulated cAMP levels that were statistically different
303 than vehicle within 2 minutes, while 1 μ M induced significant changes within 5 minutes.
304 The higher concentration of budesonide stimulated changes in cADDis fluorescence
305 that were equivalent to maximal concentrations of forskolin (10 μ M) or formoterol (not
306 shown). However, cADDis is a readily saturated sensor so high levels of cAMP may not
307 be distinguishable (9). We also examined cAMP production by other corticosteroids,
308 finding that fluticasone and prednisone (data not shown) stimulated cAMP production.
309 Fluticasone was equi-effective as budesonide although greater inter-experimental
310 variability was observed while prednisone was less efficacious and less potent.

311

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

313 Since budesonide effects on cAMP generation and bronchodilation were rapid,
314 we posited that budesonide activates a membrane-associated receptor. Other
315 laboratories have identified a mGR that can evoke immediate steroid effects in other cell
316 types (13, 23, 24). Using cell-surface biotin labeling and immunohistochemistry (Figure
317 6), we showed that HASM express mGRs that fail to translocate to the nucleus from the
318 membrane despite stimulation with a glucocorticoid (dexamethasone). As expected,
319 cytosolic GR translocated to the nucleus as shown in Figure 6.

320

321 *Budesonide augments formoterol-induced dephosphorylation of myosin light chain*

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

331

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

334 To more directly assess the role of the GR in mediating rapid cAMP responses
335 to glucocorticoids, we used siRNA to knock down GR α expression in
336 HASM. Transfection of validated siRNA sequences into HASM led to a nearly complete
337 loss of GR α immunoreactivity (detected as a doublet of approximately 94 kDa) as
338 compared to cells transfected with a scrambled siRNA (Figure
339 8A). GR α mRNA expression in control HASM was readily detected by quantitative RT-
340 PCR but was undetectable in cells transfected with GR α siRNA (not shown). We
341 then assessed cAMP levels in HASM following siRNA transfection. Forskolin-stimulated
342 cAMP responses were unaltered by GR α knockdown, implying that loss of GR α did not
343 alter AC expression or function (data not shown). Budesonide (1 μ M) or formoterol (0.1
344 nM) each elicited equivalent cAMP responses in both GR α knockdown and control cells
345 (Figure 8B and 8C). cAMP responses to simultaneous addition of budesonide and
346 formoterol were greater than either agent alone, but were similar in both control
347 and GR α knockdown conditions (Figure 8D). Knockdown of GR α was confirmed by
348 immunoblot, and showed reversal of budesonide attenuation of TNF α -induced
349 inflammatory mediator release (data not shown). These results suggest that
350 GR α expression may not be required for rapid, non-genomic signaling by GC.

351

352

353 **Discussion**

354

355 In airway smooth muscle, contractile agonists stimulate Ca²⁺-dependent
356 signaling evoking cell shortening regulated in part through increased [Ca²⁺]_i transients,
357 inhibition of sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), and pMLC inducing actin-
358 myosin cross-bridge cycling and force generation. Agents that mobilize cAMP and
359 activate protein kinase A (PKA) protect against or reverse agonist-induced
360 bronchoconstriction. In epithelial cells and neurons, glucocorticoids modulate signaling
361 pathways that promote relaxation of HASM. Dexamethasone stimulation of bronchial
362 cells attenuated [Ca²⁺]_i currents, which was reversed by SERCA inhibitors, PKA, and

363 activated adenylyl cyclase (AC) within minutes (24). Further, corticosterone treatment
364 reversed ATP-induced $[Ca^{2+}]_i$ transients in mouse HT4 neuroblastoma cells (7). In
365 primary HASM cells, budesonide stimulation alone increased cAMP levels (data not
366 shown). Interestingly, we show that simultaneous budesonide and formoterol
367 significantly reduced carbachol-induced pMLC (Figure 7), but little effect of budesonide
368 alone. Others noted existence of a mGR that may mediate non-genomic, rapid effects of
369 glucocorticoids. We demonstrate presence of mGR in HASM (Figure 6) that does not
370 translocate upon glucocorticoid stimulation. Our work suggests that expression of GR α
371 is not necessary to elicit cAMP production in response to budesonide or budesonide +
372 formoterol (Figure 8). There is a possibility that residual GR protein whose expression
373 was not attenuated by siRNA transfection may mediate the effect, but the existence of a
374 modified GR that may not be subject to knockdown of the protein may mediate the
375 effects of steroids on cAMP production/augmentation of bronchodilation. Overall, these
376 data suggest that despite the effect of glucocorticoids on attenuation of Ca^{2+} -dependent
377 pathways, glucocorticoids alone had little effect on agonist-induced bronchoconstriction
378 of small airways (data not shown) in hPCLS but rather, amplified β_2 agonist-induced
379 bronchodilation.

380 Although glucocorticoids alone induced cAMP production (data not shown), these
381 steroids had little effect on bronchodilation of small airways in hPCLS (data not shown).
382 Robust and sensitive assessment of cAMP pools is achieved using the cADDis reporter
383 (9). Although the assay shows increases in cAMP production to budesonide alone, this
384 increase in cAMP levels may be insufficient for promoting bronchodilation due to an
385 inadequate magnitude or localization of the cAMP signal despite utilizing the same
386 concentrations of budesonide as formoterol in the hPCLS bronchodilation assays (10^{-12}
387 – 10^{-5} M, data not shown). Glucocorticoids may therefore be increasing cAMP levels via
388 direct activation of $G_{\alpha s}$, activation of an unidentified GPCR, or inhibition of
389 phosphodiesterases. Others suggest that increases in intracellular cAMP elicited by
390 glucocorticoids may be due to inhibition of ABCC4, an ATP binding cassette transporter
391 that pumps cAMP into the extracellular space. In differentiated airway epithelial cells,
392 dexamethasone, but not budesonide, augmented activity of GRE-luciferase when given
393 with forskolin treatment. Additionally, blockade of ABCC4 potentiated activity of the

394 GRE-luciferase in the presence of either dexamethasone or budesonide treatment (8).
395 Unlike airway epithelial cells, β_2 agonist or forskolin treatment had little effect on
396 extracellular levels of cAMP in HASM (data not shown). However, we reported that
397 ABCC4 is expressed in HASM cells, and its expression is augmented by budesonide
398 treatment (10). In the aforementioned studies, glucocorticoid treatment was for 6-12
399 hours, conceivably the mechanisms modulating rapid glucocorticoid stimulation may
400 differ. Future studies will address whether non-genomic effects of glucocorticoids are
401 mediated by activation of ABCC4 proteins.

402 Others noted acute glucocorticoid stimulation activates signaling pathway
403 components in non-muscle cells that are associated with bronchodilatory responses in
404 smooth muscle. In bronchial epithelial cells dexamethasone inhibited agonist-induced
405 $[Ca^{2+}]_i$, with the inhibition sensitive to AC and PKA inhibition (24). Other show a non-
406 genomic glucocorticoid effect that was PKA-dependent in a neural cell line (7).
407 Accordingly, to assess mechanisms by which steroid may be augmenting agonist-
408 induced bronchodilation we examined the effect of glucocorticoids on: (1) selectivity of
409 responses we observed to the β_2AR ; (2) direct activation of $G_{\alpha s}$; and (3) whether the
410 response observed was receptor-dependent. We found that PGE_2^- , CTX-, and FSK-
411 stimulated bronchodilation of small airways (Figures 2, 3 and 4, respectively) was
412 augmented by co-administration of budesonide. Similarly, PGE_2^- , and FSK-induced
413 cAMP production in HASM (Figure 5C and D) was augmented by budesonide co-
414 administration. Despite the cADDis characterizing budesonide's effects on CTX-induced
415 cAMP production, CTX induced such saturating levels of cAMP production in HASM
416 cells making us unable to detect a synergism above CTX stimulation alone (data not
417 shown).

418 Interestingly, synergism between glucocorticoids and β_2 agonists has been
419 examined previously. A recent study noted that beclomethasone augmented
420 formoterol-induced reversal of pre-contracted airways (4), to about ~30% maximally (5).
421 Although there was synergism between the two therapeutics, the beclomethasone dose
422 was given overnight, with effects likely genomic. This is in direct contrast to what our
423 studies show, where despite budesonide inducing cAMP production in HASM, it alone
424 was unable to induce bronchodilation even at a concentration of 100 μM . Additionally,

425 the 2016 study utilized non-selective inhibitors of PKA and $G_{\alpha s}$ to establish roles for
426 those protein in beclomethasone-induced bronchodilation in lung slices. Our studies
427 have the advantage of observing cAMP production in live primary HASM in real time, a
428 system in which we can genetically manipulate components of signaling pathways to
429 more rigorously ascertain the necessity and sufficiency specific molecules in the
430 responses we observe.

431 Our studies identify mechanisms by which budesonide, a potent glucocorticoid,
432 rapidly activates cAMP production and augments agonist-mediated bronchodilation.
433 This is the first direct demonstration that glucocorticoids stimulate cAMP production in
434 airway smooth muscle. These mechanisms may explain, in part, how combination
435 therapy of β_2 agonists and ICS offer greater efficacy in comparison to either drug alone.
436 Interestingly, these beneficial effects occur separate and distinct from any anti-
437 inflammatory effects (18). Combination therapy represents a cornerstone for
438 maintenance therapy in asthma and COPD. Evidence also suggests that this
439 combination can serve as an anti-inflammatory reliever therapy (when added to
440 maintenance) that decreases exacerbation rates, improves asthma symptoms, and
441 enhances lung function as compared to higher doses of inhaled corticosteroids (ICS)
442 alone (14, 21, 22). Our data identify molecular mechanisms by which steroids and long-
443 acting β_2 agonists together improve bronchodilation.

444

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449

450

451 **Figure 1 – Acute budesonide stimulation augments formoterol-induced**
452 **bronchodilation, but does not induce bronchodilation alone.** (A) Simultaneous
453 stimulation of hPCLS with budesonide (10^{-5} M) with formoterol (10^{-12} – 10^{-6} M)
454 augments (B) maximal bronchodilation (formoterol vs. budesonide at 10^{-6} M formoterol,
455 $71.4 \pm 5\%$ vs. $89.7 \pm 7.3\%$), and increases the integrated (C) area under the curve vs.

456 formoterol alone (formoterol vs. formoterol + budesonide, 156.8 ± 25.9 vs. $257.1 \pm$
457 51.6). Data represents $n=15-25$ donors, $p<0.05$ vs formoterol alone. (D) Budesonide
458 alone does not induce bronchodilation in hPCLS. Data represents 2 (budesonide alone)
459 - 15 (formoterol) donors, 6-36 slices/condition.

460

461 **Figure 2 – Simultaneous budesonide treatment augments PGE₂-induced**
462 **bronchodilation of human small airways.** Budesonide ($10 \mu\text{M}$) was given
463 simultaneously with PGE₂ ($10^{-10} - 10^{-5}$ M) and bronchodilation was assessed.
464 Concentration-response curves (A) are represented as % dilation for the combination
465 compared to PGE₂-induced dilation alone. Maximal bronchodilation at 10^{-5} M PGE₂ (B,
466 PGE₂ vs. PGE₂ + budesonide at a maximum of 10^{-5} M PGE₂, $116.6 \pm 16.6\%$ vs. $283.3 \pm$
467 37.3%) and integrated area under the curve (C, PGE₂ vs. PGE₂ + budesonide, $264.5 \pm$
468 40 vs. 736.2 ± 209.6) of PGE₂-induced bronchodilation were significantly increased.
469 Data represents $n=3$ donors, $*p<0.05$ compared to PGE₂ stimulation alone.

470

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

480

481 **Figure 4 – Budesonide augments forskolin-induced bronchodilation of human**
482 **small airways.** Budesonide ($10 \mu\text{M}$) was given simultaneously with forskolin (Fsk, 10^{-12}
483 $- 10^{-5}$ M) and bronchodilation was assessed. Concentration-response curves to Fsk (A)
484 were plotted. Maximal bronchodilation at 10^{-5} M FSK (B, FSK vs. FSK + budesonide at
485 maximum of 10^{-5} M FSK, $83.7 \pm 8.5\%$ vs. $110.1 \pm 10.6\%$) and area under the curve (C,
486 FSK vs. FSK + budesonide, 151.9 ± 11.8 vs. 232.3 ± 29.1) were significantly increased

487 in the presence of budesonide. Data are representative of n=5 donors, * p<0.05
488 compared to control/Fsk.

489
490 **Figure 5 – Budesonide alone induces cAMP production in HASM cells, and**
491 **enhances formoterol, PGE₂, and forskolin-induced cAMP production.** HASM cells
492 were incubated with recombinant BacMam virus expressing the cADDis cAMP sensor
493 for 24 hr. After establishing baseline, fluorescence decay was monitored for 30 min after
494 addition of drug. (A) cADDis sensor fluorescent decay curves elicited by various
495 concentrations of formoterol were fit by one-phase decay non-linear regression analysis
496 (inset, 10⁻¹² – 10⁻⁶ M formoterol, log EC₅₀ of -7.78 ± 0.185). The rate (K) was multiplied
497 by the steady state change in fluorescence (plateau) for each concentration of
498 formoterol. Each point represents the mean ± SEM of n=5. (B) Budesonide alone (1 or
499 10 μM) elicits cAMP production in cells, and is compared to forskolin (10 μM)
500 stimulation. Each point represents the mean ± SEM of n=4-6 cell lines and lines
501 represent the fit by one-phase decay non-linear regression analysis. * denotes p < 0.05,
502 ** denotes p < 0.01 of each time point compared to vehicle using multiple t tests and the
503 Holm-Sidak method for correction of multiple comparisons. (C) Formoterol
504 concentration-responses curves in the presence of vehicle or 1 μM budesonide. Each
505 point represents the mean ± SEM of n=5 cell lines (log EC₅₀ formoterol vs. formoterol +
506 budesonide, -7.76 ± 0.205 vs. -8.36 ± 0.189, p=0.021). (D) PGE₂ concentration-
507 responses curves in the presence of vehicle or 1 μM budesonide. Each point represents
508 the mean ± SEM of n=4 cell lines (log EC₅₀ PGE₂ vs. PGE₂ + budesonide, -7.85 ± 0.062
509 vs. -8.78 ± 0.129, p=0.046). (E) Forskolin concentration-responses curves in the
510 presence of vehicle or 1 μM budesonide. Each point represents the mean ± SEM of n=7
511 cell lines (log EC₅₀ FSK vs. FSK + budesonide, -6.75 ± 0.168 vs. -7.67 ± 0.190,
512 p=0.005).

513
514 **Figure 6 - mGR exists in HASM.** (A) HASM from non-diseased lung donors was
515 biotinylated and purification of a membrane fraction determined the existence of a
516 membrane bound form of the glucocorticoid receptor. Presence of the epidermal growth
517 factor receptor (EGFR) and absence of GAPDH and COX IV (cytosolic proteins) were
518 used as controls for purity of membrane protein isolation. (B) HASM cells were fixed

519 with acetone following stimulation for 30 min with dexamethasone (1 μ M). Fixed cells
520 were then probed for glucocorticoid receptor and DAPI. Data are representative of 3
521 separate non-asthma donors for both (A) and (B).

522

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

530

531 **Figure 8 – Knockdown of GR α had little effect on budesonide or budesonide +**
532 **formoterol-induced cAMP production in HASM.** HASM were transfected with siRNA
533 specific for GR α or scrambled control (mock) for 72 hr. A: Lysates were collected and
534 analyzed by immunoblotting using antibodies specific for GR α or β -actin. Each lane
535 shows one of 3 separate experiments on individual cell lines. B, C and D: cADDis
536 sensor was expressed in HASM with a recombinant BacMam virus
537 then cAMP responses to budesonide (1 μ M, B), formoterol (0.1 nM, C) or both
538 budesonide and formoterol (1 μ M and 0.1 nM, respectively, D) were measured in HASM
539 were transfected with non-targeting siRNA or siRNA specific for GR α . Each point
540 represents the mean \pm SEM of $n=4-5$ cell lines.

541

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546 **References**

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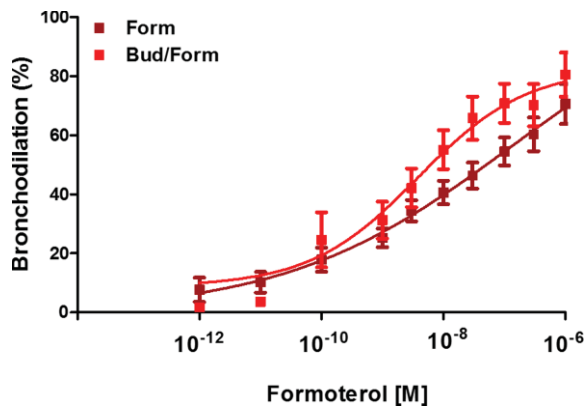
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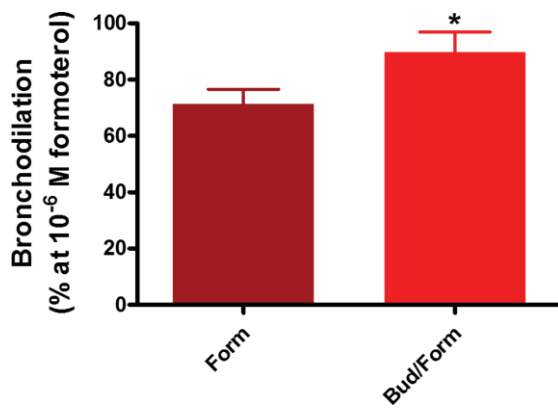
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Figure 1

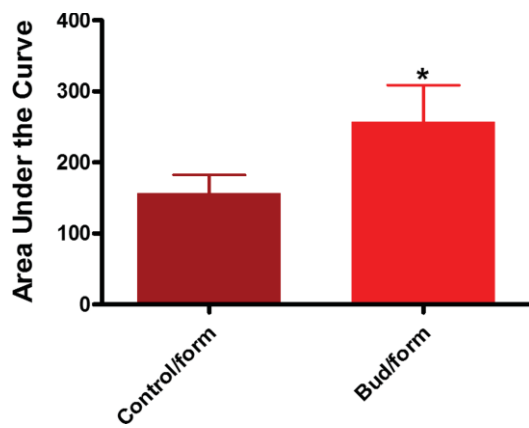
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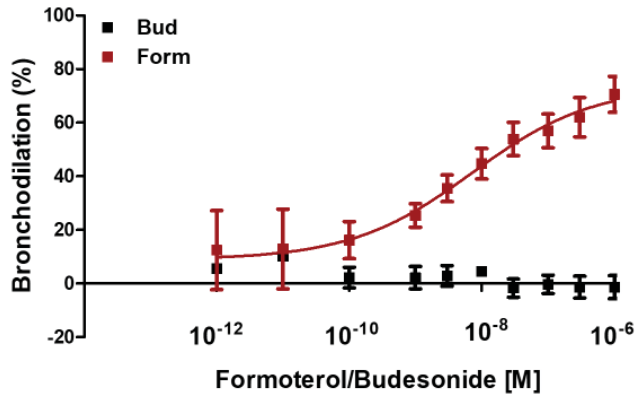
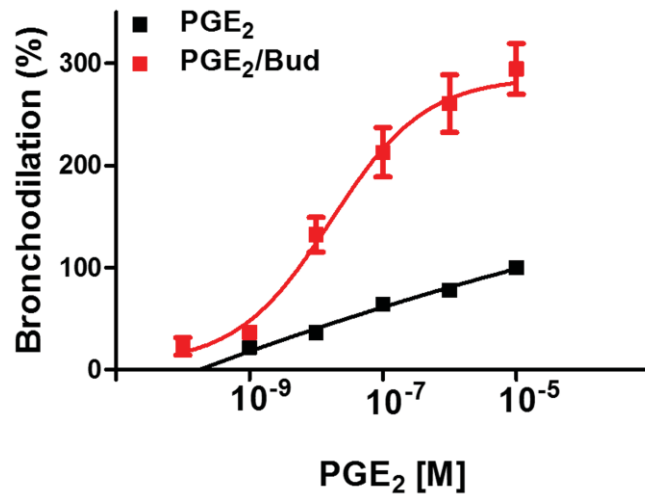
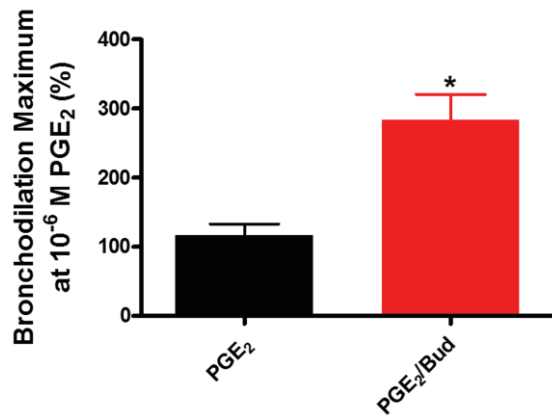


Figure 2

(A)



(B)



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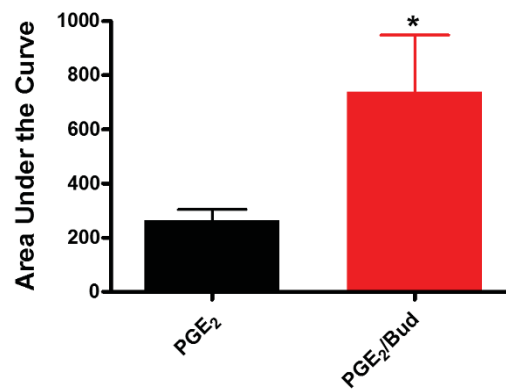
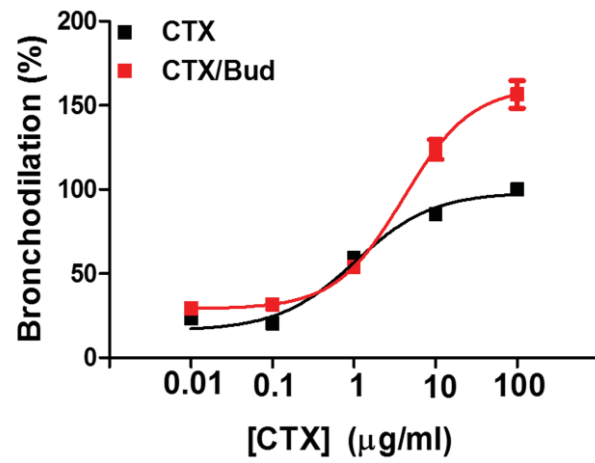
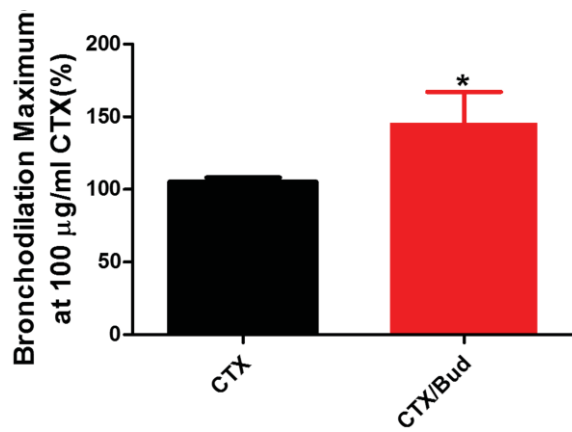


Figure 3

(A)



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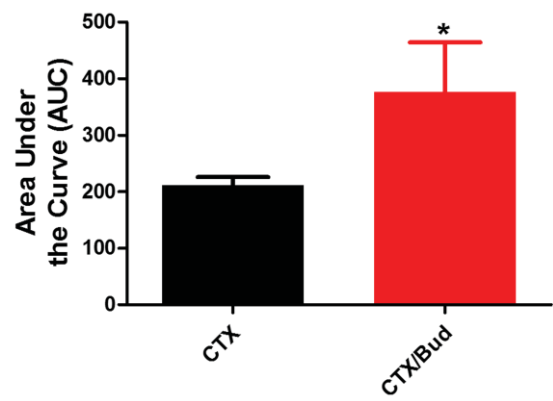
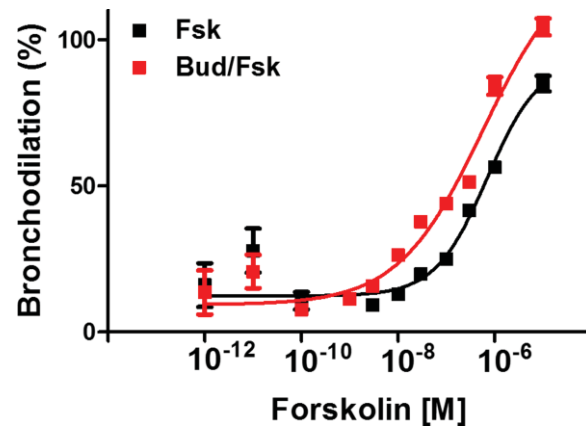
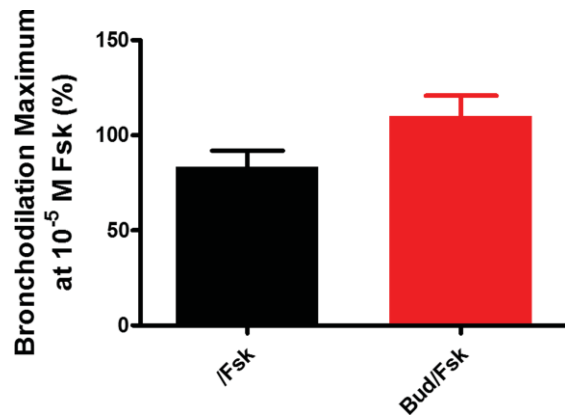


Figure 4

(A)



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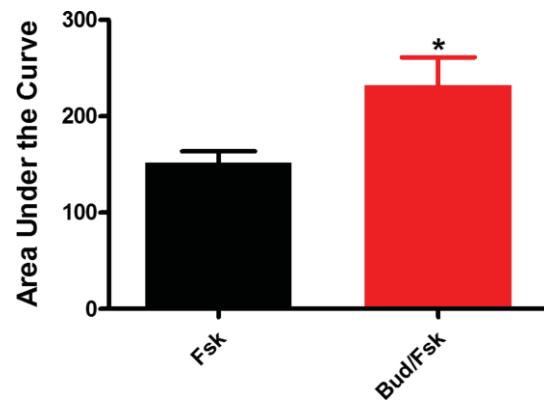
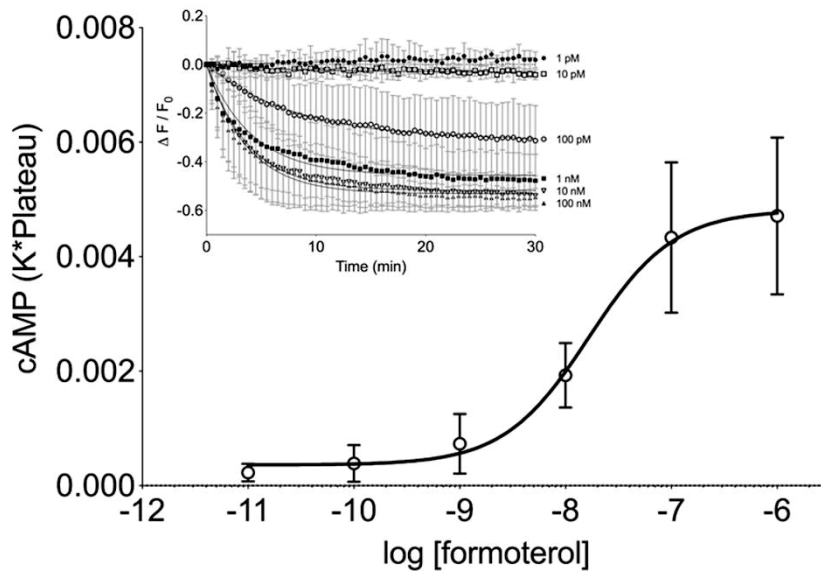
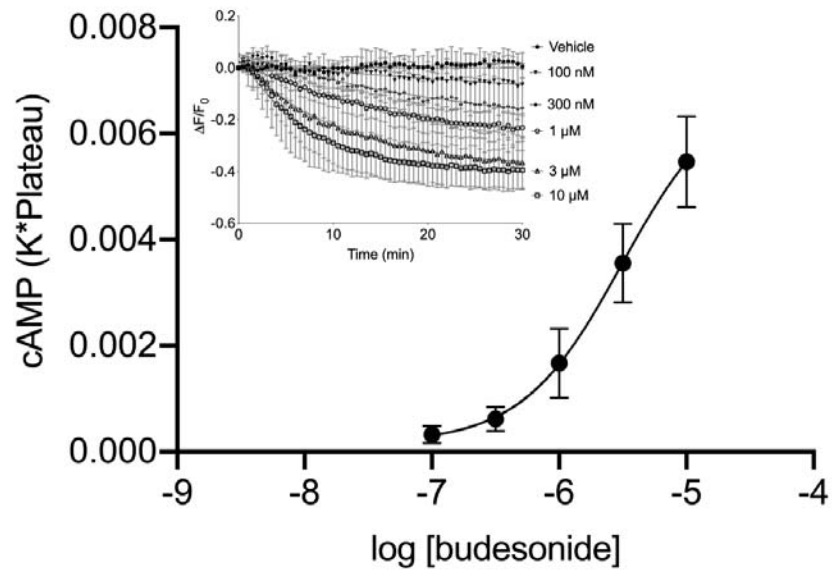


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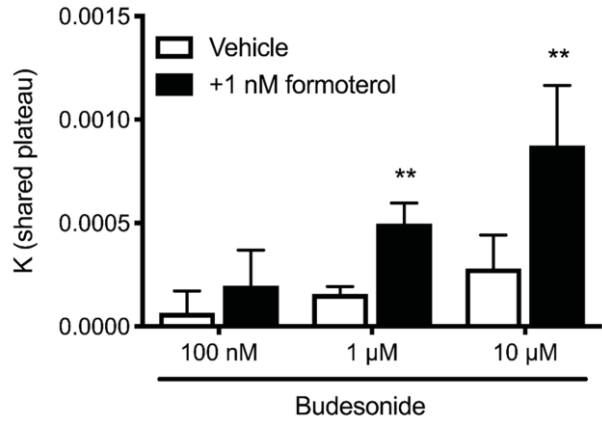
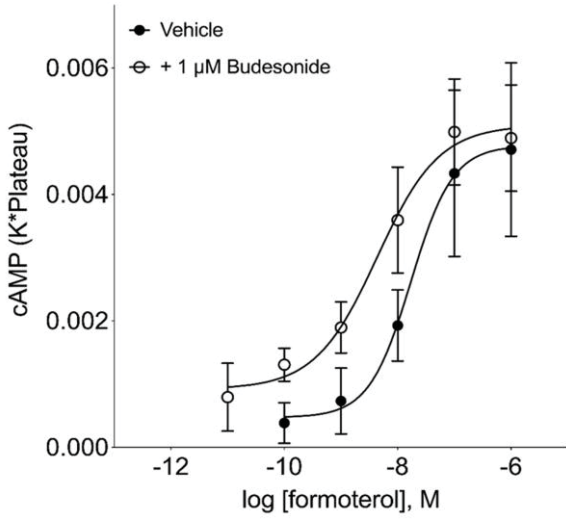
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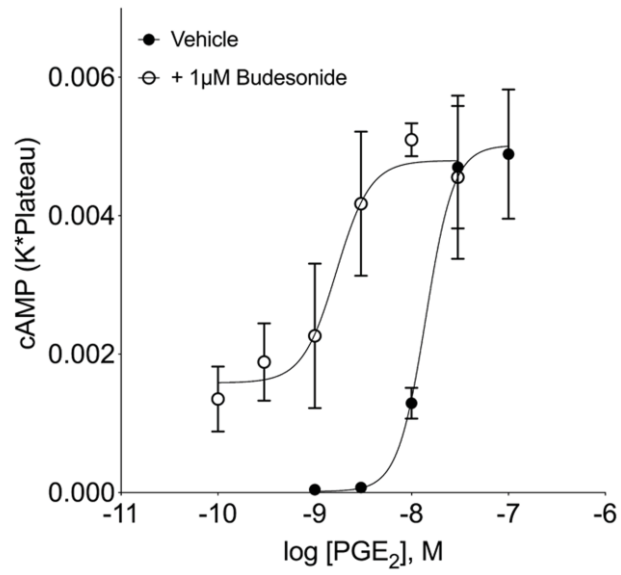
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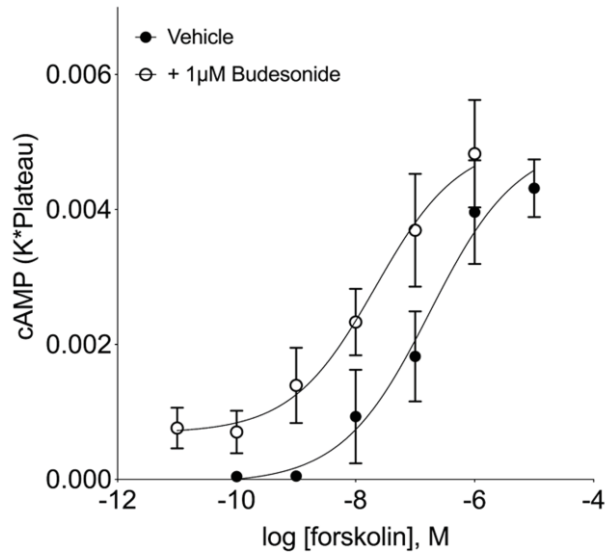
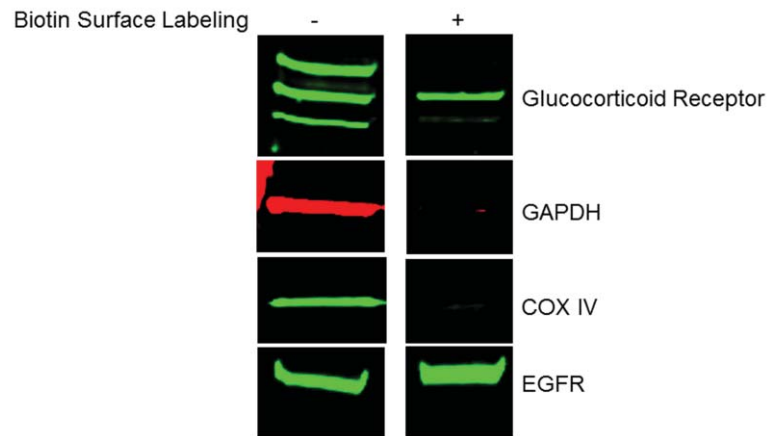


Figure 6

(A)



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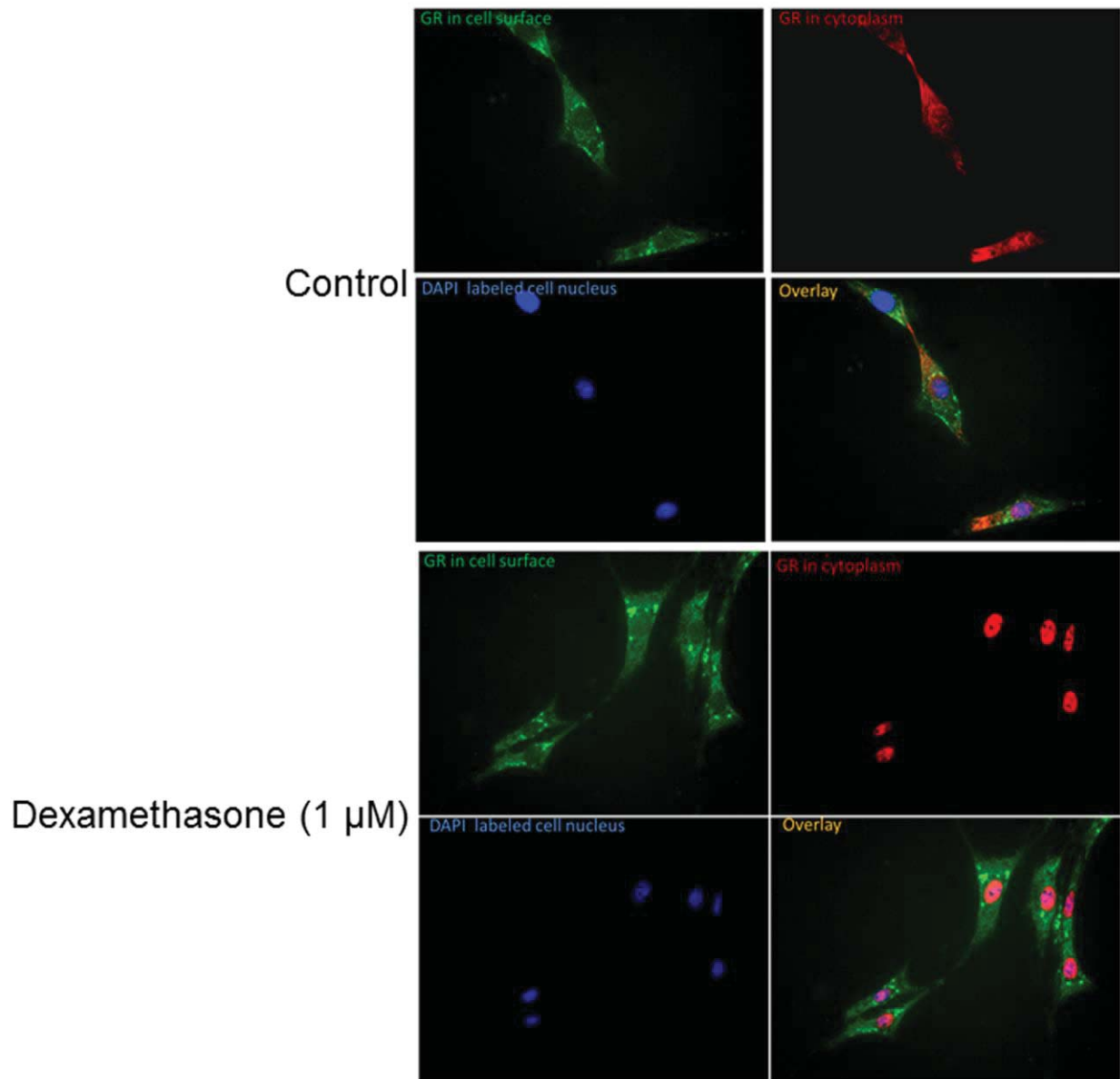
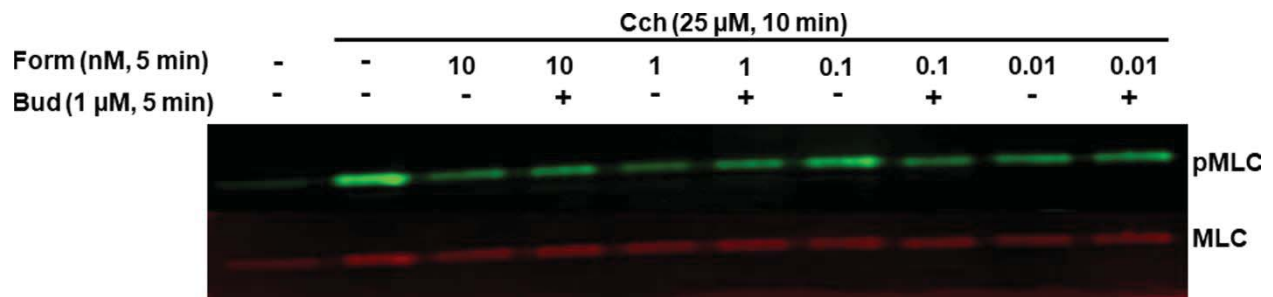


Figure 7

(A)



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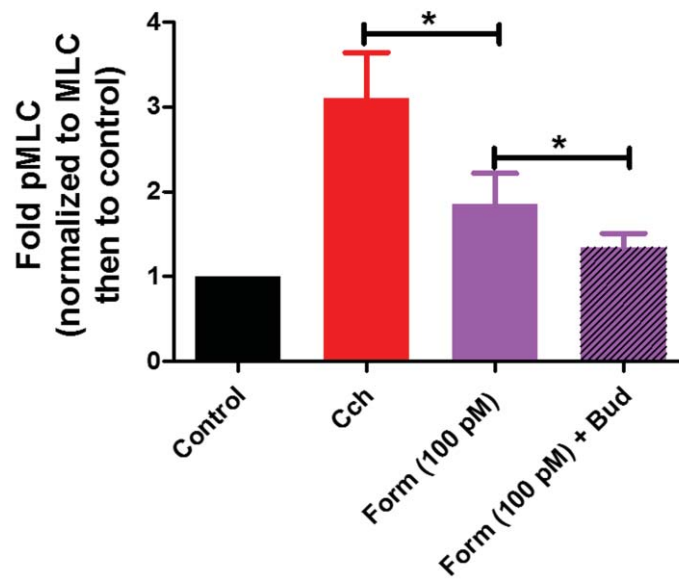


Figure 8

