

β_2 -Adrenoceptor Agonists Promote ERK1/2 Dephosphorylation in Human Airway Epithelial Cells by Canonical, cAMP-driven Signaling Independently of β -Arrestin 2

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Abbreviations: ALI, air-liquid interface; *ARRB*, arrestin gene; Arr, Arrestin; CRE, cAMP response element; DUSP, dual specificity phosphatase; ECL, electrochemiluminescence; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; GFP, green fluorescent protein; GPCR, G-protein-coupled receptor; HBEC, human bronchial epithelial cells; ICI 118,551, (2*R*,3*R*)-1-[(7-methyl-2,3-dihydro-1*H*-inden-4-yl) oxy]-3-(propan-2-ylamino)butan-2-ol; hydrochloride; L-161,982, *N*-[2-[4-[[3-butyl-5-oxo-1-[2-(trifluoromethyl)phenyl]-1,2,4-triazol-4-yl]methyl]phenyl]phenyl]sulphonyl-5-methyl-thiophene-2-carboxamide; LABA, long-acting β_2 -adrenoceptor agonist; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; MKP, mitogen-activated protein kinase phosphatase; MOI, multiplicity of infection; ONO-AE1-259, (Z)-7-[(1*R*,2*R*,3*R*,5*R*)-5-chloro-3-hydroxy-2-[(E,4*S*)-4-hydroxy-4-(1-prop-2-enylcyclobutyl)but-1-enyl]cyclopentyl] hept-5-enoic acid; ONO-AE1-329, 2-[3-[(1*R*,2*S*,3*R*)-3-hydroxy-2-[(E,3*S*)-3-hydroxy-5-[2-(methoxymethyl)phenyl]pent-1-enyl]-5-oxo-cyclopentyl]sulphonyl propylsulphonyl] acetic acid; PBS, phosphate-buffered saline; PD 098059, 2-(2-amino-3-methoxyphenyl) chromen-4-one; PDE, phosphodiesterase; PKA, cAMP-dependent protein kinase; PKI, cAMP-dependent protein kinase inhibitor; PMA, phorbol 12-myristate 13-acetate; TG4-155, (E)-*N*-[2-(2-methylindol-1-yl) ethyl]-3-(3,4,5-trimethoxyphenyl)prop-2-enamide; RNO, roflumilast *N*-oxide; SFM, serum-free medium; TNF α , tumor necrosis factor- α ; U0126, (2*Z*,3*Z*)-2,3-bis[amino-(2-aminophenyl)sulphonylmethylidene] butane dinitrile; Rap, rat sarcoma-proximate; Raf, rapidly accelerated fibrosarcoma; Ras, rat sarcoma; TPM, transcripts per million; YM-254890, [(1*R*)-1-[(3*S*,6*S*,9*S*,12*S*,18*R*, 21*S*,22*R*)-21-acetamido-18-benzyl-3-[(1*R*)-1-methoxyethyl]-4,9,10,12,16,22-hexamethyl-15-methylidene-2,5,8,11,14,17,20-hepta-oxo-1,19-dioxo-4,7,10,13,16-pentazacyclodocos-6-yl]-2-methylpropyl](2*S*,3*R*)-2-acetamido-3-hydroxy-4-methyl pentanoate.

ABSTRACT

Chronic use of β_2 -adrenoceptor agonists as a monotherapy in asthma is associated with a loss of disease control and an increased risk of mortality. Herein, we tested the hypothesis that β_2 -adrenoceptor agonists, including formoterol, *promote* biased, β -arrestin 2 (β Arr2)-dependent activation of the mitogen-activated protein (MAP) kinases, ERK1/2, in human airway epithelial cells and, thereby, effect changes in gene expression that could contribute to their adverse clinical outcomes. Three airway epithelial cell models were used: the BEAS-2B cell line, human primary bronchial epithelial cells (HBEC) grown in submersion culture and HBEC that were highly differentiated at an air-liquid interface. Unexpectedly, treatment of all epithelial cell models with formoterol decreased basal ERK1/2 phosphorylation. This was mediated by cAMP-dependent protein kinase and involved the inactivation of C-rapidly-activated fibrosarcoma, which attenuated down-stream ERK1/2 activity, and the induction of dual-specificity phosphatase-1. Formoterol also inhibited the basal expression of early growth response-1, an ERK1/2-regulated gene that controls cell growth and repair in the airways. Neither carvedilol, a β_2 -adrenoceptor agonist biased towards β Arr2, nor formoterol promoted ERK1/2 phosphorylation in BEAS-2B cells although β_2 -adrenoceptor desensitization was compromised in *ARRB2*-deficient cells. Collectively, these results contest the hypothesis that formoterol *activates* ERK1/2 in airway epithelia by nucleating a β Arr2 signaling complex; instead, they indicate that β_2 -adrenoceptor agonists *inhibit* constitutive ERK1/2 activity in a cAMP-dependent manner. These findings are the antithesis of results obtained using acutely challenged native and engineered HEK293 cells, which have been used extensively to study mechanisms of ERK1/2 activation, and highlight the cell-type-dependence of β_2 -adrenoceptor-mediated signaling.

SIGNIFICANCE STATEMENT

It has been proposed that the adverse-effects of β_2 -adrenoceptor agonist monotherapy in asthma are mediated by genomic mechanisms that occur principally in airway epithelial cells and are the result of β -arrestin 2-dependent activation of ERK1/2. This study shows that β_2 -adrenoceptor agonists, paradoxically, *reduced* ERK1/2 phosphorylation in airway epithelia by disrupting upstream Ras-C-Raf complex formation and inducing DUSP1. Moreover, these effects were PKA-dependent suggesting that β_2 -adrenoceptor agonists were not biased toward β -arrestin 2 and acted via canonical, cAMP-dependent signaling.

Introduction

Inhaled β_2 -adrenoceptor agonists are a mainstay asthma therapy and effectively relieve symptoms by promoting rapid and prolonged bronchodilation. However, regular administration of these drugs as a monotherapy is associated with tolerance (Cockcroft et al., 1993; Salpeter et al., 2006), a loss of asthma control (Drazen et al., 1996) and increased mortality (Nelson et al., 2006). The mechanism driving these undesirable clinical outcomes remains unclear, but the ability of β_2 -adrenoceptor agonists to modulate the expression of numerous pro-inflammatory genes may be an important factor (Ritchie et al., 2018; Yan et al., 2018). Indeed, β_2 -adrenoceptor agonists, administered chronically to sensitized mice, enhance the “asthma-like” pathological changes that follow allergen challenge including airway hyper-responsiveness, mucus hyper-secretion, pulmonary inflammation and increased pulmonary leukocyte burden (Lin et al., 2012; Thanawala et al., 2013). Mechanistically, Nguyen et al., (2017) have proposed that β_2 -adrenoceptor agonists stimulate $G_s\alpha$ in airway smooth muscle to effect bronchodilation and relieve symptoms but exacerbate asthma pathology by activating mitogen-activated protein (MAP) kinase signaling in a β -arrestin 2 (β Arr2)-dependent manner (*vide infra*). They also posit that this “biased agonism” is mediated by β_2 -adrenoceptors located on airway epithelial cells. This is a credible proposal because the airway epithelium promotes and regulates inflammatory processes, is the first site of action for inhaled therapies and expresses a population of efficiently coupled β_2 -adrenoceptors (Davis et al., 1990; Joshi et al., 2021; Kelsen et al., 1995; Knight and Holgate, 2003; Penn et al., 1994). Moreover, extracellular signal-regulated kinases 1 and 2 (ERK1/2) are phosphorylated to a greater degree in bronchial biopsies taken from asthmatic subjects than in healthy controls, with changes in the airway epithelium being positively correlated with indices of inflammation (Alam and Gorska, 2011; Liu et al., 2008).

Classically, β_2 -adrenoceptor agonists promote the $G_s\alpha$ -dependent stimulation of adenylyl cyclase, cAMP generation and activation of several down-stream effectors (Gerits et al., 2008; Giembycz and Newton, 2006). One target is cAMP-dependent protein kinase (PKA), which regulates MAP kinase signaling in a complex manner (Dumaz and Marais, 2005; Gerits et al., 2008). In some tissues, the MAP kinase kinase kinase, B-rapidly accelerated fibrosarcoma (Raf) is activated indirectly by PKA via the small GTP-binding protein, rat sarcoma (Ras)-proximate (Rap) 1 and promotes the sequential phosphorylation of mitogen-activated protein kinase kinase (MEK) 1/2 and ERK1/2 (Takahashi et al., 2017b; Vossler et al., 1997). In

other cell types, the B-Raf paralog, C-Raf, is phosphorylated by PKA and, thereby, rendered unable to interact with Ras, such that down-stream MEK1/2-ERK1/2 signaling is impaired (Cook and McCormick, 1993). β_2 -Adrenoceptor agonists also modulate ERK1/2 activity in a cell type-dependent manner (Lefkowitz et al., 2002). For example, ERK1/2 is activated in HEK293 cells (Daaka et al., 1997; Schmitt and Stork, 2000; van der Westhuizen et al., 2014), human dermal fibroblasts (Pullar and Isseroff, 2006), rat cardiac myocytes (Zou et al., 1999), COS-7 fibroblasts (Crespo et al., 1995) and rat dorsal root ganglion neurons (Aley et al., 2001) but inactivated in the MDA-MB-231 human breast cancer cell line (Pon et al., 2016) and J774 murine macrophages (Keränen et al., 2017). Currently, the cell-type specific factors that determine whether PKA increases or decreases MAP kinase signaling are unclear.

β_2 -Adrenoceptor agonism also leads to the recruitment of β -arrestins. These multifunctional proteins uncouple the agonist-occupied receptor from Gs resulting in homologous desensitization (Benovic et al., 1987; Goodman et al., 1996; Luttrell and Lefkowitz, 2002). They also act as scaffolds for the assembly of a myriad of signaling elements that include C-Raf, MEK1/2 and ERK1/2 (Peterson and Luttrell, 2017). Currently, the role of β -arrestins in β_2 -adrenoceptor-mediated ERK1/2 activation is controversial. While abundant data suggest that β -arrestins are obligatory (Luttrell and Gesty-Palmer, 2010; Luttrell et al., 2018; Shenoy et al., 2006), the application of genome editing technologies has recently challenged that assumption (Grundmann et al., 2018; O'Hayre et al., 2017).

In this study, we tested the hypothesis that β_2 -adrenoceptor agonists activate β Arr2-dependent C-Raf-MEK1/2-ERK1/2 signaling in airway epithelial cells and, thereby, promote changes in gene expression that could contribute to their adverse-effects in asthma. Three airway epithelial cell models were compared in this endeavor: the BEAS-2B bronchial epithelial cell line, human primary bronchial epithelial cells (HBEC) grown in submersion culture and HBEC that were highly differentiated at an air-liquid interface (ALI). HEK293 cells were examined in parallel, as they have been used extensively to interrogate the impact of β_2 -adrenoceptor agonism on MAP kinase signaling and are an instructive comparator.

Materials and Methods

Submersion Culture of HEK293 β 2 Cells. HEK293S cells stably expressing an amino-terminal, hemagglutinin-tagged human β_2 -adrenoceptor (HEK293 β 2) at a density of 3.2 pmol/mg protein were donated by Dr. Michel Bouvier (University of Montreal, PQ, Canada). Cells were grown until confluent under a 5% CO₂/air atmosphere at 37°C in Dulbecco's-modified Eagle's medium supplemented with 10% FBS, L-glutamine (2 mM) and G-418 (400 μ g/ml). Cells were cultured for a further 24 h in supplement-free, basal medium prior to experimentation.

Submersion Culture of BEAS-2B Cells. BEAS-2B cells (ATCC, Manassas, VA) were cultured for two days under a 5% CO₂/air atmosphere at 37°C in 24-well tissue culture plates (Costar Inc, Corning, NY) containing DMEM/F12 supplemented with 10% FBS (Life Technologies, Burlington, Ontario, Canada), L-glutamine (2.5 mM) and NaHCO₃ (14 mM; all Invitrogen), and for a further 24 h in serum-free medium (SFM) without supplements (Greer et al., 2013). At this time, cultures were confluent and used for experimentation.

Submersion Culture of HBEC. Ethics approval for the use of human tissues was granted by the Conjoint Health Research Ethics Board of the University of Calgary. Bronchial epithelial cells were obtained by proteinase digestion of non-transplanted, normal airways obtained from a tissue retrieval service at the International Institute for the Advancement of Medicine (Edison, NJ).

HBEC grown as monolayers in submersion culture were seeded in 12-well plates (Costar) containing bronchial epithelial cell growth medium (BEGM; PromoCell, Heidelberg, Germany) supplemented with penicillin (50 μ g/ml) and streptomycin (10 μ g/ml), and maintained for ~14 days under a 5% CO₂/air atmosphere at 37°C until ~80% confluent. Cells were cultured for a further 24 h in supplement-free, basal medium (PromoCell) prior to experimentation.

Culture of HBEC at an ALI. Highly differentiated human airway epithelial cell cultures were generated as described in detail previously (Michi and Proud, 2021). Briefly, HBEC were expanded in T75 cm² flasks (Costar) and maintained for 72 h at 37°C under a 5% CO₂/air atmosphere in PneumaCult-EX expansion medium (Stemcell Technologies, Vancouver, BC, Canada). This was replaced every 48 h until cells were 95% confluent at which time they were dissociated (TrypLE Select, Invitrogen) and resuspended in F12

medium containing 20% FBS and pelleted by centrifugation. Cells were resuspended in PneumaCult-EX expansion medium and seeded at a density of $2 \times 10^5/\text{cm}^2$ in 0.4 μm pore transwell inserts (Costar) coated with bovine collagen type I/III (Advanced BioMatrix, San Diego, CA). At 48 h, the PneumaCult-EX expansion medium was replaced with PneumaCult-ALI differentiation medium (Stemcell Technologies) containing $10\times$ supplement, fluconazole (25 $\mu\text{g}/\text{ml}$), penicillin and streptomycin (each 10 $\mu\text{g}/\text{ml}$). Cultures were fed every 48 h for 5 weeks with PneumaCult-ALI differentiation medium containing $100\times$ supplement, hydrocortisone (0.5 $\mu\text{g}/\text{ml}$) and heparin (50 $\mu\text{g}/\text{ml}$). At 14 days post-transwell seeding, cells were washed apically once per week with phosphate buffered saline (PBS) to remove mucus. Prior to experimentation, ALI cultures were maintained for 18 h in PneumaCult basal medium (Stemcell Technologies), which lacks all supplements including hydrocortisone, and washed with PBS. Drugs were administered simultaneously to both the apical and basolateral surfaces.

Stable Generation of a CRE Reporter in BEAS-2B Cells and Measurement of Luciferase Activity.

BEAS-2B cells were transfected with plasmid DNA (pADneo2-C6-BGL) to generate $6\times\text{CRE}$ luciferase reporter cells as described previously (Meja et al., 2004). Luciferase activity was measured by luminometry and expressed as a fold change relative to time-matched, vehicle-treated cells (Yan et al., 2018).

Expression of a Protein Inhibitor of PKA in HBEC and BEAS-2B Cells. Sub-confluent (70%) HBEC and BEAS-2B cells were infected with an adenovirus vector (Ad5.CMV.PKI α) containing a DNA fragment encoding the amino acid sequence of the cAMP-dependent protein kinase inhibitor protein alpha (PKI α) (Meja et al., 2004). A vector (Ad5.CMV.GFP) encoding green fluorescent protein (GFP) was used to control for any off-target effects of the virus, *per se*. Cells were cultured for 48 h at 37°C as described above and for a further 24 h in SFM. Immunofluorescence confocal microscopy and western blotting were employed to confirm expression of the transgenes.

Derivation of Clonal BEAS-2B Cells Deficient in $\beta\text{Arr}2$. Cells were subjected to CRISPR/Cas9 genome editing using a proprietary gene knockout kit (#12501, Synthego, Redwood City, CA) according to the manufacturer's protocol. Briefly, cells were grown to 50-70 % confluence in 24-well plates. A chemically modified single-guide RNA (180 pmol) encoding a sequence (5'- CGTGAAGACCTGGATGTGCTGGG-3') that targets *ARRB2* was mixed with Cas9 nuclease (20 pmol from *S. pyogenes*) in OptiMEM media (#31985070,

Thermo Scientific, Waltham, MA) containing 1 μ l Lipofectamine Cas9 Plus reagent (#CMAX00008, Thermo Scientific). The mixture was incubated at room temperature for 10 min, added to OptiMEM medium containing 1.5 μ l Lipofectamine CRISPRMAX transfection reagent (Thermo) and transfected into BEAS-2B cells in the presence of serum. At 48 h, cells were seeded into 96-well plates and single clones were isolated by limited dilution. Clones were cultured for 10-14 days and subjected to western blotting to confirm the absence of β Arr2. Three positive clones (C_1 , C_2 , C_3) were identified and the deletion of *ARRB2* was verified by PCR genotyping using primers (forward: 5'-GCTAG GGAAGTGAAATGGGC-3'; reverse: 5'-TCACGGTGAAGAAGAAGGGG-3') that flanked both deleted and undeleted regions. PCR products were subcloned into the pEGFP-C2 vector (Takara Bio, Mountain View, CA) at the EcoR I and Kpn I sites; Sanger sequencing of plasmid DNA was then performed at the Centre for Genome Engineering, University of Calgary. Figure S1 shows the sequencing of C_1 , which revealed the insertion of a thymine nucleotide resulting in a frameshift mutation and the generation of a premature stop codon.

Knockdown of *DUSP1* and *ARRB1*. Parental BEAS-2B cells were grown to 60-70% confluence in 12-well plates before being transfected with siRNAs. Two *DUSP1*-targeting siRNAs (SI00374801: 5'-TAGCG TCAAGACATTTGCTGA-3'; SI00374808: 5'-CTGTACTATCCTGTAAATATA-3') or a non-targeting siRNA control (SI03650325: 5'-AATTCTCCGAACGTGTCACGT-3') (all Qiagen) were mixed with 3 μ l of Lipofectamine RNAiMax (#13778150, Thermo Scientific) in 100 μ l of OptiMEM and incubated at room temperature for 5 min. Cells were cultured for 24 h in the presence of DMEM/F12 supplemented with 10% FBS containing each siRNA at a final concentration of 25 nM as indicated. Cells were then incubated for an additional 24 h in SFM prior to experimentation. In β Arr1 knockdown experiments, pools of four *ARRB1*-targeting siRNAs (SI02776921: 5'-CTCGACGTTCTGCAAGGTCTA-3'; SI02643977: 5'-CGGTGTGGA CTATGAAGTCAA-3'; SI00058961: 5'-CACCAACAAGACGGTGAAGAA-3'; SI00058954: 5'-TACCA ATCTCATAGAACTTGA-3') or non targeting control siRNAs (SI03650325: 5'-AATTCTCCGAACGTG TCACGT-3'; SI04380467: 5'-AAGCAGCACGACTTCTTCAAG-3'; SI1022064: 5'-CGGCAAGCTGAC CCTGAAGTTCAT-3'; SI1027280: (proprietary) (all Qiagen) were transfected into BEAS-2B or HEK293 β 2 cells using lipofectamine RNAiMax at final concentrations of 0.1, 1 or 10 nM.

RNA Extraction and Gene Expression. Total RNA was extracted using Nucleospin RNA mini kits (Macherey-Nagel Inc., GmbH & Co., Duren, Germany) and reverse transcribed to cDNA using a qScript cDNA synthesis kit as described by the manufacturer (Quanta Biosciences, Gaithersburg, MD). The abundance of mRNA encoding *DUSP1* and early growth response 1 (*EGR1*) was measured by real-time PCR using a StepOnePlus instrument (Applied Biosystems) as described previously (Joshi et al., 2017; Yan et al., 2018) and expressed either as a ratio to *GAPDH* or as a fold-change relative to vehicle-treated cells as described. Primer sequences were as follows: *DUSP1* (forward, 5'-CGCGCAAGTCTTCTTCCTCA-3'; reverse, 5'-GATGCTTCGCCTCTGCTTCA-3'), *EGR1* (forward, 5'-ACCTGACCGCAGAGTTTTT-3'; reverse, 5'-GAGTGGTTTGGCTGGGGTAA-3') and *GAPDH* (forward, 5'-ATGGAAATCCATCACCATCTT-3'; reverse, 5'-CAGCATCGCCCCACTTG-3').

Western Immunoblot Analysis. Confluent BEAS-2B cells, HBEC and HEK293β2 cells were lysed in 1× Laemmli sample buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 125 mM Tris HCl, pH 6.8) supplemented with phosphatase inhibitors (Sigma-Aldrich) and 1× complete protease inhibitor cocktail (Roche, Basal, Switzerland). Cell lysates were size fractionated on SDS polyacrylamide gels, electrotransferred onto reinforced 0.2 μm nitrocellulose membranes (GE Healthcare, Waukesha, WI) and blocked with 5% milk in Tris-buffered saline containing 1% Tween 20. Membranes were probed with primary antibodies against total (t) ERK1/2 (cs-4695), phospho (p) ERK1/2 (cs-9101), tMEK1/2 (cs-9122), pMEK1/2 (cs-9154), C-Raf (cs-53745), pSer²⁵⁹-C-Raf (cs-9421), pSer³³⁸-C-Raf (cs-9427), βArr1 (cs-12697), βArr2 (cs-3857), EGR1 (cs-4153), GFP (cs-2555) (Cell signaling Technology, Danvers, MA), DUSP1 (sc-1102), Elk-1 (sc-365876), pElk-1 (sc-8406), PKIα (sc-1943) (Santa Cruz Biotechnology, Dallas, TX), pSer⁴³-C-Raf (ab-150365), pSer⁶²¹-C-Raf (ab-157201) (Abcam, Cambridge, MA) or GAPDH (MCA4739, Bio-Rad, Hercules, CA) overnight at 4°C. Membranes were incubated with horseradish peroxidase-linked secondary immunoglobulin (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 1 h at room temperature and detected by enhanced chemiluminescence (Thermo Scientific). Images were acquired using a ChemiDoc Touch imaging system (Bio-Rad) and analysed with ImageLab software (Bio-Rad). Band volumes of interest were normalised as indicated in each figure or presented as a fold change from unstimulated levels at time zero.

Measurement of ERK1/2 Phosphorylation by Electrochemiluminescence (ECL). Phospho-ERK1/2 (Thr²⁰²/Tyr²⁰⁴; Thr¹⁸⁵/Tyr¹⁸⁷) and total ERK1/2 in cell lysates prepared from confluent BEAS-2B cells, HBEC or HEK293β2 cells treated with the drug of interest or vehicle were measured by ECL using the MesoScale Discovery whole cell lysate multi-spot kit (#K15107D-2) with a MESO QuickPlex SQ 120 instrument (MesoScale Discovery, Gaithersburg, MD).

Determination of cAMP Mass. Confluent wild type and βArr2-deficient BEAS-2B cells at 37°C were treated with the drug of interest or vehicle. The culture medium was decanted and cells were lysed with HCl (0.1M). After neutralization with NaOH, cAMP mass was measured by ECL using a 96-well assay kit (#K150FDD-2, MesoScale Discovery) according to the manufacturer's instructions and quantified using Discovery workbench 4.0 software (MesoScale Discovery). Total protein in each sample was measured using a BCA protein assay (#23227, Thermo Scientific) and cAMP was expressed in units of pg/μg protein.

Gene Expression Profiling. HBEC in submersion culture were treated with formoterol (1 nM) or vehicle for 1, 2, 6 and 18 h. The relative expression of *DUSP* isoforms and components of the βArr- and G_{βγ}-signaling pathways were determined by RNA-sequencing as described previously (Joshi et al., 2019) and are expressed in transcripts per million (TPM). These data are freely available via NCBI's Gene Expression Omnibus (accession code pending). The effects of formoterol (1 nM), salmeterol (100 nM) and indacaterol (100 nM) on gene expression changes in BEAS-2B cells were mined from previous microarray or RNA-sequencing data generated by the authors (accession codes: GSE106710, GSE115830 and GSE126981).

Curve Fitting. Monophasic, agonist concentration-response curves were fit by least-squares, non-linear, iterative regression to the following equation (Prism 6, GraphPad Software Inc, San Diego, CA):

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

where E is the effect, E_{min} and E_{max} are the basal and maximum responses, respectively, $[A]$ is the molar concentration of agonist, EC_{50} is the molar concentrations of agonist that produces $(E_{max} - E_{min})/2$, and n is the Hill coefficient. Agonist potency is expressed as $-\log_{10} EC_{50}$ (pEC₅₀).

The time-dependent phosphorylation and dephosphorylation of elements of the C-Raf signaling cascade in response to drug treatment were fit to one-phase exponents of the form

$$E = E_0 + (p - E_0) \cdot (1 - e^{-kx}) \text{ and } E = (E_0 - p) \cdot e^{-kx} + p$$

respectively, where x is time, E is the response at time x , E_0 is the response when $x = 0$, k is the rate constant and p is the plateau at infinite time; for dephosphorylation p may be greater than, or equal to, a value of zero. The time taken for drug treatment to generate 50% of the maximal response ($t_{1/2}$) is given by $\ln(2)/k$.

Drugs and Analytical Reagents. *R,S*-salmeterol xinafoate, *R,R*-formoterol fumarate and L-161,982 were donated by GlaxoSmithKline (Stevenage, UK), AstraZeneca (Mölndal, Sweden) and Merck Frosst, Inc (Montreal, PQ, Canada), respectively. *R,S*-carvedilol hydrochloride, *R,S*-alprenolol hydrochloride and ICI 118,551 were purchased from Tocris Bioscience (Toronto, ON, Canada). ONO-AE1-259 and ONO-AE1-329 were donated by ONO Pharmaceuticals (Osaka, Japan). *R,S*-carazolol hydrochloride and *R,S*-Vilanterol were from Toronto Research Chemicals (North York, ON, Canada). *R*-indacaterol maleate, roflumilast *N*-oxide (RNO), TG4-155 and YM-254890 were from, Gilead Sciences (Seattle, WA), Altana (Konstanz, Germany), ChemDiv (San Diego, CA) and Focus Biomolecules (Plymouth, PA), respectively. Forskolin, *R,S*-atropine sulphate, mepyramine maleate, PD 098059, U0126 and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma-Aldrich (St. Louis, MO). All drugs were dissolved in DMSO and diluted to the required working concentration in aqueous medium. The final concentration of DMSO was $\leq 0.2\%$ and did not affect any outcome measured. Histamine dihydrochloride, carbachol chloride and G-418 were purchased from Sigma-Aldrich and dissolved in sterile water. Epidermal growth factor (EGF) and tumor necrosis factor- α (TNF α) were obtained from R & D Systems (Minneapolis, MN) and dissolved in PBS containing 0.1% BSA.

Statistics. Data are presented as either the mean \pm s.e. mean or, more typically, as box and whisker plots (showing all data points) of N independent measurements. Significant changes in luciferase reporter activity and gene expression were determined by using Student's two-tailed, paired t -test or repeated measures, one-way ANOVA, as indicated. When the ANOVA F -test P value was <0.05 , differences between groups were determined by using Tukey's multiple comparisons test without Greenhouse-Geisser correction. The time-

dependent change in ERK1/2 phosphorylation between two treatments was by repeated measures, two-way ANOVA followed by Sidak's multiple comparisons test. The null hypothesis was rejected when $P < 0.05$.

Results

Formoterol and Salmeterol Promoted ERK1/2 Phosphorylation in HEK293β2 Cells. In agreement with several previous studies (Luttrell et al., 2018; Shenoy et al., 2006; van der Westhuizen et al., 2014), western blotting determined that ERK1 and ERK2 were partially phosphorylated in unstimulated HEK293β2 cells (Fig. 1). The long-acting β₂-adrenoceptor agonist (LABA), formoterol (1 nM), produced a transient increase in the phosphorylation of both ERK isoforms relative to vehicle-treated cells matched for time, which peaked at 5 min (~8-fold increase) and then declined to a new steady state that at 60 min was ~2-fold above the pre-stimulated level (Fig. 1A). This effect was abolished in cells pre-treated (30 min) with ICI 118,551 (1 μM), a competitive and selective β₂-adrenoceptor antagonist (Fig. 1B). The ability of formoterol to phosphorylate ERK1/2 was concentration-dependent with an EC₅₀ measured at 5 min of 5 pM (Fig. 1C). Similarly, treatment of cells with EGF (10 ng/ml; 30 min) produced a robust phosphorylation of ERK1/2 that was greater than that produced by formoterol (Fig. 1A). Comparable data were obtained in HEK293β2 cells treated with a structurally dissimilar LABA, salmeterol (SFig. 2A & B). Total ERK1/2 levels were unaffected by these interventions. ≥

Formoterol and Salmeterol Promoted ERK1/2 Dephosphorylation in Airway Epithelial Cells. In unstimulated BEAS-2B cells and HBEC, western blotting similarly detected a basal level of pERK1 and pERK2 (Fig. 2; SFig. 3 & 4). However, in both epithelial cell models formoterol (1 nM) produced a time-dependent and sustained dephosphorylation of these ERK isoforms (t_{1/2} ~10 min) that had plateaued to a level that was at 10-30% of the control at 60 min (Fig. 2A & B). In cells pre-treated (30 min) with ICI 118,551 (1 μM), ERK1/2 phosphorylation was abolished indicating that this was a β₂-adrenoceptor-mediated effect (Fig. 2C & D). At the 60 min time-point, formoterol dephosphorylated ERK1/2 in BEAS-2B and HBEC in a concentration-dependent manner with EC₅₀s of 12 pM and 22 pM, respectively (Fig. 2E & F). Comparable data were produced with salmeterol (100 nM) and another LABA, indacaterol (100nM), indicating that ERK1/2 dephosphorylation was a class effect of β₂-adrenoceptor agonists (SFig. 3 & 4). In contrast, exposure of BEAS-2B cells and HBEC to EGF (10 ng/ml; 30 min), TNFα (10 ng/ml; 60 min) and PMA (100 nM; 30 min) enhanced ERK1/2 phosphorylation indicating that the basal levels measured in unstimulated cells were not maximal (Fig. 2; SFig. 3 & 4). Total ERK1/2 expression was unaffected by these interventions (Fig. 2; SFig. 3 & 4).

The human airway epithelium has a pseudostratified columnar architecture, which largely influences its mucosal defense properties and function. HBEC isolated from the main stem bronchi of human lung donors are composed of basal or “progenitor cells” and are grown as non-differentiated monolayers, which renders them a less ideal model. Farther removed from HBEC is the Ad12/SV40-immortalized BEAS-2B cell line, which was originally cloned from HBEC (Lechner et al., 1982). To corroborate the data obtained using HBEC and BEAS-2B, experiments were performed using HBEC cultured for 5 weeks at ALI, which resemble the structure of the airway epithelium *in vivo*. At this time, the epithelium was highly differentiated and contained specialised elements including ciliated and goblet cells. Consistent with HBEC grown in submersion culture and the BEAS-2B cell line, ERK1 and ERK2 were also phosphorylated in unstimulated ALI cultures (Fig. 2G). Moreover, exposure of these cells to formoterol (1 nM; 60 min) and EGF (10 ng/ml; 60 min) decreased and increased, respectively the phosphorylation of both ERK isoforms (Fig. 2G).

Formoterol Promoted ERK1/2 Dephosphorylation in Airway Epithelial Cells Determined by ECL.

The ability of formoterol and EGF to modulate ERK1/2 phosphorylation was also investigated by ECL using the MesoScale Discovery platform. This alternative, quantitative, approach, which measures the fraction of the total ERK1/2 pool in an active state, confirmed the results obtained by western blotting. Thus, in BEAS-2B cells formoterol dephosphorylated ERK1/2 in a time-dependent manner ($t_{1/2} = 6.7$ min) that by 30 min had plateaued to a new steady state that was at ~25% of the unstimulated level (Fig. 2H). Similarly, formoterol reduced the pERK1/2 level in HBEC to 20% of the unstimulated level at 60 min, whereas in HEK293 β 2 cells it was increased by 3.3-fold at 5 min (Fig. 1I). Epidermal growth factor (10 ng/ml; 30 min) increased ERK1/2 phosphorylation in all three cell models, which was again consistent with the immunoblotting results (Fig. 2H). Of note, ECL established that basal ERK1/2 phosphorylation in HEK293 β 2 cells was 25- and 40-fold lower than in BEAS-2B cells and HBEC, respectively (Fig. 2H). Nevertheless, formoterol (1 nM) still enhanced ERK1/2 phosphorylation in HEK293 β 2 cells pre-treated (10 min) with a concentration of EGF (1 ng/ml) that increased pERK1/2 to levels similar to those measured in unstimulated BEAS-2B cells (Fig. 2J).

β_2 -Adrenoceptor Agonists Promoted ERK1/2 Dephosphorylation in Airway Epithelial Cells by Canonical, cAMP-dependent Signaling. In HBEC, the adenylyl cyclase activator, forskolin (10 μ M), promoted a time-dependent and sustained dephosphorylation of ERK1/2 ($t_{1/2}$ ~11 min) that had declined and plateaued to 25% of the unstimulated level by 30 min (Fig. 3A). A cAMP-dependent mechanism was also implied in BEAS-2B cells treated with the selective EP₂- and EP₄-receptor agonists ONO-AE1-259 and ONO-AE1-329, respectively (Suzawa et al., 2000). These ligands promote canonical, cAMP-dependent signaling in human airway epithelial cells but are partial agonists (Joshi et al., 2021) and were, therefore, used in cells pre-treated with the PDE4 inhibitor, RNO (1 μ M). Exposure of BEAS-2B cells to ONO-AE1-259 and ONO-AE1-329 (both 1 μ M; 60 min) reduced basal pERK1/2 levels, which was blocked by the EP₂- and EP₄-receptor antagonists TG4-155 (Jiang et al., 2012) and L-161,982 (Machwate et al., 2001), respectively (both 1 μ M; 30 min pre-treatment) (Fig. 3B).

The role of canonical cAMP signalling was further interrogated by infecting HBEC and BEAS-2B cells with adenovirus vectors that encode PKI α , a selective inhibitor of PKA (Glass et al., 1986), or a control protein, GFP, at MOIs ranging from 1 to 100 (Fig. 3C & D). At a MOI of 30, >95% of cells expressed either the GFP or PKI α transgenes (Meja et al., 2004) (Fig. 3C & D). Moreover, the activation by formoterol (1 nM; 6 h) of a CRE reporter stably transfected into BEAS-2B cells was prevented by PKI α with MOIs causing 50% and 95% inhibition of 2.6 and 10, respectively, whereas GFP-expressing cells were unaffected. In contrast, in the presence of RNO (1 μ M), formoterol (1 nM)- and forskolin (10 μ M)-induced cAMP accumulation in BEAS-2B cells was augmented by PKI α (Fig. 3D). Consistent with a previous report, this effect may be due to the inhibition of a PKA-activated PDE that is distinct from PDE4 (Violin et al., 2008). In both epithelial cell models, the expression of PKI α but not GFP (MOI = 25) abolished formoterol-induced ERK1/2 dephosphorylation (Fig. 3E & F). Comparable results were obtained in HBEC and BEAS-2B cells treated with salmeterol (SFig. 5) indicating that LABA-induced ERK1/2 dephosphorylation in human airway epithelial cells was dependent upon canonical, cAMP-dependent signaling and involved the activation of PKA.

Induction of *DUSP1* Contributed to LABA-induced ERK1/2 Dephosphorylation in Airway Epithelial Cells. Ten MAP kinase phosphatases (MKPs) have been identified that form part of the larger, dual-

specificity phosphatase (DUSP) superfamily (Theodosiou and Ashworth, 2002). As ERK1 and ERK2 are substrates for several MKPs, it was hypothesised that one or more of these enzymes may contribute to β_2 -adrenoceptor-mediated ERK1/2 dephosphorylation in airway epithelial cells. RNA-sequencing identified 18 DUSP mRNAs that were common to BEAS-2B cells and HBEC (Fig. 4A). Of those, only *DUSP1* (*MKP1*), *DUSP4* (*MKP2*), *DUSP8* (*hVH5*) and *DUSP10* (*MKP5*) were induced by LABAs in both cell models (Fig. 4A). Since *DUSP1* mRNA was profoundly increased by formoterol at 1 h (Fig. 4A) and is an established negative regulator of MAP kinase signaling (Lang and Raffi, 2019; Sun et al., 1993), its role in mediating ERK1/2 dephosphorylation was explored.

The induction of *DUSP1* determined by gene-expression profiling was validated by PCR over a time frame of 0 to 6 h. Formoterol (1 nM) rapidly increased *DUSP1* expression in BEAS-2B cells. This was detectable at 15 min (a time when dephosphorylation of ERK1/2 was evident), peaked at 60 min and had returned to unstimulated levels by 4-6 h (Fig. 4B). In BEAS-2B and HBEC, DUSP1 protein levels increased with kinetics that mirrored the changes in mRNA; for salmeterol (100 nM) the level increased rapidly, peaked at 60 min and had returned to baseline by ~4 h (Fig. 4C & D). At the 1 h time-point, formoterol and salmeterol increased DUSP1 expression in BEAS-2B cells in a concentration-dependent manner with EC₅₀ values of 78 pM and 2.5 nM, respectively (Fig. 4E & F). The induction of *DUSP1* mRNA by salmeterol and formoterol in both epithelial cell models was mimicked by forskolin (10 μ M) and prevented in cells infected with Ad5.CMV.PKI α (MOI = 25) but not the control virus (Fig. 4G). Pre-treatment (30 min) of BEAS-2B cells with ICI 118,551 (1 μ M) inhibited the increase of *DUSP1* induced by salmeterol whereas the effect of forskolin was unchanged (Fig. 4G).

Transfection of BEAS-2B cells with *DUSP1*-targeting siRNAs (25 nM) markedly reduced (by >95%) the peak expression of DUSP1 protein induced by salmeterol (100 nM) that occurred at 1 h and, in the same cells, reduced ERK1/2 dephosphorylation by 30-50%; in contrast, a control siRNA (25 nM) had no significant effect (Fig. 4H). Collectively, these results indicated that the PKA-dependent induction of DUSP1 played a minor role in β_2 -adrenoceptor-mediated ERK1/2 dephosphorylation in human airway epithelial cells.

Phosphorylation of C-Raf Contributed to LABA-induced ERK1/2 Dephosphorylation in BEAS-2B

Cells. The protein kinases, B-Raf and C-Raf activate and inhibit, respectively downstream MEK1/2-ERK1/2 signaling in response to an increase in cAMP (Dumaz and Marais, 2005). Given the modest role of DUSP1 in LABA-induced ERK1/2 dephosphorylation (*vide supra*), the possible contribution of C-Raf in this response was explored using BEAS-2B cells. There are 13 known phosphorylation sites in C-Raf that regulate catalysis (Fig. 5A). Three of those residues (Ser⁴³, Ser²³³ and Ser²⁵⁹) independently inhibit kinase activity when phosphorylated by PKA by preventing C-Raf from interacting with the small GTP-binding protein, Ras (Cook and McCormick, 1993; Dumaz and Marais, 2005). To determine if Ser⁴³ and Ser²⁵⁹ were phosphorylated by formoterol with kinetics that would account for ERK1/2 dephosphorylation, western blotting was performed using phospho-specific antibodies. The phosphorylation of Ser²³³ was not determined because antibodies are unavailable. The amino acids Ser³³⁸ and Ser⁶²¹ were also probed as they are markers of C-Raf activation (Ghosh et al., 2015; Takahashi et al., 2017a; Yip-Schneider et al., 2000).

In BEAS-2B cells, western blotting determined Ser²⁵⁹, Ser³³⁸ and Ser⁶²¹ were phosphorylated constitutively in unstimulated cells relative to Ser⁴³ (Fig. 5B). Formoterol (1 nM) rapidly increased the phosphorylation of Ser⁴³ and Ser²⁵⁹ with similar $t_{1/2}$ values (~30 s). These events preceded the dephosphorylation of MEK1/2 ($t_{1/2}$ = 1.2 min) and ERK1/2 ($t_{1/2}$ = 6 min), which was consistent with the sequential activation of the C-Raf-MEK1/2-ERK1/2 signaling cascade (Fig. 5B & C). Conversely, formoterol reduced the basal phosphorylation of the C-Raf activation marker Ser³³⁸ with slower kinetics ($t_{1/2}$ = 4.7 min) whereas the level of pSer⁶²¹ was unchanged (Fig. 5B & C). A different profile of serine phosphorylation changes was seen in BEAS-2B cells treated with EGF (10 ng/ml; 30 min). As predicted, this mitogen produced a robust activation of the C-Raf-MEK1/2-ERK1/2 signaling cascade, which was associated with increased levels of pSer⁴³ and pSer³³⁸ and reduced phosphorylation of Ser²⁵⁹ (SFig. 6).

At 60 min, changes in formoterol-induced serine phosphorylation were prevented in cells infected with Ad5.CMV.PKI α but not the GFP-expressing control virus (Fig. 5D & E). The dephosphorylation of MEK1/2 and ERK1/2 was also blocked by this intervention indicating that formoterol had inactivated C-Raf in a PKA-dependent manner and attenuated downstream MAP kinase signaling.

In contrast to formoterol, the MEK1/2 inhibitors, U0126 and PD 098059 (both 10 μ M), had no effect on the phosphorylation status of C-Raf or MEK1/2 but markedly reduced basal ERK1/2 phosphorylation. PD 098059, which selectively targets MEK1 (Alessi et al., 1995; Dudley et al., 1995), was less effective than U0126 (Fig. 5D & E) suggesting that MEK2 was the dominant isoform that controlled ERK1/2 activity in BEAS-2B cells.

Formoterol Inhibited the Expression of EGR1 in Airway Epithelial Cells. Extracellular signal-regulated kinases 1 and 2 are the terminal kinases in the Ras-C-Raf-MEK1/2 signaling cascade and regulate a diverse repertoire of downstream targets that include the transcription factor, Elk-1 (Ünal et al., 2017). To establish a functional correlate of LABA-induced ERK-1/2 dephosphorylation, the activation of Elk-1 and the expression of an Elk-1-sensitive gene, *EGR1* (Khachigian and Collins, 1998; Pagel and Deindl, 2011), which is known to be regulated by β_2 -adrenoceptor agonists in airway epithelial cells (Yan et al., 2018), were determined. *EGR1* is a master regulator of cell cycle transition (Meloche and Pouyssegur, 2007), is highly expressed in the lungs of individuals with a variety of respiratory diseases (Ning et al., 2004; Yasuoka et al., 2009; Zhang et al., 2000) including asthma (Goleva et al., 2008) and plays a central role in fibrosis and wound healing (Bhattacharyya et al., 2013; Wu et al., 2009).

In unstimulated BEAS-2B cells, HBEC and ALI cultures *EGR1* was constitutively expressed in agreement with previous reports (Joshi et al., 2019; Yan et al., 2018). Formoterol (1 nM) reduced basal *EGR1* mRNA and protein expression in all three cell models by >50% after 1 h and/or 2 h of exposure (Fig. 6A & B). Kinetic analysis in BEAS-2B cells determined that repression of *EGR1* transcripts was time-dependent ($t_{1/2}$ ~50 min) and had declined to ~50% and ~13% of the unstimulated level by 1 h and 2 h, respectively (Fig. 6C). The dual MEK1/2 inhibitor, U0126 (10 μ M), alone and in combination with formoterol, likewise repressed *EGR1* with kinetics that were similar to formoterol (Fig. 6C). Baseline *EGR1* mRNA was also reduced (by ~50% at 1 h) in HEK293 β 2 cells exposed to U0126 (Fig. 6D), indicating that this gene was regulated similarly across these cell types. In contrast, formoterol (1 nM) up-regulated *EGR1* expression in HEK293 β 2 cells at 1 h (Fig. 6D), which was consistent with its ability to enhance, rather than inhibit, basal ERK1/2 phosphorylation (Fig. 1).

At 2 h, U0126 and PD 098059 (both 10 μ M), had markedly reduced pElk-1 levels and the constitutive expression of EGR1 (Fig. 6E) although PD 098059 was a relatively weak inhibitor as it was of ERK1/2 phosphorylation (Figs. 5 & 6E). Formoterol (1 nM) also dephosphorylated ERK1/2 and Elk-1 and repressed EGR1 protein expression. These effects were abolished in cells infected with Ad5.CMV.PKI α but not the GFP-expressing control virus establishing a role for canonical, cAMP signaling in β_2 -adrenoceptor-mediated *EGR1* repression (Fig. 6E). In contrast, and consistent with the data in figure 4H, siRNA-induced DUSP1 knockdown had either a modest or no effect in protecting cells against these formoterol-induced changes that depended on the outcome (i.e., ERK1/2 vs. Elk-1 and EGR1) measured (Fig. 6E).

Distinct effects of Carvedilol, Carazolol and Alprenolol on ERK1/2 Phosphorylation between HEK293 β_2 and BEAS-2B Cells. Carvedilol is a biased agonist at the β_2 -adrenoceptor and apparently promotes ERK1/2 phosphorylation in HEK293 β_2 cells by non-canonical signaling that involves the recruitment of β Arr2 (Wisler et al., 2007). The data presented in figure 7A are consistent with those findings. Thus, carvedilol (10 μ M) produced a rapid and transient increase in ERK1/2 phosphorylation that peaked at 5 min (~4.5-fold increase), declined, reaching a nadir at ~15 min, and then increased again to a new steady-state that at 60 min was 3.5-fold above the unstimulated levels (Fig. 7A). Two structurally-related β_2 -adrenoceptor “antagonists”, carazolol and alprenolol (both 10 μ M), also increased ERK1/2 phosphorylation in HEK293 β_2 cells (responses peaked between 1 to 5 min) but their effects were transient and had returned to baseline levels by 15 min (Fig. 7B & C). Pre-treatment (30 min) of HEK293 β_2 cells with ICI 118,551 (100 nM) abolished carvedilol-, alprenolol- and carazolol-induced ERK1/2 phosphorylation (SFig. 7).

Unlike in HEK293 β_2 cells, basal ERK1/2 phosphorylation in BEAS-2B cells was unaffected by carvedilol, carazolol or alprenolol (each 10 μ M) after 1, 5, 15, 30 and 60 min of treatment (Fig. 7D-F).

β Arr2 Constrained Formoterol-induced cAMP Accumulation and ERK1/2 Dephosphorylation in BEAS-2B Cells. The inability of carvedilol and related ligands to promote ERK1/2 phosphorylation in BEAS-2B cells was enigmatic given that epithelial cells express a high density of efficiently-coupled β_2 -adrenoceptors (Davis et al., 1990; Kelsen et al., 1995; Penn et al., 1994) at which partial agonists are predicted to display efficacy (Yan et al., 2018). Moreover, *ARRB2* transcripts determined by RNA-

sequencing were abundant in BEAS-2B cells and HBEC (TPMs: ~35 and ~9, respectively) and predominated (≥ 30 -fold) over *ARRB1*, the other non-visual arrestin (Fig. 8A). In BEAS-2B cells, these findings were validated at the protein level with β Arr2 being identified by western blotting (Fig. 8B). A band, corresponding to β Arr1 was also labelled in BEAS-2B cells. However, this was only detected using an ultrasensitive chemiluminescent substrate (i.e., SignalFire EliteTM), which can detect proteins in the femtogram range (SFig. 8A). In contrast, a very strong β Arr1 signal was detected in HEK293 β 2 cells (SFig. 8B). In both cell types, gene silencing confirmed that these bands were β Arr1 (SFig. 8B).

To examine if β Arr2 played a role in β_2 -adrenoceptor-mediated signaling in BEAS-2B cells, three clones (C_1 , C_2 and C_3) deficient in this protein were derived by using CRISPR/Cas9 genome editing technology (Fig. 8B). In parental and clonal cells pre-treated with the PDE4 inhibitor, RNO (1 μ M; 30 min), formoterol increased cAMP mass in a concentration-dependent manner with similar EC_{50} values (2.2 nM and 2.8 nM, respectively) (Fig. 8C). However, the change in maximal response was 2-fold greater in cells deficient in β Arr2 (Fig. 8C). This effect was replicated when the time-course of cAMP accumulation was determined in response to a supra-maximally-effective concentration of formoterol (100 nM) although the kinetics were similar ($t_{1/2}$ = 1.5-2 min) in both clonal and parental cells (Fig. 8C). In contrast, *ARRB2* deletion had no effect on RNO- or forskolin-induced cAMP generation (Fig. 8E) confirming the selectivity of β Arr2 in promoting agonist-induced β_2 -adrenoceptor desensitization (Fig. 8D).

In unstimulated *ARRB2*^{-/-} cells, ERK1 and ERK2 remained partially phosphorylated although western blotting and ECL determined that the level was significantly ($P < 0.05$) lower (~30%) than in wild type cells (Fig. 8F). Nevertheless, in all clones formoterol (1nM) produced a time-dependent and sustained dephosphorylation of ERK1/2 that had plateaued at 60 min to a level that was 25% of the control (Fig. 8G; SFig. 9). Kinetically, the rate of dephosphorylation occurred more rapidly in *ARRB2*-deficient cells ($t_{1/2}$ = 3.7 min) than in their parental counterparts ($t_{1/2}$ = 11.0 min) (Fig. 8G) presumably because β Arr2-mediated desensitization had been compromised (Fig. 8C & D). Western blotting and ECL determined that the deletion of *ARRB2* had no effect on ERK1/2 phosphorylation induced by EGF (10 ng/ml; 30 min) (Fig. 8F & G).

Effect of Formoterol on the Expression of Signaling Components Required for β Arr- and $G_{\beta\gamma}$ -dependent ERK1/2 Activation. The possibility that key signaling elements were either absent or expressed at limiting levels in untreated human primary airway epithelial cells such that a functional β Arr2-signaling complex could not assemble was examined by RNA-sequencing. This analysis was extended to include components of the $G_{\beta\gamma}$ -signaling pathway, which can promote ERK1/2 phosphorylation in a β Arr-independent manner (Crespo et al., 1995; O'Hayre et al., 2017).

As shown in supplemental figure 10, mRNAs encoding 27 signaling elements implicated in β_2 -adrenoceptor-mediated ERK1/2 phosphorylation were identified in untreated HBEC. These included β Arr1, β Arr2, Ras isoforms, C-Raf, MEK1, MEK2, ERK1 and ERK2, which are involved in β Arr-signaling, and 16 components of the $G_{\beta\gamma}$ -signaling pathway (i.e., $G_{\beta 1-\beta 5}$, $G_{\gamma 3-\gamma 5}$, $G_{\gamma 10-\gamma 12}$, the tyrosine kinase, Src, the Shc1 and Shc2 adaptor proteins and the son of sevenless guanine nucleotide exchange factors, Sos1 and Sos2). The majority (22) of these were expressed at levels similar to or greater than transcripts encoding the β_2 -adrenoceptor (SFig. 10A).

Treatment of HBEC with formoterol (1 nM) for 1, 2, 6 and 18 h did not induce or repress any of these mRNAs relative to vehicle-treated cells matched for time (SFig. 10B). Likewise, formoterol failed to induce mRNAs encoding G_{γ} subunits that were not detected in unstimulated HBEC (i.e., $G_{\gamma 1}$, $G_{\gamma 2}$, $G_{\gamma 6-8}$) under identical experimental conditions. Comparable data were derived from BEAS-2B cells treated with formoterol (data not shown; see GSE115830).

Histamine and Carbachol Promoted Gq-dependent ERK1/2 Phosphorylation in BEAS-2B Cells.

Additional studies were performed to determine if ERK1/2 could be phosphorylated in airway epithelial cells by activators of GPCRs that signal independently of Gs. For this purpose, the airway-relevant agonists, histamine and carbachol, were examined together with the signaling roles of Gq and β Arr2. Exposure of BEAS-2B cells to histamine or carbachol (both 10 μ M) produced a transient increase in ERK1/2 phosphorylation (Fig. 8H & I). The responses induced by both agonists peaked at 5 min (2- to 2.5-fold increase relative to vehicle matched for time) and then gradually declined over the next 25 min towards the baseline levels (Fig. 8H & I). ERK1/2 phosphorylation was abolished in BEAS-2B cells pre-treated (30 min) with mepyramine or atropine (both 1 μ M) indicating that histamine and carbachol were acting through

the H₁-receptor and a muscarinic receptor subtype, respectively (Fig. 8H & I). Likewise, histamine- and carbachol-induced ERK1/2 phosphorylation was completely inhibited in cells pre-treated (60 min) with the Gq-inhibitor, YM-254890 (1 μM) (Fig. 8J & K) whereas the effect of EGF (10 ng/ml; 30 min) was unchanged (Fig. S11). Mepyramine, atropine and YM-254890 had not affect basal pERK1/2 levels (SFig. 11).

In *ARRB2*^{-/-} BEAS-2B cells, the time-to-peak and magnitude of histamine- and carbachol-induced ERK1/2 phosphorylation was unchanged relative to parental cells (Fig. 8L & M). However, the *rate* of ERK1/2 dephosphorylation was protracted. For histamine, the *t*_{1/2} was increased from 6.1 to 12.9 min in parental and clonal cells, respectively (Fig. 8L). A similar, but less pronounced, increase in *t*_{1/2} was seen with carbachol (parental: 4.5 min; clonal: 7.9 min) (Fig. 8M) implying that βArr2 had exerted a negative regulatory influence on histamine H₁- and muscarinic receptor-mediated signaling.

Discussion

Chronic, β_2 -adrenoceptor agonist monotherapy in asthma is associated with adverse clinical outcomes that may be partially driven by changes in pro-inflammatory gene expression. It has been proposed that this occurs principally in airway epithelial cells and involves the β Arr2-dependent nucleation of the C-Raf-MEK1/2-ERK1/2 signaling complex (Nguyen et al., 2017). However, in the present study, the LABAs formoterol and salmeterol paradoxically *reduced* pERK1/2 and pMEK1/2 levels in airway epithelial cells by activating the G α -adenylyl cyclase-cAMP signaling cascade (Fig. 9). Mechanistically, evidence for two PKA-regulated mechanisms was obtained: disruption of Ras-C-Raf complex assembly and induction of DUSP1. At a genomic level, ERK1/2 dephosphorylation lead to the inactivation of the transcription factor, Elk-1, and repression of a representative Elk-1-regulated gene, *EGR1*. Together, these findings indicate that acute exposure of airway epithelial cells to LABAs does not promote β Arr2-dependent ERK1/2 phosphorylation and down-stream gene expression changes; instead, cAMP-regulated mechanisms predominate, consistent with the conventional view (Yan et al., 2018; Zhang et al., 2005).

Antithetical Regulation of ERK1/2 Activity by β_2 -Adrenoceptor Agonists. In three human airway epithelial cell models, the structurally dissimilar LABAs formoterol, salmeterol and indacaterol promoted a time-dependent dephosphorylation of ERK1/2 that was mediated by the β_2 -adrenoceptor. Based on these data, the likelihood that ERK1/2 would be similarly inactivated in the airway epithelium of asthmatic subjects after taking an inhaled β_2 -adrenoceptor agonist should be entertained. In contrast, and consistent with several previous reports (Luttrell et al., 2018; Shenoy et al., 2006; van der Westhuizen et al., 2014), formoterol and salmeterol increased basal ERK1/2 phosphorylation in HEK293 β_2 cells. Thus, the impact of β_2 -adrenoceptor agonists on MAP kinase signaling is cell-type dependent and should not be generalized. Indeed, the β_2 -adrenoceptor-mediated regulation of ERK1/2 activity is not even consistent across human airway epithelial cell lines (Nishimura et al., 2002) indicating the need to exercise caution in selecting a suitable system to model human primary cells.

Mechanistically, formoterol- and salmeterol-induced ERK1/2 dephosphorylation in BEAS-2B cells and HBEC was abolished by PKI α suggesting that this was mediated by canonical, cAMP-dependent signaling. The adenylyl cyclase activator, forskolin, and the EP $_2$ - and EP $_4$ -receptor agonists, ONO-AE1-259 and

ONO-AE1-329, respectively likewise decreased pERK1/2 levels, which strengthens this conclusion. EP₄-receptor agonists acting via the cAMP-PKA axis also decrease ERK1/2 activity in chondrocytes and neutrophils (Fushimi et al., 2007; Mizuno et al., 2014), which is consistent with the signaling paradigm reported here. However cAMP-independent ERK1/2 phosphorylation and dephosphorylation have also been reported (Gerits et al., 2008) emphasizing again the context-dependence of this response.

Early growth response 1 is one of several genes regulated by Elk-1 and related E twenty-six domain transcription factors. Many of these are downstream substrates of ERK1/2 (Buchwalter et al., 2004; Ünal et al., 2017) and their expression is regulated by interventions that change ERK1/2 activity. In HEK293β2 cells and all epithelial cell models, MEK1/2 inhibitors reduced constitutive Elk-1 phosphorylation and repressed *EGR1* gene expression confirming that basal Elk-1-EGR1 signaling was maintained by ERK1/2. In contrast, formoterol, increased and decreased Elk-1-regulated EGR1 expression in HEK293β2 and airway epithelial cells, respectively, consistent with its opposing effects on ERK1/2 phosphorylation.

The role of EGR1 in asthma pathology and the consequences of repression produced by formoterol are unclear. A beneficial effect might be predicted given its association with several human fibrotic pulmonary disorders and that airway inflammation and mucus hyper-secretion following exposure to particulate matter were inhibited in *EGR1* knockout mice (Xu et al., 2018). However, a reduction in EGR1 could be detrimental under certain circumstances. Indeed, the lung pathology that develops in transgenic mice that over-express transforming growth factor β was more severe in animals that also lacked *EGR1* (Kramer et al., 2009). Given the central role of ERK1/2 in cell cycle regulation (Meloche and Pouyssegur, 2007), one effect of chronic β₂-adrenoceptor agonist therapy could be to arrest cell growth and repair by inhibiting constitutive gene expression programs that are actively maintained by pERK1/2.

Multiple Mechanisms Contribute to β₂-Adrenoceptor-mediated ERK1/2 Dephosphorylation Human Airway Epithelial Cells. BEAS-2B cells were used as a representative epithelial cell model to explore how formoterol and related LABAs caused ERK1/2 dephosphorylation. Initially, the participation of DUSP1 was investigated because it is up-regulated by β₂-adrenoceptor agonists (Kang et al., 2016; Manetsch et al., 2012; Tsvetanova and von Zastrow, 2014), is a classical target for the PKA-regulated transcription factor, CRE-binding protein (Zhang et al., 2005) and can dephosphorylate ERK1/2 in intact cells (Caunt and

Keyse, 2013; Moosavi et al., 2017). DUSP1 was also rapidly induced in BEAS-2B cells and primary HBEC over the timeframe when ERK1/2 dephosphorylation was on-going. Gene silencing established that under conditions of >95% DUSP1 protein knockdown, β_2 -adrenoceptor-mediated ERK1/2 dephosphorylation was inhibited by ~30-50% although down-stream Elk-1 phosphorylation and EGR1 repression were unaffected. While the ~5% DUSP1 remaining in these cells may have been sufficient to ensure that most of the ERK1/2 pool was still dephosphorylated in response to agonist, other mechanisms were implicated. The observation that basal MEK1/2 phosphorylation was reduced by formoterol in a PKI α -sensitive manner indicates that a signaling component(s) upstream of ERK1/2 was also a PKA substrate. Indeed, the kinetics of MEK1/2 dephosphorylation were rapid ($t_{1/2} = 1.2$ min) in BEAS-2B cells and consistent with the slower and causal inactivation of ERK1/2 ($t_{1/2} = 6.7$ min). There is good evidence that C-Raf regulates ERK1/2 activity by physically linking Ras to MEK1/2 (Dougherty et al., 2005; Lavoie and Therrien, 2015). Moreover, PKA can disrupt this nucleation by phosphorylating C-Raf at Ser⁴³ and Ser²³³/Ser²⁵⁹, which weakens the Ras-C-Raf interaction by steric hindrance and stabilizes the kinase in an inactive conformation, respectively (Dhillon et al., 2002; Dougherty et al., 2005; Dumaz and Marais, 2003; Lavoie and Therrien, 2015). In agreement with these prior data, formoterol similarly enhanced the basal phosphorylation of C-Raf in BEAS-2B cells at Ser⁴³ and Ser²⁵⁹. This occurred rapidly ($t_{1/2} \sim 0.5$ min), was dependent on PKA and preceded the dephosphorylation of MEK1/2 and ERK1/2. Formoterol also reduced the basal phosphorylation of a C-Raf activation marker, Ser³³⁸ (Takahashi et al., 2017a). Taken together, these data suggest that the primary means by which β_2 -adrenoceptor agonists inactivated MEK1/2-ERK1/2-Elk-1-EGR1 signaling in airway epithelial cells was by disrupting the link between Ras and MEK1/2 through the PKA-dependent phosphorylation of C-Raf (Fig. 9).

Agonist-induced Desensitization of the β_2 -Adrenoceptor on Human Airway Epithelial Cells was Mediated by β Arr2. In BEAS-2B cells and HBEC, β Arr2 was the predominant, non-visual arrestin with a relatively minor representation from β Arr1. Functionally, formoterol-induced cAMP accumulation was enhanced in BEAS-2B cells deficient in β Arr2 when compared to their parental counterparts. These data support the established role of β Arr2 in mediating agonist-induced homologous desensitization of the β_2 -adrenoceptor although it remains unclear whether β Arr1 is more or less important in this regard. While both arrestins can restrain coupling of the agonist-bound β_2 -adrenoceptor to Gs, the assembly of the C-Raf-

MEK1/2-ERK1/2 signaling complex is suggested to be more dependent on β Arr2 (Luttrell et al., 2018; Penn et al., 2001; Tohgo et al., 2003). Nevertheless, β Arr2 has been reported to promote β_2 -adrenoceptor desensitization in murine embryonic fibroblasts (Baillie et al., 2007), consistent with the results of the present study.

No Evidence for β_2 -Adrenoceptor-mediated, β Arr2-dependent ERK1/2 Phosphorylation in Human Airway Epithelial Cells. Having established that β Arr2 was expressed and functional in BEAS-2B cells, the possibility that ERK1/2 is activated by a non-canonical, G-protein-independent mechanism was investigated. Carvedilol, a partial β_2 -adrenoceptor agonist biased towards β Arr2 (Liu et al., 2012; Wisler et al., 2007) was used for this purpose as it activates ERK1/2 in HEK293 β_2 cells (Luttrell et al., 2018; Wisler et al., 2007; this study). However, consistent with data gained in another epithelial cell line (Peitzman et al., 2015), carvedilol did not affect basal pERK1/2 levels in BEAS-2B cells. While this may indicate the absence of β Arr2-dependent signaling, relatively low β_2 -adrenoceptor density (*cf.* HEK293 β_2 cells) may have rendered carvedilol inactive. Indeed, the *Law of Mass Action* dictates that a decrease in receptor number will reduce potency and efficacy especially of a partial agonist like carvedilol. This explanation suggests that ERK1/2 phosphorylation may be realised with biased agonists of high intrinsic efficacy and, possibly, with carvedilol under conditions of β_2 -adrenoceptor over-expression. Low receptor density could also explain why alprenolol (Liu et al., 2012; Wisler et al., 2007) and carazolol (unpublished) promoted G α -dependent ERK1/2 phosphorylation in HEK293 β_2 cells but not in the BEAS-2B cell line.

The increase in formoterol-induced cAMP accumulation in β Arr2-deficient cells was associated with an accelerated rate at which ERK1/2 was dephosphorylated ($t_{1/2}$ was reduced from 9.7 to 4 min). While this kinetic discrepancy is consistent with compromised β_2 -adrenoceptor desensitization, such data can also be explained by the ablation of β Arr2-dependent ERK1/2 phosphorylation that occurs rapidly (≤ 5 min) in some cell types after agonist stimulation (Shenoy et al., 2006). However, in BEAS-2B cells and HBEC, this interpretation is inconsistent with the finding that PKI α abolished ERK1/2 dephosphorylation without revealing latent activation at any time-point.

Additional signaling roles for β Arr2 were explored by investigating the effect of carbachol and histamine on ERK1/2 phosphorylation in *ARRB2*^{-/-} and wild-type BEAS-2B cells. Histamine and carbachol produced

transient increases in ERK1/2 phosphorylation that were dependent upon Gq. In agreement with muscarinic M₃-receptor signaling in HEK293 cells (Luo et al., 2008), neither carbachol- nor histamine-induced ERK1/2 phosphorylation was inhibited in *ARRB2*^{-/-} BEAS-2B cells. In fact, in both experiments the rate of decline of the pERK1/2 signal was reduced in cells lacking βArr2. This extended kinetic is consistent with exaggerated Gq-signaling presumably because the histamine H₁- and muscarinic-receptors were no longer susceptible to homologous desensitization. These data are contrary to the siRNA-mediated knockdown of βArr2 reported in myometrial cells, in which histamine H₁-receptor-mediated ERK1/2 activation was abolished (Brighton et al., 2011), indicating that this form of GPCR regulation is system dependent.

Cell-type and Context-dependent Regulation of ERK1/2 Activity by β₂-Adrenoceptor Agonists. Why are LABAs unable to promote βArr2-dependent ERK1/2 phosphorylation in airway epithelial cells? The idea that essential components required to nucleate a functional βArr-signaling complex were absent or limiting was considered but seems unlikely. Indeed, mRNAs encoding 27 key proteins implicated in βArr- and G_{βγ}-regulated signaling were expressed in HBEC and BEAS-2B cells at levels similar to, or in excess of, *ADRB2*, which encodes the β₂-adrenoceptor. Moreover, EGF promoted robust ERK1/2 phosphorylation in all three epithelial cell models, where many of these same signaling intermediates are obligatory. Alternatively, basal phosphorylation of ERK1/2 in airway epithelial cells may have been maximal. Indeed, ECL determined that pERK1/2 levels were considerably higher (25- to 40-fold) in BEAS-2B cells and HBEC than in HEK293β2 cells. However, that idea could not be reconciled with the activation of ERK1/2 by EGF, TNFα, PMA, histamine and carbachol. The results with the latter two agonists are noteworthy because they illustrate that the G-protein to which a given GPCR preferentially couples dictates if ERK1/2 activity is increased or decreased. Furthermore, formoterol augmented ERK1/2 phosphorylation in HEK293β2 cells in which the baseline was elevated by EGF to a level similar to that measured in unstimulated BEAS-2B cells. The reason for high, constitutive C-Raf/MEK1/2/ERK1/2 signaling in unstimulated BEAS-2B cells (*cf.* HEK293β2s) is unknown. Cell culture conditions were similar, which suggests that this is not the cause. However, the release of an autocrine factor(s) that activates this pathway should be considered. Alternatively, sufficient βArr2 may have been plasma membrane-associated in unstimulated airway epithelia to assemble an active C-Raf-signaling complex independently of an agonist-bound GPCR (Jafri et al., 2006; Terrillon and Bouvier, 2004). The ~30% reduction in basal pERK1/2 levels

in clonal cells lacking β Arr2 is consistent with this idea. Thus, β_2 -adrenoceptor agonists may be unable to increase ERK1/2 phosphorylation in human airway epithelial cells because of constitutive, β Arr-dependent signaling.

The discrepancy between the findings reported here and those derived from murine models of asthma merits discussion. Notwithstanding a species difference, context-dependent factors may play a role. Indeed, β_2 -adrenoceptor agonists were administered to mice chronically or repeatedly prior to allergen challenge (Lin et al., 2012; Thanawala et al., 2013), which could have resulted in an adaptation of the signaling that would typically follow acute agonist exposure. In particular, chronic administration could promote genomic effects in the airway epithelium that modify the regulation of β Arr2. However, mRNAs encoding key components involved in β Arr2- and $G_{\beta\gamma}$ -dependent ERK1/2 phosphorylation were unchanged in HBEC and BEAS-2B cells exposed to formoterol for up to 18 h. While these negative data do not necessarily extrapolate to mice subjected to chronic agonist exposure they, nevertheless, argue against such a mechanism in human primary airway epithelial cells. They also raise the prospect that β_2 -adrenoceptor agonists exacerbate the asthma-like pathology in murine models of asthma by canonical mechanisms that regulate β Arr2 activity, rather than biased agonism. Indeed, cAMP increases ERK1/2 activity in many cell types where β Arr2 may play an indispensable role.

Conclusions. The experiments described herein failed to unearth evidence for β_2 -adrenoceptor-mediated, β Arr2-dependent signaling in airway epithelia; neither conventional (e.g., formoterol) nor biased (e.g., carvedilol) agonists increased pERK1/2 levels whereas robust phosphorylation was evident in HEK293 β 2 cells. In fact, β_2 -adrenoceptor agonists paradoxically dephosphorylated ERK1/2 in all three human cell models examined including highly differentiated primary cultures. This antithetical behavior of formoterol and related ligands is striking and underscores the cell-type-dependence of β_2 -adrenoceptor-mediated signaling. It also emphasises the need in drug discovery to evaluate lead candidates in the therapeutic target of interest or in systems where receptor density and coupling to downstream effectors are similar. The failure of carvedilol to phosphorylate ERK1/2 in BEAS-2B cells, where receptor number for this ligand may have been limiting, highlights this dilemma and has been acknowledged previously (Luttrell et al., 2018; Wisler et al., 2007). Thus, β Arr2 may assemble a functional C-Raf-MEK1/2-ERK1/2 signaling

complex in native human airway epithelial cells, but only in response to biased β_2 -adrenoceptor agonists with high intrinsic efficacy.

Authorship Contributions

Participated in research design: Giembycz, Hamed.

Conducted experiments: Hamed, Joshi, Kooi, Michi.

Performed data analysis: Giembycz, Hamed, Joshi.

Wrote or contributed to the writing of the manuscript: Giembycz, Hamed.

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Footnotes

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The authors state no conflict of interest.

Figure Legends

Fig. 1. Formoterol promotes ERK1/2 phosphorylation in HEK293 β 2 cells. (A & B). Confluent cells were treated with vehicle (Veh) or formoterol (Form; 1 nM), or formoterol in the absence and presence of ICI 118,551 (ICI; 1 μ M; 30 min pre-incubation) for 1, 5, 15, 30 and 60 min. EGF (10 ng/ml; 30 min) was used as a positive control. (C). Cells were exposed to increasing concentrations of formoterol for 5 min ranging from 10^{-13} M to 10^{-9} M. Cell lysates were prepared and total (t) and phosphorylated (p) ERK1/2 were measured by western blotting. pERK1/2 band volumes were quantified, normalized to tERK1/2 and expressed as fold change from the not stimulated (NS) baseline level at time zero (defined by the dashed horizontal line where NS = 1). The concentration of formoterol that produced half maximal response (pEC₅₀) is also shown. Data are the mean \pm s.e. mean of *N* independent determinations.

Fig. 2. Formoterol promotes ERK1/2 dephosphorylation in airway epithelial cells. BEAS-2B cells (A, C & E), HBEC (B, D & F) and ALI cultures (G) were treated with vehicle (Veh), formoterol (Form), or formoterol in the absence and presence of ICI 118,551 (ICI; 1 μ M; 30 min pre-incubation) for the times and at the concentrations indicated. EGF or TNF α (both 10 ng/ml) were included as positive controls. Cell lysates were prepared and total (t) and phosphorylated (p) ERK1/2 were measured by western blotting. As described in the legend to figure 1. Alternatively, BEAS-2B cells, HBEC and HEK293 β 2 (H & I) were exposed to vehicle, formoterol or EGF for the times and the concentrations indicated or not stimulated (NS) as indicated. pERK1/2 was measured by ECL and presented as either a fold change from the NS level at time zero (defined by the dashed horizontal line where NS = 1; H) or a fraction of the tERK1/2 pool (I). (J). HEK293 β 2 cells were treated with EGF (1 ng/ml) for 10 min. Formoterol (1 nM) or vehicle was added at time zero and pERK1/2 levels were quantified at 5, 15, 30, 60 and 120 min by western blotting. The time required for pERK1/2 to decline to 50% of the maximal dephosphorylation ($t_{1/2}$) and the concentration of formoterol that produced half maximal response (pEC₅₀) are also shown. Data are the mean \pm s.e. mean or box and whisker plots of *N* independent determinations. Statistical analysis was by student's paired *t*-test (G) and by repeated measures, two-way ANOVA followed by Sidak's multiple comparisons test (J). ^a*P* < 0.05, significantly different from NS and from time-matched, EGF-treated samples as indicated.

Fig. 3. Formoterol promotes ERK1/2 dephosphorylation in airway epithelial cells by canonical, cAMP-dependent signaling. **(A)** Effect of forskolin on pERK1/2 levels in HBEC. Confluent cells were treated with vehicle (Veh) or forskolin (Forsk; 10 μ M) for 1, 5, 15, 30 and 60 min along with EGF (10 ng/ml; 30 min) as a positive control. **(B)** Effect of EP₂- and EP₄-receptor agonists on pERK1/2 levels in BEAS-2B cells. Confluent cells were pre-treated (30 min) with or without the EP₂- or EP₄-receptor antagonists, TG4-155 and L-161,982 (both 1 μ M), respectively and then exposed to the corresponding agonist (ONO-AE1-259 - EP₂; ONO-AE1329 - EP₄; both 1 μ M) for 1 h. The PDE4 inhibitor, RNO (1 μ M) was present throughout. **(C)** Expression of PKI α and GFP in HBEC. Cells were infected with increasing MOIs of Ad5.CMV.PKI α and Ad5.CMV.GFP. After 48 h, the transgenes were detected by western blotting, using GAPDH as a loading control, and immunofluorescence confocal microscopy, respectively. The image shows cells before and after infection with the GFP-expressing virus (MOI=30); GFP⁺ cells are shown in green; nuclei are stained blue with 4',6-diamidino-2-phenylindole. **(D)** Functional impact of PKI α and GFP in BEAS-2B cells. Cells were infected with Ad5.CMV.PKI α or Ad5.CMV.GFP at the MOIs shown. Expression of PKI α was determined by western blotting. Formoterol (1 nM)- and forskolin (10 μ M)-induced cAMP accumulation was measured at 10 min in cells pre-treated with RNO (1 μ M). Similarly, formoterol-induced CRE-reporter activation (in the absence of RNO) was measured at 6 h by luminometry. **(E & F)** Effect of PKI α and GFP on the time-courses of formoterol-induced ERK1/2 dephosphorylation in HBEC and BEAS-2B cells. Confluent cells were infected with Ad5.CMV.PKI α or Ad5.CMV.GFP (MOI = 25) for 48 h. Formoterol (1 nM) was added for the indicated times along with EGF (10 ng/ml; 30 min) as a positive control. In all panels the pERK1/2, PKI α and GFP were measured by western blotting and normalised to GAPDH or tERK1/2 as indicated or expressed as fold change from the not stimulated (NS) baseline level at time zero (defined by the dashed horizontal line where NS = 1). The time required for pERK1/2 to decline to 50% of the maximal dephosphorylation ($t_{1/2}$) and the MOI of Ad5.CMV.PKI α that produced 50% inhibition of reporter activation (IC₅₀) are also shown. Data are the mean \pm s.e. mean or box and whisker plots of *N* independent determinations. Statistical analysis was by repeated measures one-way ANOVA followed by Tukey's multiple comparison test. ^a*P* < 0.05.

Fig. 4. DUSP1 plays a minor role in β_2 -adrenoceptor-mediated ERK1/2 dephosphorylation. **(A)** Heat-map comparing the mean fold change on a log₂ scale of the 18 *DUSP* mRNAs that were co-regulated by

formoterol (Form), indacaterol (Ind), vilanterol (Vil) and salmeterol (Salm) in both BEAS-2B cells and HBEC. Transcripts are ranked from most induced (red) to most repressed (blue) in cells treated with formoterol (Form; 1 nM; 1 h) against which all other interventions are aligned. Only *DUSP1*, *DUSP4*, *DUSP8* and *DUSP10* (indicated with arrows) were increased in both variants. **(B-F)** Time-course and concentration-dependence of DUSP1 protein and mRNA induction. BEAS-2B cells and HBEC were exposed for 1 h to increasing concentrations of formoterol (from -12M to -7M) or salmeterol (from -10M to -6M) or with a fixed concentration of formoterol (1 nM) or salmeterol (100 nM) for 0.25, 0.5, 1, 2, 4 and 6 h. Expression of DUSP1 mRNA and protein was then determined by real-time PCR and western blotting, respectively. **(G)**. Effect of ICI 118,551 and PKI α on *DUSP1* induction. BEAS-2B cells and HBEC were pre-treated (30 min) with ICI 118,881 (ICI; 1 μ M) or infected with Ad5.CMV.PKI α or Ad5.CMV.GFP (MOIs = 25). Salmeterol (Salm; 100 nM), formoterol (Form; 1 nM) or forskolin (Forsk; 10 μ M) was added for 1 h and DUSP1 mRNA transcript measured by real-time PCR. **(H)** Effect of DUSP1 knockdown on salmeterol-induced ERK1/2 phosphorylation in BEAS-2B cells. Cells were transfected with two *DUSP1*-targeting siRNAs or a non-targeting control (both 25 nM) for 48 h. Salmeterol (100 nM) was added for 60 min and the expression of DUSP1 and pERK1/2 was measured by western blotting. In panels **B-H**, DUSP1 and pERK1/2 were normalised to GAPDH or tERK1/2 as indicated or expressed as fold change from the not stimulated (NS) baseline level at time zero (defined by the dashed horizontal line where NS = 1). Data are the mean \pm s.e. mean or box and whisker plots of *N* independent determinations. Statistical analysis was by repeated measures one-way ANOVA followed by Tukey's multiple comparison test. $^aP < 0.05$.

Fig. 5. Phosphorylation of C-Raf mediates formoterol-induced ERK1/2 dephosphorylation in BEAS-2B cells. **(A)** Domain structure of C-Raf. C-Raf is a 648 amino acid protein kinase containing three conserved regions (CR1, CR2 and CR3). CR1 is located at the regulatory N-terminus and features the Ras-binding domain (RBD) and the cysteine-rich domain (CRD) domain, which are necessary for membrane recruitment and linking Ras to MEK1/2, respectively. CR2 is also found in the regulatory region and provides sites for the binding of inhibitory 14-3-3 proteins, which maintain C-Raf in an auto-inhibited conformation. The kinase domain is embedded within CR3 towards the C-terminus. At least 13 phosphorylation sites have been identified that regulate C-Raf activity. Residues marked with a red asterisk are phosphorylated by PKA and lead to kinase inactivation. Residues marked with green and blue asterisk are activating

phosphorylation sites and ERK1/2 feedback sites, respectively. **(B & C)** Effect of formoterol on C-Raf phosphorylation. BEAS-2B cells were treated with formoterol (Form; 1 nM) or vehicle for 1, 5, 15, 30 and 60 min. **(D & E)** Effect of PKA inhibition on formoterol-induced C-Raf phosphorylation. BEAS-2B cells were infected (MOI = 25) with either Ad5.CMV.PKI α (PKI α) or Ad5.CMV.GFP (GFP) for 48 h. Formoterol (Form; 1 nM) was added for 1 h and cell lysates were subjected to western blotting for pC-Raf (at Ser⁴³, Ser²⁵⁹, Ser³³⁸ and Ser⁶²¹), pMEK1/2 and pERK1/2. Alternatively, cells were treated (1 h) with the MEK1/2 inhibitors, PD 098059 or U0126 (both 10 μ M). Band volumes were quantified, normalised to tC-Raf, tMEK1/2 and tERK1/2 as indicated and expressed as a fold change from the not stimulated (NS) baseline at time zero (defined by the dashed horizontal line where NS = 1). Phospho-Ser²⁵⁹ appear as a doublet on SDS polyacrylamide gels and both bands were quantified. Data are the mean \pm s.e. mean or box and whisker plots of *N* independent determinations. Statistical analysis was by repeated measures one-way ANOVA followed by Tukey's multiple comparison test. ^a*P* < 0.05, significantly different from unstimulated cells; ^b*P* < 0.05, significantly different from formoterol-stimulated cells expressing GFP.

Fig. 6. Effect of formoterol on ERK1/2-Elk-1-EGR1 signaling in airway epithelial cells. **(A & B)** Effect of formoterol on the expression of EGR1. HBEC, BEAS-2B cells and ALI cultures were exposed to formoterol (Form; 1 nM; 1 and 2h) or EGF (10 ng/ml; 1 h). **(C)** Time-course of *EGR1* repression in BEAS-2B cells. Cells were treated with formoterol (1 nM), U0126 (10 μ M) and both drugs in combination for 15, 30, 60 and 120 min. **(D)** Effect of formoterol and U0126 on *EGR1* mRNA expression in HEK293 β 2 cells. Cells were treated with U0126 (10 μ M) or formoterol (1 nM) and *EGR1* mRNA was measured at 1 and/or 2h as indicated. **(E)** Effect of PKI α over-expression and of DUSP1 knockdown on formoterol-induced ERK1/2 and Elk-1 dephosphorylation and on the repression of EGR1. BEAS-2B cells were infected (MOI = 25) with either Ad5.CMV.PKI α (PKI α) or Ad5.CMV.GFP (GFP) for 48 h. Alternatively, cells were transfected with *DUSP-1*-targetting siRNA-2 or a control siRNA (Fig. 4). Formoterol (1 nM) was added for 2 h. PD 098059 and U0126 (both 10 μ M) were included as positive controls. In all panels, mRNA level and protein expression were measured by real-time PCR and western blotting, respectively. Levels were normalised to GAPDH, tERK1/2 or tElk-1 or expressed as a fold change from the not stimulated (NS) level at time zero (NS = 1), 1 h or 2 h as indicated. Data are presented as the mean \pm s.e. mean or as box and whisker plots of *N* independent measurements. Statistical analysis was by repeated measures one-way

ANOVA followed by Tukey's multiple comparison test. ^{a-e}*P* < 0.05, significantly different from ^aNS, ^bGFP, ^cformoterol+PKI α , ^dControl siRNA and ^eDUSP1 siRNA.

Fig. 7. Distinct effects of carvedilol, carazolol and alprenolol on ERK1/2 phosphorylation between airway epithelial and HEK293 β 2 cells. HEK293 β 2 (**A-C**) and BEAS-2B cells (**D-F**) were treated with carvedilol, carazolol and alprenolol (each 10 μ M) for 1, 5, 15, 30 and 60 min. EGF (10 ng/ml; 30 min) was used throughout as a positive control. Cell lysates were prepared and total (t) and phosphorylated (p) ERK1/2 were measured by western blotting. Band volumes were quantified and processed as described in the legend to figure 1. Each panel shows a representative western blot and the mean data (\pm s.e. mean) of *N* independent determinations.

Fig. 8. Effect of genetic deletion of *ARRB2* on agonist-induced cAMP accumulation and ERK1/2 phosphorylation in BEAS-2B cells. (**A**) *ARRB1* and *ARRB2* mRNA abundance. Total RNA was extracted from unstimulated BEAS-2B cells and HBEC, subjected to RNA-sequencing and non-visual arrestin variants were expressed as TPM. (**B**) Representative western blot of β Arr2 protein expression in wild type (WT) and three clones (C₁, C₂, C₃) lacking β Arr2 derived in BEAS-2B cells by CRISPR/Cas9 genome editing. (**C**) Effect of β Arr2 deletion on formoterol-induced cAMP accumulation. Clonal and parental BEAS-2B cells were pre-incubated (30 min) with the PDE4 inhibitor, RNO (1 μ M) and exposed to increasing concentrations of formoterol (from -10M to -7M; 15 min) or with a fixed concentration of formoterol (100 nM) for 1, 5, 10, 15 and 30 min. cAMP mass was measured by ECL and expressed as a fold change from the not stimulated (NS) baseline level at time zero (defined by the dashed horizontal line where NS = 1). (**D**) Schematic of the mechanism by which β_2 -adrenoceptor-mediated signalling is constrained by β Arr2. Agonist-binding leads to phosphorylation of the receptor by G-protein-coupled receptor kinases (GRK). β Arr2 is then recruited and uncouples the receptor from G α s such that downstream signaling is impaired. (**E**) Effect of β Arr2 deletion on RNO- and forskolin-induced cAMP accumulation. Wild-type and clonal cells were treated (30 min) with RNO (1 μ M) and forskolin (10 μ M) was added for a further 15 min. cAMP mass was measured by ECL and expressed as a fold change as described in panel C. (**F**) Effect of β Arr2 deletion on basal and EGF-induced ERK1/2 phosphorylation. Wild-type and clonal BEAS-2B cells were left untreated or exposed to EGF (10 ng/ml; 30 min).

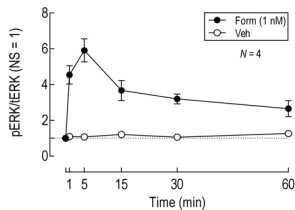
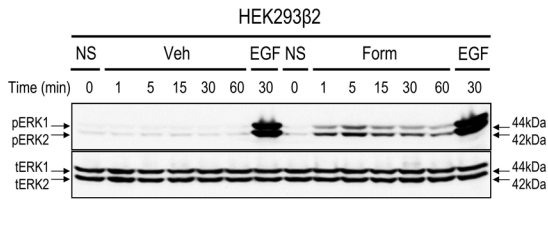
Phosphorylated (p) ERK1/2 was measured by western blotting and ECL and expressed as a ratio to total (t) ERK1/2 or as a fraction of the tERK1/2 pool, respectively. (G) Effect of β Arr2 deletion on the time-course of formoterol-induced ERK1/2 dephosphorylation. Wild-type (WT) and clonal BEAS-2B cells were exposed to formoterol (1 nM) for 1, 5, 15, 30 and 60 min. The decay in the pERK1/2 signal was fit to a single exponent from which $t_{1/2}$ values were derived assuming the same maximum degree of dephosphorylation in both cell types. The red arrow indicates the decrease in $t_{1/2}$ in *ARRB2*^{-/-} cells. (H & I) Effect histamine and carbachol on ERK1/2 phosphorylation in BEAS-2B cells. Confluent cells were exposed to vehicle (Veh), histamine (Hist), carbachol (CCh; both 10 μ M), or histamine and carbachol in cells pre-treated (30 min) with mepyramine (Mep) and atropine (Atr; both 1 μ M), respectively for 5, 10, 15 and 30 min. (J & K) Effect of Gq inhibition on the time-course of histamine and carbachol-induced ERK1/2 phosphorylation in BEAS-2B cells. Confluent cells were exposed to histamine (Hist) or carbachol (CCh; both 10 μ M) for 5, 10, 15 and 30 min in the absence and presence of the Gq inhibitor YM-254890 (YM; 1 μ M; 60 min pre-incubation). (L & M) Effect of β Arr2 deletion on the time-course of histamine- and carbachol-induced ERK1/2 dephosphorylation. Wild-type (WT) and clonal BEAS-2B cells deficient in β Arr2 (*ARRB2*^{-/-}) were exposed to histamine or carbachol (both 10 μ M) for 5, 10, 15 and 30 min. The decay in the pERK1/2 signal, beginning at the 5 min time-point (S), was fit to a single exponent from which $t_{1/2}$ values were derived. It was assumed that the degree of phosphorylation at 5 min and the maximum degree of dephosphorylation were the same in both cell types and did not fall below the not stimulated (NS) baseline value of 1. The red arrows indicate the increase in $t_{1/2}$ in *ARRB2*^{-/-} cells. In panels G, L and M, the western blots were generated with C₁ while the kinetics show the mean results of experiments using all clones. EGF (10 ng/ml; 30 min) was used throughout as a positive control. Western blots for C₂ and C₃ are presented in Sfigure 9. Membranes were also probed for β Arr2 to confirm deletion in clonal cells. Data are presented as the mean \pm s.e. mean or as box and whisker plots of *N* independent measurements. ^a*P* < 0.05, significantly different from WT.

Fig. 9. Schematic representation of the effect of β_2 -adrenoceptor agonists on ERK1/2 phosphorylation in human airway epithelial cells. β_2 -Adrenoceptor agonists dephosphorylate ERK1/2 (ERK) by canonical Gas/adenylyl cyclase/cAMP/PKA signaling that involves (1) the inhibition of constitutive C-Raf activity leading to the sequential inactivation of MEK and ERK (major mechanism), and (2) induction of DUSP-1, a

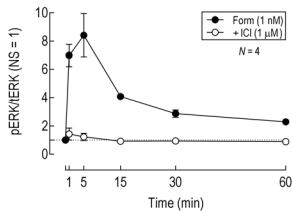
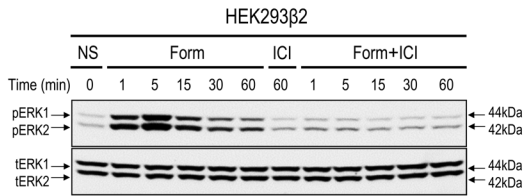
MKP that directly dephosphorylates ERK (minor mechanism). ERK dephosphorylation is associated with repression of *EGR1* gene transcription due to the dephosphorylation of the ERK-sensitive transcription factor, Elk-1, which is bound to its cofactor serum response factor (SRF) at the promoter region of *EGR1*. β_2 -Adrenoceptor agonism also promotes β Arr2-mediated homologous desensitization of the agonist-occupied receptor but does not promote β Arr2-dependent C-Raf/MEK/ERK signaling.

Figure 1

A



B



C

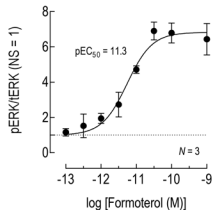
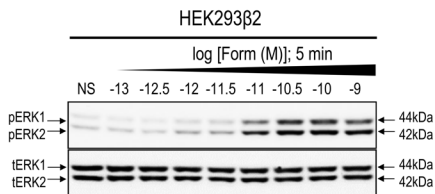


Figure 2

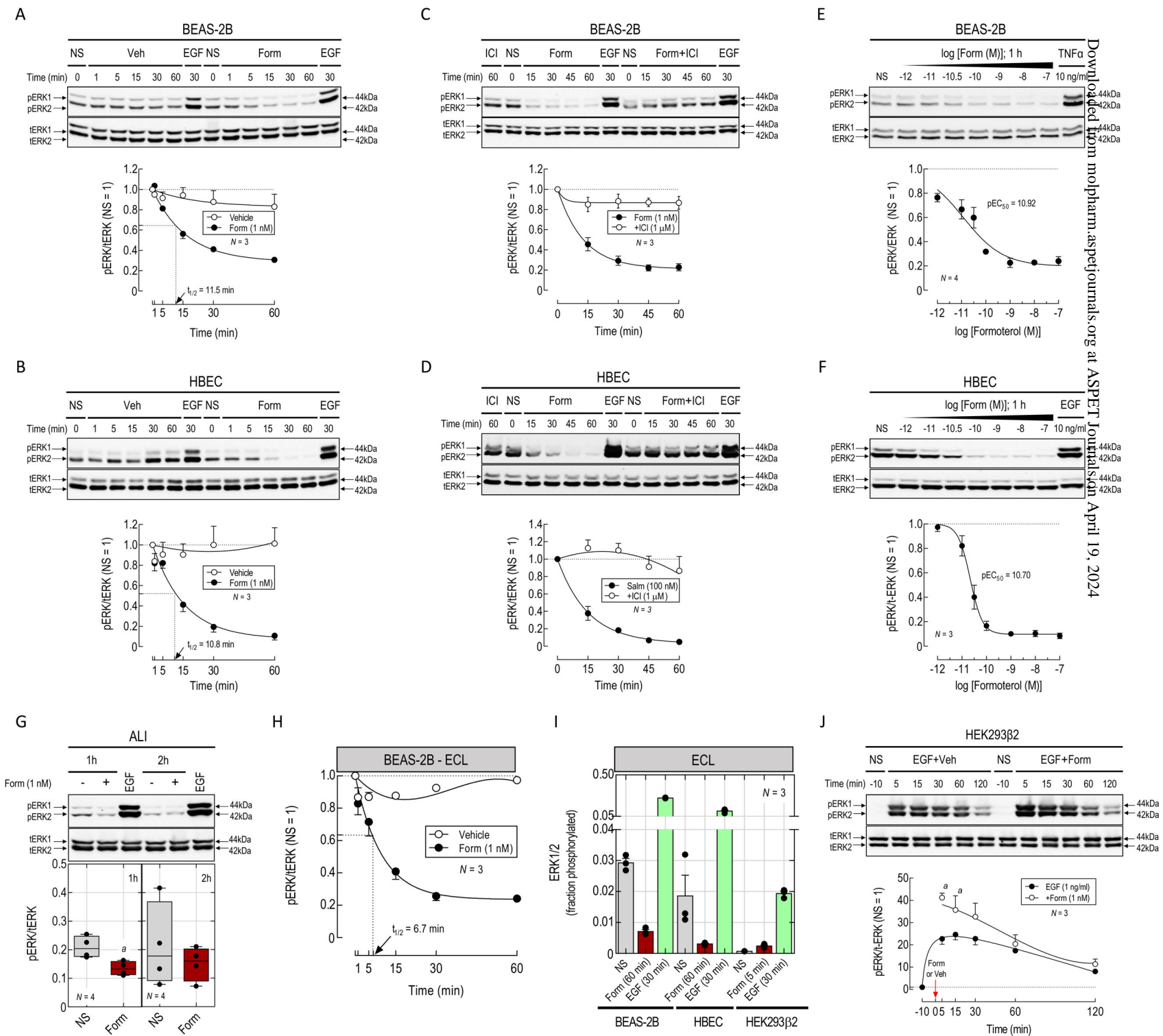
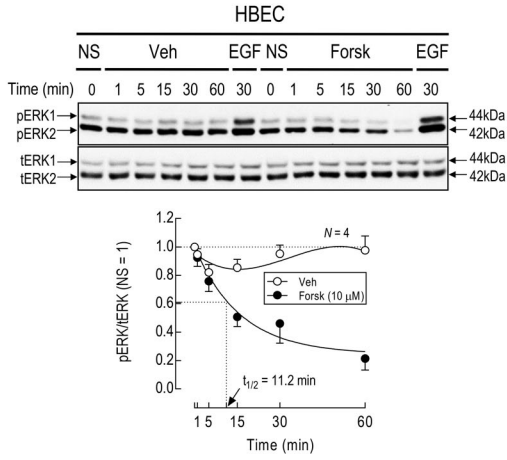
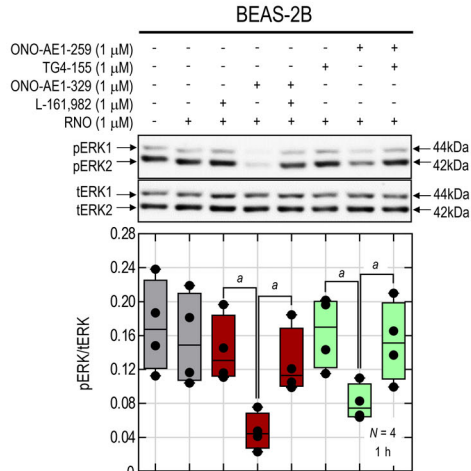


Figure 3

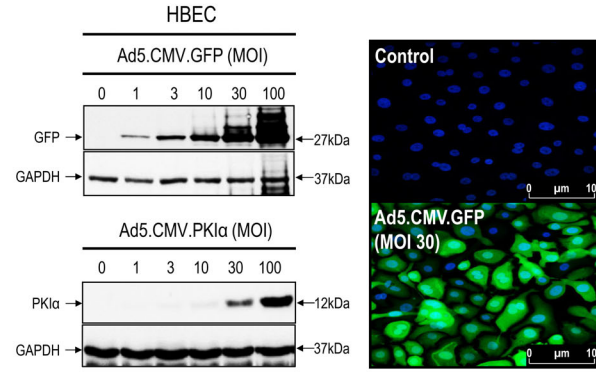
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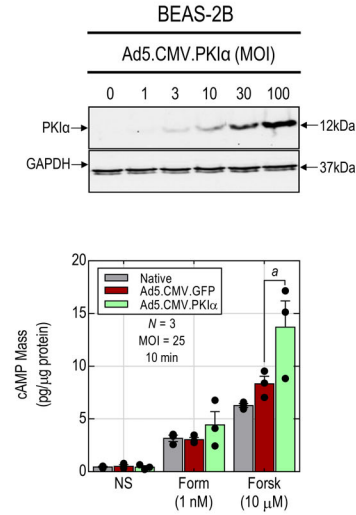
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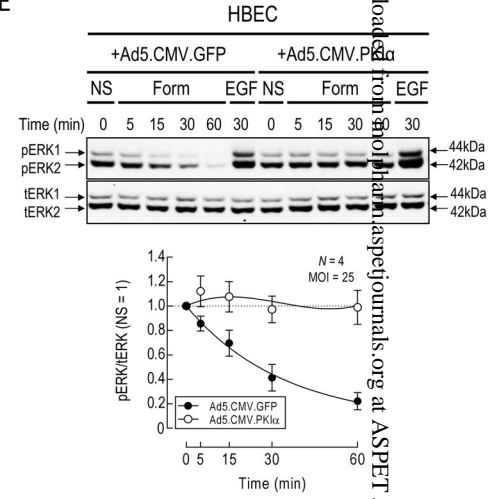
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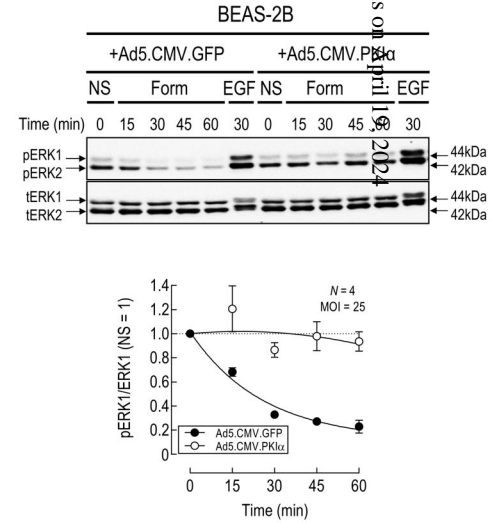
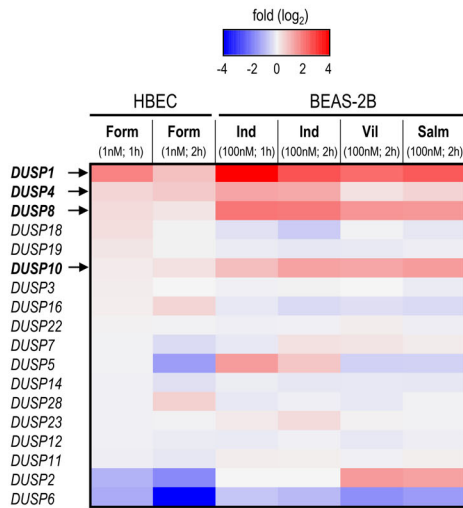
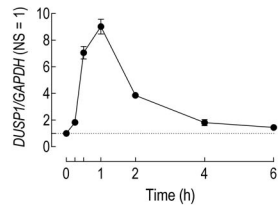


Figure 4

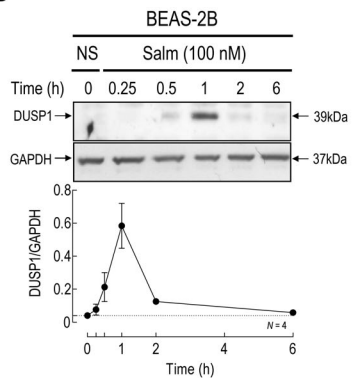
A



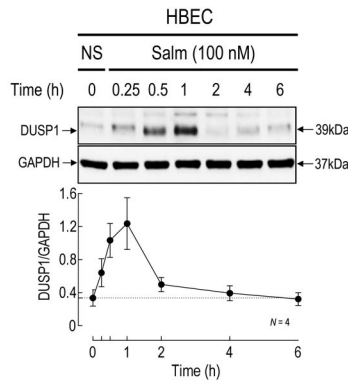
B



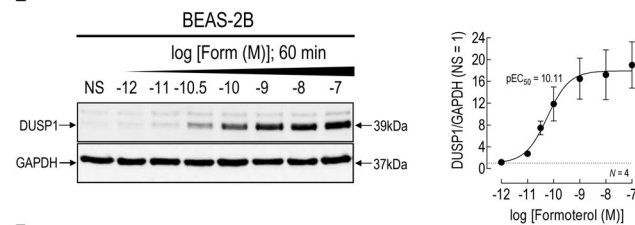
C



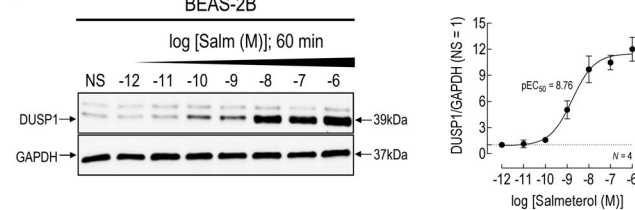
D



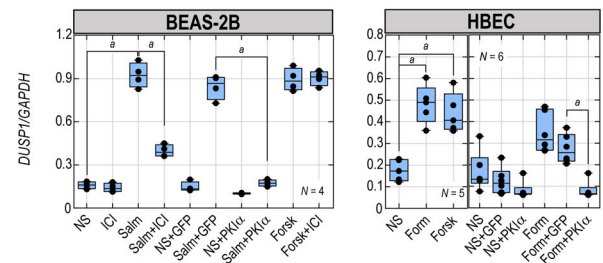
E



F



G



H

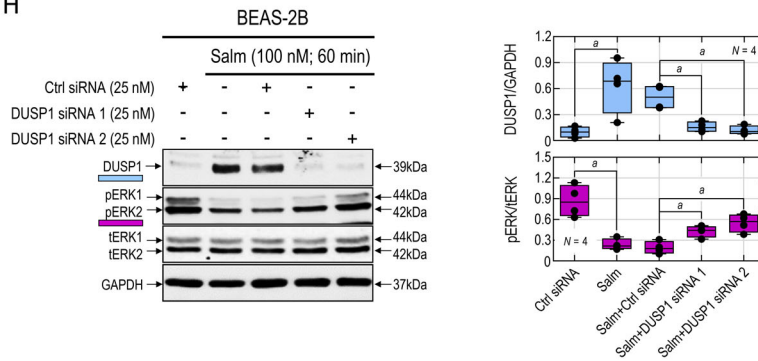
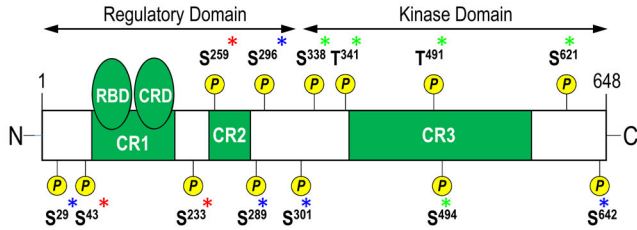
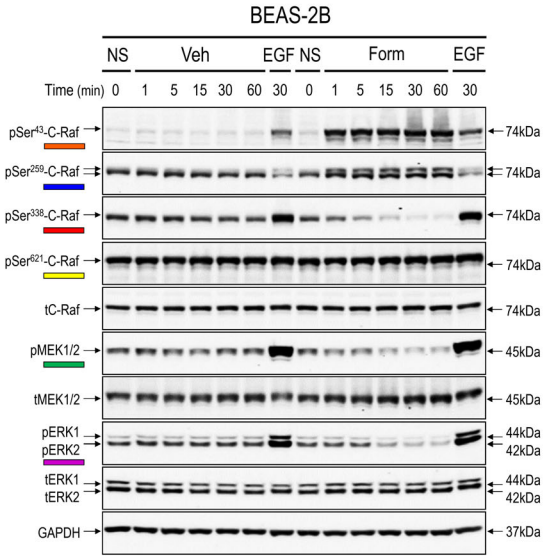


Figure 5

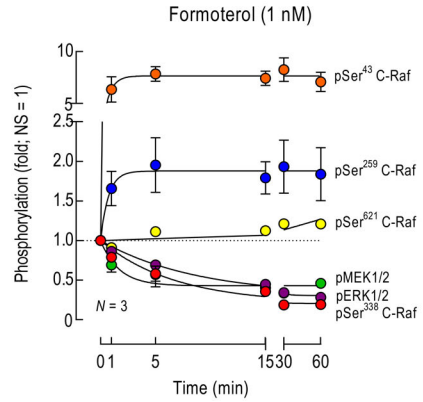
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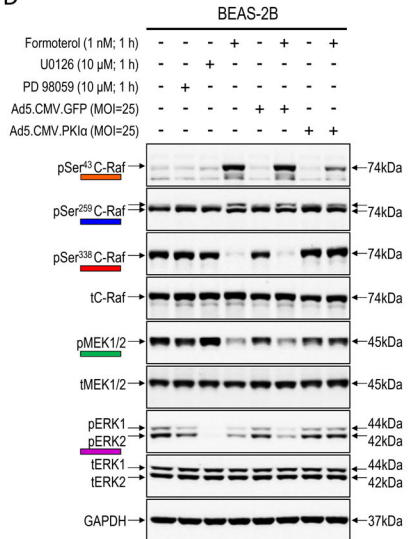
B



C



D



E

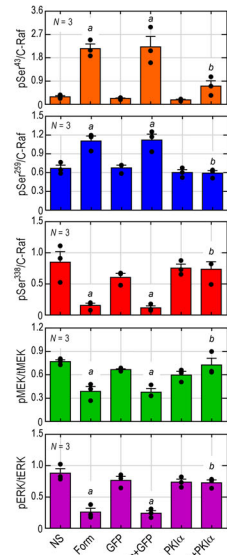
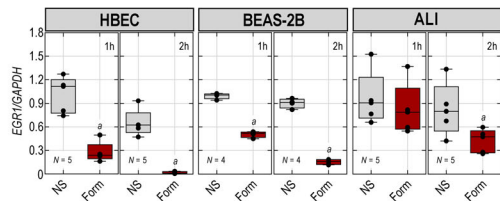
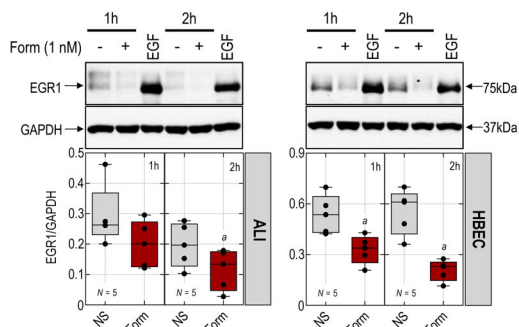


Figure 6

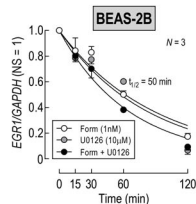
A



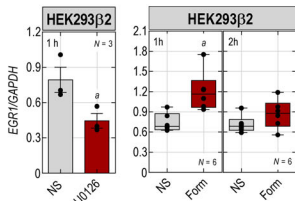
B



C



D



E

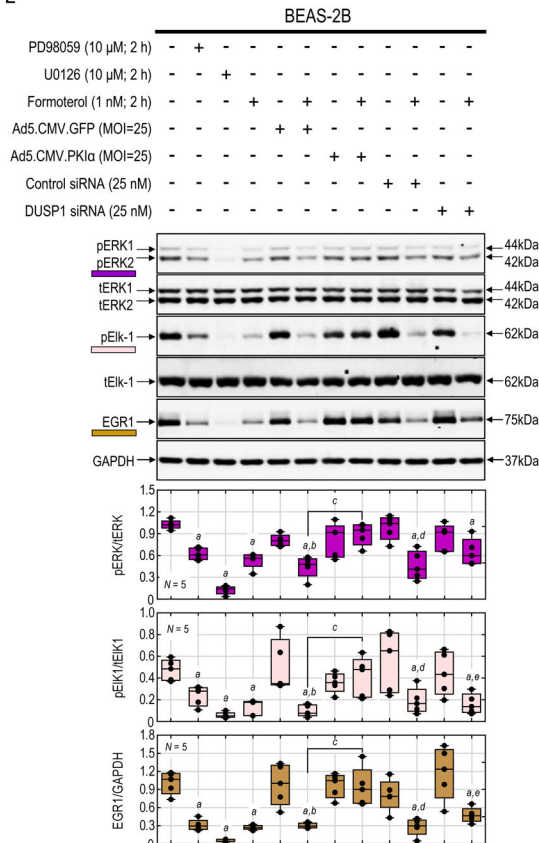
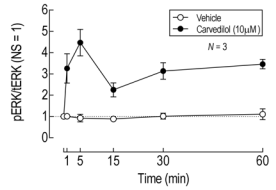
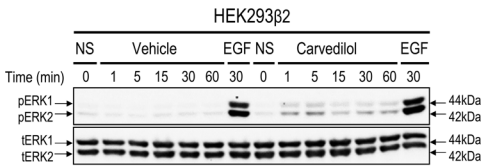
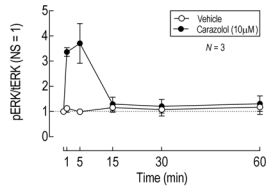


Figure 7

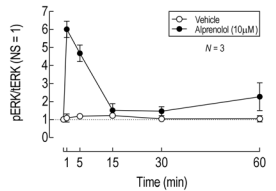
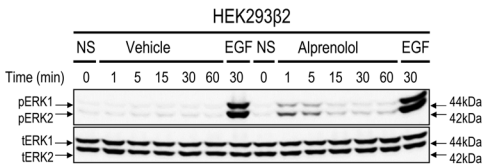
A



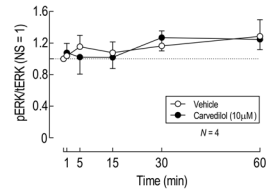
B



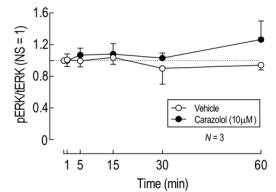
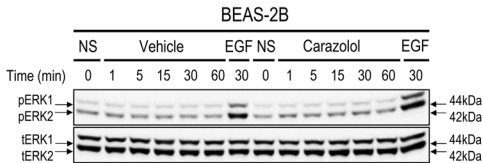
C



D



E



F

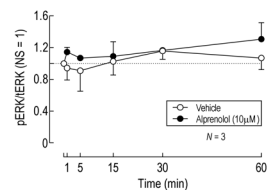


Figure 8

