Cytokines Regulate β-2-Adrenergic Receptor Responsiveness in Airway Smooth Muscle via Multiple PKA- and EP2 Receptor-Dependent Mechanisms†

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ABSTRACT: β_2AR desensitization in airway smooth muscle (ASM) mediated by airway inflammation has been proposed to contribute to asthma pathogenesis and diminished efficacy of β-agonist therapy. Mechanistic insight into this phenomenon is largely conceptual and lacks direct empirical evidence. Here, we employ molecular and genetic strategies to reveal mechanisms mediating cytokine effects on ASM β_2AR responsiveness. Ectopic expression of inhibitory peptide (PKI-GFP) or a mutant regulatory subunit of PKA (RevAB-GFP) effectively inhibited intracellular PKA activity in cultured human ASM cells and enhanced β_2AR responsiveness by mitigating both agonist-specific (β-agonist-mediated) desensitization and cytokine (IL-1β and TNF-α)-induced heterologous desensitization via actions on multiple targets. In the absence of cytokine treatment, PKA inhibition increased β_2AR-mediated signaling by increasing both β_2AR−G protein coupling and intrinsic adenylyl cyclase activity. PKI-GFP and RevAB-GFP expression also conferred resistance to cytokine-promoted β_2AR−G protein uncoupling and disrupted feed-forward mechanisms of PKA activation by attenuating the induction of COX-2 and PGE_2. Cytokine treatment of tracheal ring preparations from wild-type mice resulted in a profound loss of β-agonist-mediated relaxation of methacholine-contracted rings, whereas rings from EP2 receptor knockout mice were largely resistant to cytokine-mediated β_2AR desensitization. These findings identify EP2 receptor- and PKA-dependent mechanisms as the principal effectors of cytokine-mediated β_2AR desensitization in ASM.

β-Agonists inhibit contractile force development in airway smooth muscle (ASM)[1] by stimulating cAMP-dependent protein kinase (PKA) activity via the β-2-adrenergic receptor (β_2AR)−Gs-adenylyl cyclase pathway. PKA phosphorylates numerous intracellular proteins in ASM to reduce both Ca^{2+} flux and the sensitivity of the contractile apparatus to intracellular calcium (*1*). A long-held (albeit somewhat controversial) belief in Airway Biology is that airway inflammation contributes to β_2AR dysfunction and thereby influences asthma pathogenesis or the response to inhaled β-agonist therapy. Such a loss of β_2AR function could promote bronchial hyperreactivity (due to an imbalance between pro-contractile and pro-relaxant cellular signaling) or a reduction of the prophylactic or rescue effects of β-agonists (*2, 3*). Indeed, numerous studies examining contractile state in ASM cells (*4*−*7*), tissue (*8*−*10*), or in vivo (*11*−*15*) models have identified a loss of the relaxant effect of β-agonists following induction of allergic inflammation or treatment with agents (e.g., cytokines) present in the inflammatory milieu of the airway.

The mechanisms by which inflammatory agents promote diminished β_2AR function in ASM are not established but the prevailing hypothesis has been shaped by our understanding of fundamental mechanisms of β_2AR desensitization and studies that demonstrate inflammatory mediators can activate signaling pathways associated with these mechanisms. β_2AR desensitization can be characterized as either homologous (agonist-specific) or heterologous (nonagonist-specific) (see Penn and Benovic (*1*) for a comprehensive review). Homologous desensitization involves the phosphorylation of the *agonist-occupied* receptor, typically by G protein-coupled receptor (GPCR) kinases (GRKs), which serves to diminish β_2AR coupling with the heterotrimeric G protein Gs. GRK-phosphorylated β_2AR is subsequently bound by one of a family of arrestin proteins, which not only completely uncouples the β_2AR from Gs but also initiates receptor internalization for the purpose of either receptor degradation or receptor recycling. Heterologous

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[1] Abbreviations: ASM, airway smooth muscle; β_2AR, β-2-adrenergic receptor; cAMP, cyclic AMP; PKA, cAMP-dependent protein kinase; CRE, cAMP response element; CYP, cyanopindolol; COX-2, cyclooxygenase-2; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; PGE, prostaglandin E; FSK, forskolin; ISO, isoproterenol; IL-1β, interleukin-1-beta; MCh, methacholine; PKC, protein kinase C; PDE, phosphodiesterase; TNF-α, tumor necrosis factor-alpha.

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desensitization does not require agonist-occupied β_2AR, yet involves the phosphorylation of the β_2AR by second messenger kinases such as PKA or protein kinase C (PKC). Any agent capable of activating second messenger kinases has the potential to promote heterologous desensitization. Studies using COS-7 or HEK-293 cells examining recombinant β_2-ARs in which consensus PKA phosphorylation sites are mutated have demonstrated the capacity of PKA to mediate β_2AR desensitization by agents such as prostaglandins (PGE_1 or PGE_2, which activate Gs-coupled EP2 or EP4 receptors) or forskolin (FSK) (a direct activator of adenylyl cyclase) (see Tran et al. (*16*) and references therein). Thus, in many cell types, PKA has the capacity to function as both an effector and negative-feedback regulator of β_2ARs.

PGE_2 is a product of cellular phospholipids; rapid pulmonary metabolism of PGE_2 limits its steady-state levels in the lung and renders its actions primarily to those of an autocrine/paracrine factor. Numerous resident or infiltrating airway cell types represent sources of paracrine PGE_2 capable of affecting ASM (*17*). The relevance of autocrine PGE_2 as a modulator of ASM β_2AR responsiveness and β-agonist-dependent effects has been asserted by multiple studies demonstrating that treatment of ASM cells with the cytokine interleukin-1-beta (IL-1β) causes increased expression of cyclooxygenase-2 (COX-2) and consequently increased PGE_2 synthesis (*18*−*20*). Studies using ASM cultures from various species have identified IL-1β-mediated inhibition of serum-stimulated growth (*21*, *22*), as well as decreased β_2AR-mediated cAMP production (*5*, *7*, *22*, *23*) and inhibition of cell contraction/tension (*5*−*7*, *24*, *25*). IL-1β-mediated growth inhibition, GPCR hyporesponsiveness, and COX-2 induction can all be inhibited by glucocorticoid treatment (*7*, *19*, *23*, *26*), and indomethacin pretreatment can similarly attenuate the PGE_2 synthesis and functional consequences elicited by IL-1β treatment (*5*, *21*, *23*). Because exogenous PGE_2 can mimic the effects of IL-1β treatment, the collective evidence suggests induced COX-2-dependent PGE_2 synthesis leading to EP receptor activation of PKA as the principal mechanism by which IL-1β alters ASM growth and GPCR function.

However, the specific roles of PKA, PGE_2, and EP receptors in cytokine-induced β_2AR desensitization in ASM have never been directly demonstrated, primarily because specific inhibition of PKA and EP receptor subtypes has been problematic. The significance of this problem as it relates to questions of PKA function in lung and ASM has been recently asserted in Dent (*27*) and Spicuzza et al. (*28*) and appears to require approaches beyond those involving frequently used pharmacological agents whose lack of specificity has become increasingly apparent (*28*, *29*). With respect to the role of EP receptors in cytokine/PGE_2 -dependent effects, a lack of useful antagonists for EP receptor subtypes has thwarted investigation into this question.

Here, we employ molecular and genetic strategies to selectively target PKA and EP receptors, respectively, to identify multiple mechanisms by which these proteins mediate modes of regulation of β_2AR signaling and function in ASM.

EXPERIMENTAL PROCEDURES

Cell Culture. Human ASM cultures were established as described by Panettieri et al. (*30*) from human tracheae obtained from lung transplant donors. Fourth to eighth passage cells were grown in Ham's F12 media supplemented with 10% fetal bovine serum (FBS) to confluence in either 10 cm or 6 well (for subsequent immunoblot analysis) or 24 well (for cAMP accumulation assays in intact cells) plates, then were growth-arrested in serum-free Ham's F12 containing 0.1% bovine serum albumin (*22*, *31*).

Construct Generation. For generation of a cAMP response element (CRE)-stimulated luciferase reporter construct, 2 μg each of the oligonucleotides 5′ tcgatagcctgacgtcagagag 3′ (containing the consensus CRE sequence *tgacgtca*) and its reverse complement were phosphorylated with T4 kinase, annealed, ligated into Δ56FosdE (*32*), and transformed into DH5α cells. A clone identified with three CRE inserts all in the 5′ to 3′ orientation was subsequently identified and used in the present study.

The open reading frames of PKI (from pcDNA3PKI, provided by Tung Chan, Thomas Jefferson University) and RevAB (a mutant regulatory PKA subunit (*33*), provided as pcDNA3RevAB by G. Stanley McKnight, University of Washington) were PCR-cloned into the HindIII/SalI sites of pEGFP-N1 to generate pEGFPN1-PKI and pEGFPN1-RevAB, encoding the GFP chimeras PKI-GFP and RevAB-GFP. HindIII/NotI fragments of pEGFPN1-PKI and pEGFPN1-RevAB, containing sequences encoding PKI-GFP and RevAB-GFP, respectively, were each subcloned into pLNCX2 to enable generation of retrovirus (described below).

Synthesis of TAT-Conjugated PKI. Peptides of TAT (YGRKKRRQRRR), PKI (CGRTGRRNAI), and TAT-PKI fusions were generated and purified by high-performance liquid chromatography as described previously (*34*). ASM cultures were pretreated with 0.1−1 μM (final) TAT-PKI or TAT-TAT peptide control as described previously for cardiac myocytes (*34*).

Immunoblot Analyses. After treatment/stimulation as described below, cells were lysed in 50 mM Tris buffer (pH 8.0) containing 150 mM NaCl, 20 mM NaF, 5 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 10 mM *p*-nitrophenyl phosphate, 1 mM benzamidine, 0.1 M PMSF, and 1% (v/v) NP-40. Thirty micrograms of whole cell protein from the soluble fraction was electrophoresed on 10% SDS polyacrylamide gels, transferred to nitrocellulose membranes, and subsequently probed with antibodies that recognize vasodilator-stimulated phosphoprotein (VASP) (BD Biosciences, San Jose, CA), phospho-Ser 157 VASP (Alexis Biochemicals, San Diego, CA), COX-2 (Cayman Chemical, Ann Arbor, MI), GFP, or β-actin (Sigma, St. Louis, MO), followed by the appropriate (infrared fluorophore-conjugated) AlexaFluor or Rockland secondary antibodies for detection and quantification on the Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE).

Infection Procedures. Retrovirus for expression of GFP, PKI-GFP, and RevAB-GFP was produced by transfecting GP2-293 cells with pLNCX2-CRE-Luc, pLNCX2-GFP, pLNCX2-PKI-GFP, or pLNCX2-RevAB-GFP, each with pVSV-G Vector which encodes the pantropic (VSV-G) envelope protein. Supernatants were harvested 48 h after transfection and used to infect human ASM cultures plated

at a density of 2×10^6 cells/cm^2 24 h previously. Virus was either concentrated or applied directly to ASM cells, with effective virus concentrations established by immunoblot analysis. Infected cells typically exhibited 50−70% GFP expression within 48 h (direct visualization by fluorescent microscopy), and selection to homogeneity with 250 μg/mL G418 was rapid (7 days).

Radioligand Binding. β_2AR density in human ASM cultures was assessed by [^{125}I]cyanopindolol (CYP; 2000 Ci/mmol, Amersham Biosciences Piscataway, NJ) binding to ASM cells similar to that described previously (*35*). Specific binding, calculated as [(total bound [^{125}I]CYP) − ([^{125}I]CYP bound in the presence of 1 μM alprenolol)] was measured in triplicate tubes for each, using a saturating concentration (300 pM) of [^{125}I]CYP.

Assay of cAMP Accumulation and Luciferase Activities. For assay of cAMP accumulation, cells plated in 24 well plates were grown to near confluence in Ham's F12/10% FBS, washed and fed Ham's F12/0.1% bovine serum albumin for 24 h, then pretreated for either (1) 30 min with vehicle, 100 nM PGE_2, or 10 μM FSK or (2) 18 h with vehicle, 20 U/mL IL-1β, or 20 U/mL IL-1β + 10 ng/mL TNF-α. Following pretreatment, cells were washed three times with cold PBS and subsequently stimulated with phosphate-buffered saline (PBS) containing 300 μM ascorbic acid, 1 mM RO-20-1724 (phosphodiesterase inhibitor), and either vehicle (basal), (−)isoproterenol (ISO), or FSK at the indicated concentrations for 10 min at 37 °C. cAMP was isolated and quantified by radioimmunoassay as described previously (*35*). For luciferase assays, cells were transfected as described above with 3XCRE-Luc and plated into 96 well plates. Cells were subsequently growth-arrested, stimulated with vehicle, 1 μM ISO, 1 μM PGE_2, or 10 μM FSK for 8 h, then quenched directly in the well with a reaction mix containing firefly luciferase substrate as per manufacturer's instructions (Bright-Glo Luciferase Assay System, Promega, Madison, WI). Relative Light Units (RLU) per well were quantified by a Turner Biosystems (Sunnyvale, CA) microplate luminometer.

PGE_2 Isolation and Quantification. PGE_2 from the harvested culture media was purified using C18 columns (*36*) and subsequently quantified by radioimmunoassay as described previously (*22*).

Treatment of Murine Tracheal Ring Preparations and Analysis of Contractile Properties ex Vivo. Age-matched wild-type (EP2+/+) and EP2 receptor knockout (EP-/-) mice on a C57BL/6 background (*37*) were euthanized by CO_2 inhalation, and tracheae were rapidly excised and prepared as described previously (*38*). Tracheae were washed three times at 30 min intervals in serum-free Ham's F12 media then incubated 18 h in 300 μL of Ham's F12 media containing vehicle, 20 U/mL IL-1β, or 20 U/mL IL-1β + 10 ng/mL TNF-α. Rings were then washed in PBS and suspended longitudinally by a plexiglass rod using a stainless steel pin in a double-jacketed, glass organ bath (Radnoti Tissue-Organ Bath Systems, ADI Instruments, Colorado Springs, CO) perfused with 10 mL of Krebs−Henseleit solution containing 1 μM indomethacin, maintained at 37 °C, as described previously (*38*). Ring tension (preload) was set to 0.5 g after repeated washing. After rings were initially contracted with 10 μM methacholine (MCh, 10 μM representing 80% of maximal contractile force independently established in analyses of the dose-dependent response to MCh), they were washed three times, readjusted to 0.5 g tension, and stimulated again with 10 μM MCh. Following development of steady-state tension, ISO, PGE_2 (0.001−10 μM), or FSK (0.01−100 μM) was added to the bath progressively and a level of steady-state tension established for each concentration. Recordings were monitored using PowerLab data acquisition and Chart Software (ADI Instruments). IC_{50} values and maximal relaxation effect (expressed as a percentage of maximal inhibition induced by 200 μM papaverine added at the end of dose−response determination) were calculated and compared among groups. Force generation was calculated as milligrams of tension per milligram of tracheal ring weight.

Data Presentation and Statistical Analysis. Data points from assays of cAMP accumulation and [^{125}I]CYP binding experiments represent the mean ± standard error (SE) values of individual data points averaging duplicate or triplicate measurements. Statistically significant differences among groups were assessed by *t*-test for paired samples, with p values < 0.05, sufficient to reject the null hypothesis.

RESULTS

Establishing PKA Activation and Inhibition in ASM Cells. To establish the role of PKA in mediating the effects of cytokines on β_2AR responsiveness and function in ASM cells, initial experiments characterized the efficacy of several putative pharmacologic PKA inhibitors in cultured ASM. Because of potential difficulties in interpreting results from an in vitro assay of cellular PKA activity, intracellular PKA activity was assessed by examining the phosphorylation state of endogenous VASP in ASM cells. Phosphorylation of VASP by PKA at Ser 157 causes a significant mobility shift in VASP (*39*). This shift is readily detected in immunoblots (Figure 1A), and the percentage of VASP distributing to the slower migrating band correlates with the efficacy of Gs-coupled receptor agonists in promoting intracellular cAMP accumulation ((*40*) and Penn, unpublished observations). Interestingly, we found that numerous agents (including the mimetic substrate PKI peptide conjugated to the TAT sequence, as well as myristoylated PKI, KT5720, and RpcAMP), reported to inhibit PKA activity in both cell-free assays and in intact cells, failed to do so in ASM cells stimulated with either the β_2AR agonist ISO or the direct activator of adenylyl cyclase, FSK. These agents similarly failed to inhibit ISO- and FSK-stimulated CRE-Luc activity in ASM cells (data not shown). Only the isoquinoline H-89 and, to a lesser extent, the related compound H-7 were able to inhibit the VASP shift induced by acute treatment with ISO or FSK. However, nonspecific effects of H-89 and H-7, including actions as βAR receptor antagonists (*29*) render these compounds unsuitable for analysis of PKA-dependent effects on β_2AR regulation.

Consequently, we sought to develop a molecular strategy for effectively inhibiting PKA in ASM cells. Because the failure of TAT-conjugated and myristoylated PKI resulted from an apparent lack of cell permeability, we therefore cloned the sequence encoding PKI into pEGFPN1 in frame with GFP to generate pEGFPN1-PKI (encoding PKI-GFP). Similarly, we cloned a mutant regulatory subunit of PKA (RevAB, capable of binding and inhibiting cAMP-dependent

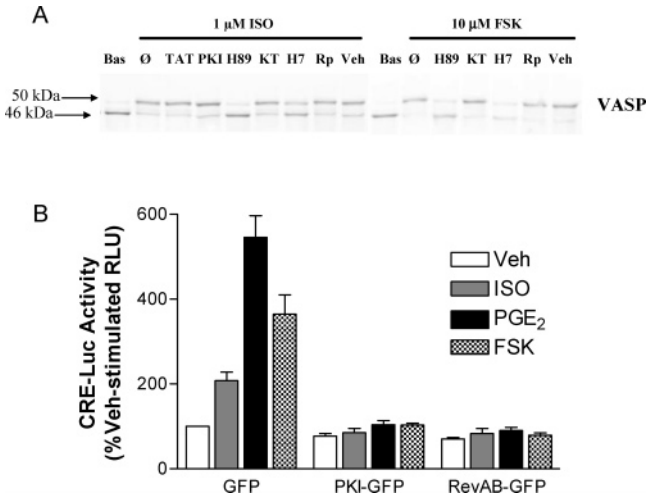

FIGURE 1: Effect of putative PKA inhibitors on PKA activity in human ASM cells. (A) Cells grown to confluence on 12 well plates and growth-arrested for 24 h were pretreated for 30 min with the following agents: no treatment (Ø); 1 μM TAT-PKI (TAT); 10 μM myristoylated PKI (mPKI); 10 μM H-89, 10 μM KT5720 (KT); 100 μM RpcAMP (Rp); or vehicle (0.1% DMSO). Either vehicle (0.01% EtOH), 1 μM ISO, or 10 μM FSK was then added to wells for 10 min. Media were aspirated, the cells washed once in cold PBS, and cell lysates harvested for immunoblot analysis. Immunoblots were probed for detection of VASP and visualized using the Odyssey Infrared Imaging System. Results did not vary with more extended pretreatment (up to 3 h) of cells with TAT, mPKI, KT, or Rp (not shown). (B) Cells were cotransfected with Δ56FosdE3XCRE-Luc and pEGFPN1-based constructs encoding GFP, PKI-GFP, or RevAB-GFP as described in Experimental Procedures. Twenty-four hours after transfection, cells were plated in 96 well plates, growth-arrested for 24 h, and then stimulated with vehicle, 1 μM ISO, 1 μM PGE_2, or 10 μM FSK for 8 h. Reactions were quenched directly in the well with a reaction mix containing firefly luciferase substrate as described in Experimental Procedures. Data represent mean ± SE values ($n = 4$).

activation of the catalytic subunit binding partners of PKA RI and RII regulatory subunits) (*33*) into pEGFPN1 to generate pEGFPN1-RevAB (encoding RevAB-GFP). These constructs were transfected into ASM cultures, and their expression was confirmed by direct visualization of GFP fluorescence and immunoblotting for GFP or the R subunit of PKA. Although efficacy in inhibiting PKA activity was suggested in analyses of CRE-Luc activity in which each construct was cotransfected with a CRE-Luc reporter (Figure 1B), transfection efficiency was typically too low to enable PKA inhibition in a sufficient percentage of cells (not shown). To overcome this limitation and enable a more comprehensive analysis of PKA-dependent effects in ASM, we sought to generate stable lines of ASM expressing PKI-GFP, RevAB-GFP, or GFP. We therefore generated retroviral constructs for expression of GFP, PKI-GFP, and RevAB-GFP. Virus was propagated in GP2-293 cells, and media containing virus were used to infect ASM cultures. Cultures infected with each of the viruses exhibited robust expression in the majority of cells within 2 days of infection, and near-homogeneous populations expressing each construct were established by selection within 7 days (Figure 2). In lines expressing PKI-GFP or RevAB-GFP, acute treatment with PGE_2 or FSK caused only a small percentage of VASP to shift relative to that observed for the GFP-expressing lines (Figure 3A), and agonist-stimulated CRE-Luc activity in these cells was abolished (not shown).

Effects of Stable PKI-GFP and RevAB-GFP Expression on β_2AR Responsiveness and Desensitization. Having established the capacity of PKI-GFP and RevAB-GFP to strongly inhibit PKA activity in ASM cells, we assessed β_2-AR responsiveness under multiple conditions in which PKA could function as a mediator of desensitization. Stable expression of PKI-GFP and RevAB-GFP had multiple, complex effects on β_2AR−adenylyl cyclase transmembrane signaling and its regulation. Under control (vehicle pretreatment) conditions, cAMP production induced by stimulation with 10 nM or 1 μM ISO treatment was significantly greater in PKI-GFP- and RevAB-GFP-expressing cells relative to that in the GFP group (Figure 3B,C). The effect of PKA inhibition in increasing cAMP accumulation was much more pronounced in cells stimulated with a low concentration of ISO (10 nM) with values in PKI-GFP- and RevAB-GFP-expressing cells being more than 2-fold greater than those in the matched GFP group. These data are consistent with analyses of recombinant β_2ARs in artificial expression systems that conclude that β-agonist-induced β_2AR desensitization that occurs at low levels of receptor occupancy is largely PKA-dependent (*41*, *42*). FSK-stimulated cAMP accumulation was also significantly greater (∼45−55%) in PKI-GFP- and RevAB-GFP-expressing cells, demonstrating upregulated activity of adenylyl cyclase. However, normalization of data to FSK-stimulated values (Figure 3D,E) reveals an increase in β_2AR responsiveness in PKI-GFP- and RevAB-GFP-expressing cells independent of any effect on adenylyl cyclase activity per se.

Thirty minutes pretreatment with 100 nM PGE_2 or 10 μM FSK caused significant β_2AR desensitization in the GFP lines, and this loss was significantly inhibited in cells expressing PKI-GFP or RevAB-GFP. The relevant loss of ISO-stimulated cAMP accumulation and the attenuating effects of PKI-GFP or RevAB-GFP were more prominent in cells stimulated with 10 nM ISO (Figure 3D,E). Similar results were obtained in experiments using cells transiently transfected with GFP, PKI-GFP, and RevAB-GFP then sorted to homogeneity (GFP-positive cells) by flow cytometry as described previously (*31*) (data not shown).

Pretreatment of cells for 18 h with IL-1β or IL-1β + TNF-α resulted in β_2AR desensitization that was attenuated in PKI-GFP- and RevAB-GFP-expressing cells (Figures 4 and 5). In ASM cells cultured in the absence of serum or growth factors, cytokines induce a sensitization of adenylyl cyclase activity that mitigates the loss of ISO-stimulated cAMP production (*22*). Expression of PKI-GFP or RevAB-GFP had minimal effect on this cytokine-promoted increase in FSK-stimulated cAMP production (which was observed in all lines), suggesting that PKA does not mediate adenylyl cyclase sensitization induced by cytokines (Figure 4C). When regulatory effects on adenylyl cyclase activity (conferred by PKI-GFP or RevAB-GFP expression or cytokine treatment) are accounted for by normalization of data to FSK-simulated cAMP production (Figures 4D,E and 5), attenuation of the loss of ISO-stimulated cAMP production is still apparent in PKI-GFP- and RevAB-GFP-expressing cells, suggesting that PKA modulates the β_2AR locus and β_2AR-G protein coupling in cytokine-mediated β_2AR desensitization.

Effects of PKI-GFP and RevAB-GFP on Other Putative PKA-Mediated Mechanisms of β_2AR Desensitization. Results from Figure 3 reflecting the effects of acute PKA activation

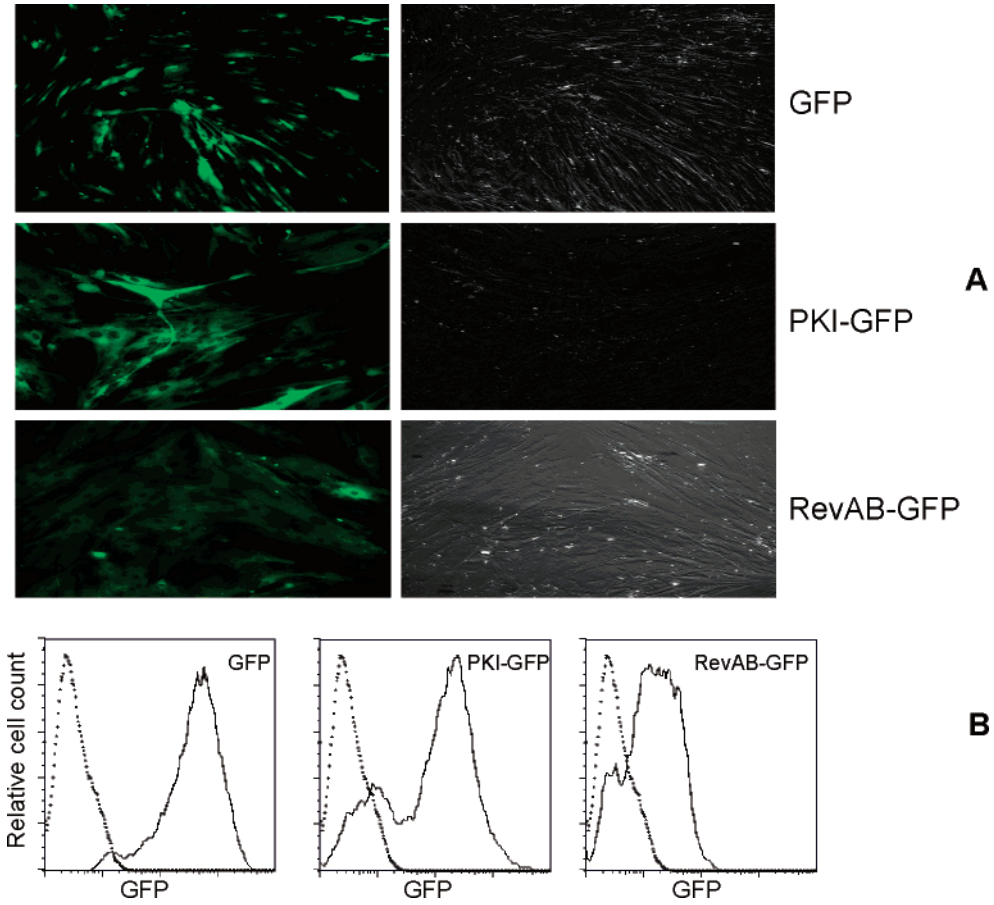

FIGURE 2: Stable expression of GFP, PKI-GFP, and RevAB-GFP in ASM cultures. GFP, PKI-GFP, and RevAB-GFP were introduced by retroviral infection as described in Experimental Procedures. Cells were selected using 250 μg/mL G418 for 7 days and visualized directly by fluorescent microscopy (A) or analyzed for cell fluorescence intensity by flow cytometry (B) as described previously for ASM cells (*31*). (A) Panels on left represent FITC images; panels on right represent Hoffman modulation light field images. (B) Dotted lines represent cellular autofluorescence in non-GFP expressing ASM lines.

suggest direct effects of PKA on the β_2AR contributing to its desensitization. Results from Figure 4 suggest a similar mechanism applies during chronic cytokine treatment which is associated with COX-2 and PGE_2 induction. An additional potential mechanism by which PKA could promote cytokine-induced β_2AR desensitization is via the regulation of cytokine-induced COX-2 and PGE_2 induction (thereby influencing the stimulus for its (PKA) activation). In HEK 293 cells expressing recombinant EP2 receptors, autocrine PGE_2 produced by COX-2 stimulates PKA activation, which in turn provides a (CREB-dependent) stimulus for further COX-2 induction (*43*, *44*). A similar feed-forward mechanism may exist in ASM given the inhibitory effects of indomethacin and CRE consensus site ablation on IL-1β-stimulated COX-2 reporter activity assessed in ASM cells (*45*, *46*). Analysis of the effects of PKI-GFP or RevAB-GFP expression demonstrates that inhibition of PKA causes an ~50% reduction in the induction of COX-2 caused by treatment with IL-1β or IL-1β + TNF-α and that this reduction is associated with similar reductions in culture supernatant levels of PGE_2 (Figure 6). Because PGE_2-mediated activation of PI3K via EP4 receptor activation has also been identified as an additional mechanism of feed-forward amplification of COX-2 induction (*43*, *44*), this potential mechanism was also examined. However, pretreatment of ASM cultures with the PI3K inhibitor wortmannin did not inhibit IL-1β- or IL-1β + TNF-α-induced COX-2 levels (data not shown), consistent with our previous findings suggesting minimal expression of EP4 receptors in ASM (*31*, *38*). These data suggest an EP2 receptor-mediated positive-feedback loop for PKA that contributes to effects of IL-1β or IL-1β + TNF-α on β_2AR responsiveness.

We next sought to examine whether a loss of whole cell β_2ARs (downregulation) is associated with cytokine-induced β_2AR desensitization, and the regulatory effect of PKA inhibition on this putative mechanism. Radioligand binding studies revealed that whole cell β_2AR density in the GFP lines (20 $\pm$ 3 fmol/mg protein) was unaffected by IL-1β treatment, with a small reduction (20 $\pm$ 4%, $p < 0.05$, $n = 4$) caused by treatment with IL-1β + TNF-α. Vehicle- and IL-1β-treated PKI-GFP and RevAB-GFP lines exhibited similar levels of β_2AR density relative to those determined for the GFP lines, but treatment with IL-1β + TNF-α had no significant effect (data not shown).

No differences in the levels of Gαs protein were observed among GFP-, PKI-GFP-, and RevAB-GFP l-expressing cells, nor did cytokine treatment alter these levels in any of the lines (data not shown). These findings suggest that PKI-GFP- and RevAB-GFP-dependent increases in β_2AR−Gs coupling

[2] Whereas human ASM βARs are represented almost exclusively by the β_2AR subtype (*35*, *47*), studies using β_1AR and β_2AR knockout mice (*48*) suggest that the β_1AR can contribute to the relaxant effect of β-agonists on murine ASM.

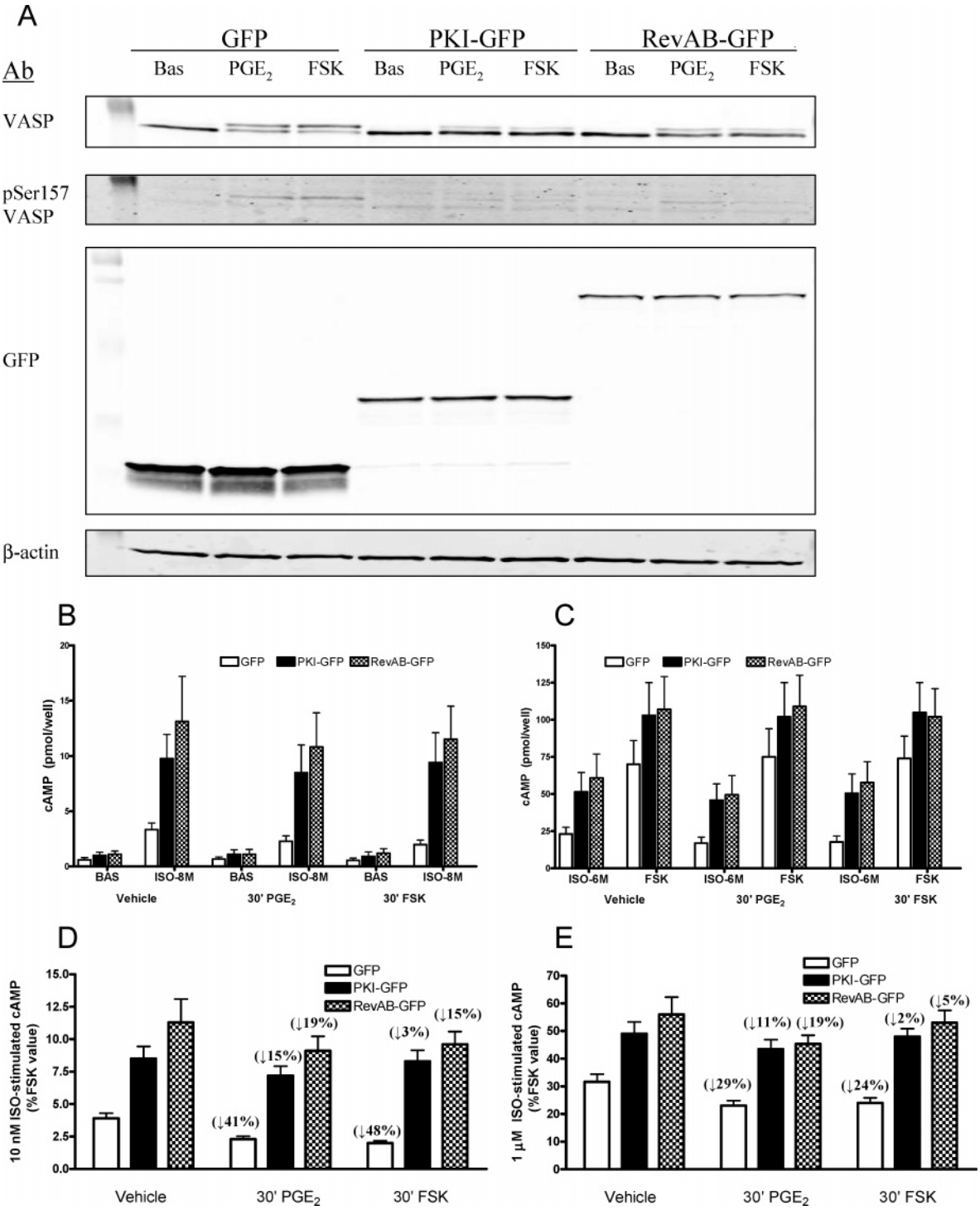

FIGURE 3: Effects of stable PKI-GFP and RevAB-GFP expression on acute heterologous desensitization of β_2ARs in ASM. ASM stably expressing GFP, PKI-GFP, or RevAB-GFP were grown to confluence in 10 cm (A) or 24 well plates (B−E), cultured an additional 24 h in serum-free media, then pretreated 30 min with vehicle, 100 nM PGE_2, or 10 μM FSK. Cells were washed extensively in cold PBS then challenged for 10 min with vehicle, 10 nM or 1 μM ISO, or 100 μM FSK. (A) Cell lysates were harvested and subject to immunoblot analysis of VASP, pSer157VASP, GFP, and β-actin. Levels of the ~50 kDa VASP species in PKI-GFP-expressing cells stimulated by PGE_2 or FSK were reduced 72 ± 6% and 84 ± 9%, respectively, relative to values in GFP-expressing cells, and by 82 ± 5% and 89 ± 3%, respectively, in RevAB-GFP-expressing cells (mean ± SE values, $n = 3$). (B and C) Absolute cAMP generation (pmol/well). (D and E) Values of 10 nM and 1 μM ISO-stimulated cAMP production, respectively, normalized to the value of FSK-stimulated cAMP production for that group. Data represent mean ± SE values ($n = 6$), generated using six separate sets of stable lines generated from six different ASM cell cultures.

indicated in Figure 4D,E are a result of modulation (decreased PKA-mediated phosphorylation) of the receptor locus.

Role of the EP2 Receptor in Cytokine-Mediated Desensitization of βAR Function in ASM. Given the lack of useful EP receptor ligands, we employed a genetic approach to examine the role of EP receptors in the functional consequences of βAR[2] desensitization induced by cytokines. Tracheae were excised from age-matched wild-type (EP2+/+) and EP2R knockout (EP-/-) mice, the surrounding

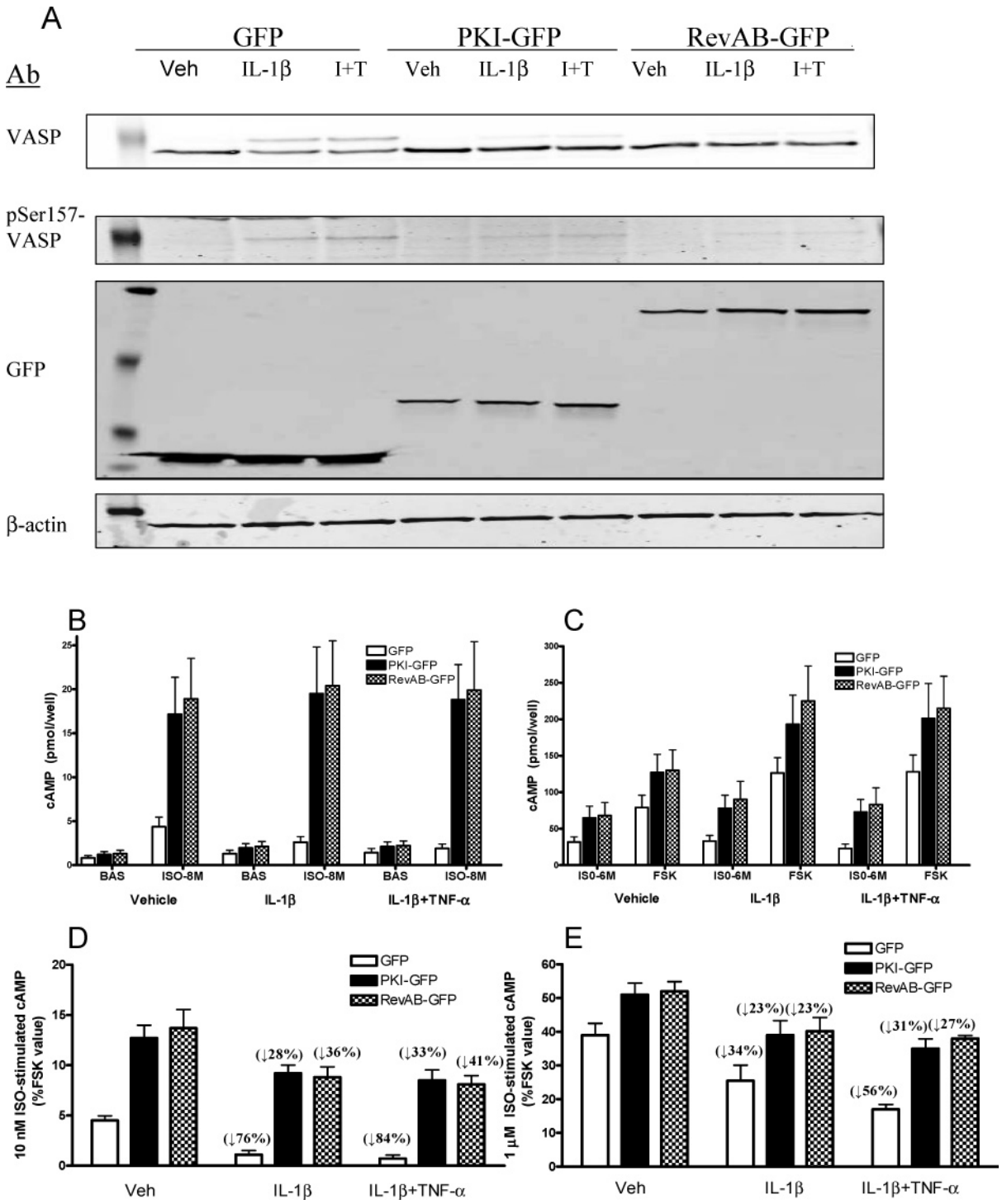

FIGURE 4: Effects of PKI-GFP and RevAB-GFP on cytokine-mediated regulation of PKA and β_2AR responsiveness in ASM. Cells stably expressing GFP, PKI-GFP, or RevAB-GFP were grown to confluence in 10 cm (A) or 24 well plates (B−E), cultured an additional 24 h in serum-free media, then treated 18 h with vehicle, IL-1β, or IL-1β + TNF-α. (A) Cell lysates were harvested and subjected to immunoblot analysis of VASP, pSer157VASP, GFP, and β-actin. Levels of the ~50 kDa VASP species in PKI-GFP-expressing cells stimulated by IL-1β or IL-1β + TNF-α were reduced 80 ± 3% and 81 ± 3%, respectively, relative to values in GFP-expressing cells, and by 80 ± 3% and 85 ± 4%, respectively, in RevAB-GFP-expressing cells (mean ± SE values, $n = 6$). (B and C) Cells were washed extensively, then challenged with the indicated agents for 10 min. (D and E) Values of 10 nM and 1 μM ISO-stimulated cAMP production, respectively, normalized to the value of FSK-stimulated cAMP production for that group. Data represent mean ± SE values ($n = 6$), generated using six separate sets of stable lines generated from six different ASM cell cultures.

connective tissue and epithelium were removed, and rings were treated in a 24 well dish for 18 h with vehicle, IL-1β, or IL-1β + TNF-α. Rings were then mounted in an organ bath system, and βAR-mediated relaxation of MCh-stimulated tension development was assessed. Methacholine-stimulated tension development was not significantly altered by cytokine treatment (see Figure 7 caption). In rings from EP2+/+ mice, treatment with IL-1β resulted in a significant loss of the maximal relaxant effect of ISO (vehicle-treated = 30% reduction in maximal tension development versus 15% in IL-1β-treated rings) (Figure 7A). Conversely, treatment of rings from EP2-/- mice with IL-1β resulted in only

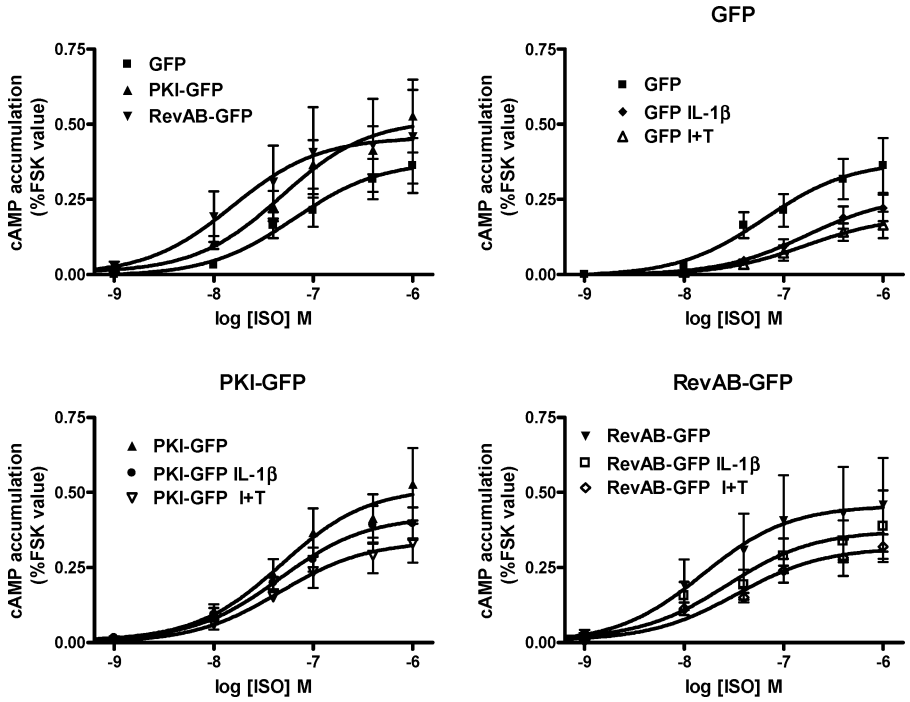

FIGURE 5: PKI-GFP and RevAB-GFP effects on dose-dependent response to ISO in cytokine-treated ASM. ASM stably expressing GFP, PKI-GFP, or RevAB-GFP were cultured and treated with vehicle or cytokines for 18 h as described in Figure 8B−E. Cells were washed and challenged with ISO at concentrations ranging from 10^{-9} to 10^{-6} M, or 100 μM FSK. Data presented are values normalized to the value of FSK-stimulated cAMP production for that group and represent mean ± SE values ($n = 4$). Calculated EC_{50} values (for vehicle-, IL-1β-, and IL-1β + TNF-α-treated cells): GFP (74, 117, 119 nM); PKI-GFP (44, 32, 52 nM); RevAB-GFP (26, 26, 37 nM).

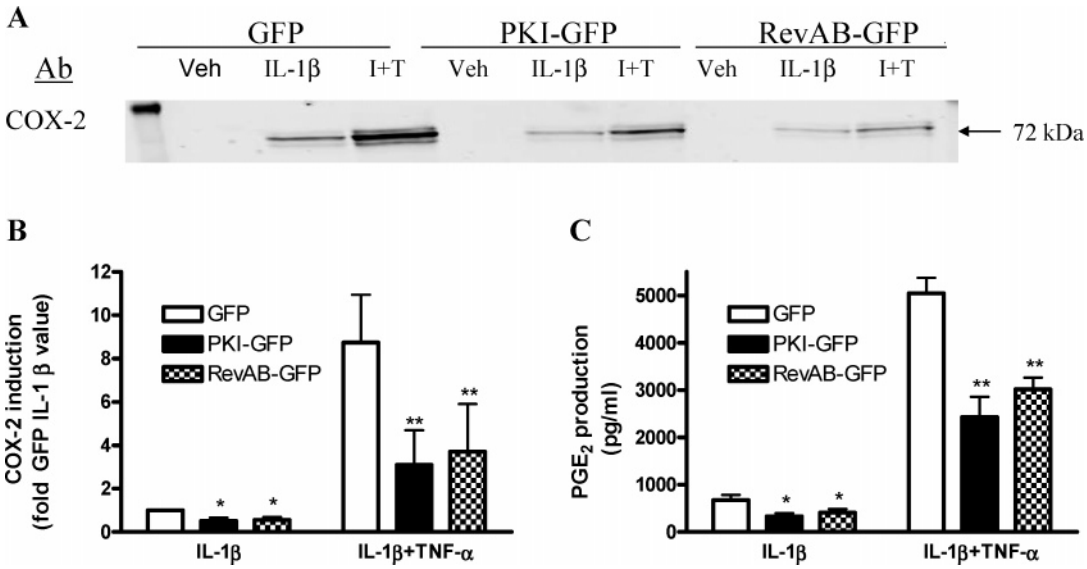

FIGURE 6: Effects of PKI-GFP and RevAB-GFP on cytokine-mediated COX-2 and PGE_2 induction. ASM stably expressing GFP, PKI-GFP, or RevAB-GFP were cultured and treated with vehicle or cytokines for 18 h. Supernatants were harvested for isolation and quantification of PGE_2, and cell lysates were generated for immunoblot analysis of COX-2 protein as described in Experimental Procedures. (A) Representative blot of regulation of COX-2 expression. (B) Band intensities reflecting COX-2 expression were directly quantified by the Odyssey Imaging system, and mean ± SE values from four independent experiments were reported. (C) PGE_2 production in culture supernatants in GFP-, PKI-GFP-, and RevAB-GFP-expressing cells following 18 h treatment with vehicle, IL-1β, or IL-1β + TNF-α (mean ± SE values, $n = 4$). *, $p < 0.05$, IL-1β treatment, GFP vs PKI-GFP or RevAB; **, $p < 0.05$, IL-1β + TNF-α treatment, GFP vs PKI-GFP or RevAB.

a slight loss of the ISO-mediated relaxant effect (vehicle-treated = 31% reduction versus 24% in IL-1β-treated rings). Relative to that observed with IL-1β treatment, treatment of rings from EP2+/+ mice with IL-1β + TNF-α resulted in a slightly greater loss of the ISO-mediated relaxant effect (maximal relaxation 28% in vehicle-treated versus 11% in IL-1β + TNF-α-treated) (Figure 7B). Although IL-1β + TNF-α-treated rings from EP2-/- mice also exhibited a loss of ISO-mediated relaxation (maximal relaxation 29% in vehicle-treated versus 20% in IL-1β + TNF-α-treated), the loss was less pronounced than that observed in the matched EP2+/+ mice. In tracheae from EP2+/+ mice, 18 h treatment with IL-1β resulted in a significant but not complete loss of PGE_2-mediated relaxation of MCh-contracted rings (Figure 7C), perhaps reflecting the relative resistance of EP2 receptors to agonist-specific desensitization

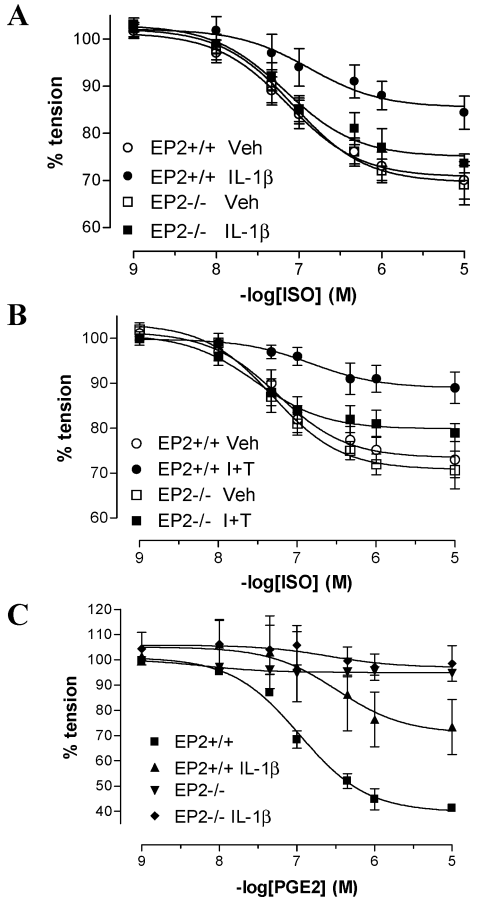

FIGURE 7: Effects of cytokine treatment on ISO-mediated relaxation of tracheal rings from EP2 receptor knockout and wild-type mice. (A−D) Tracheae were excised from EP2 receptor knockout (EP2 -/-) and age-matched wild-type (EP2 +/+) mice and treated 18 h with vehicle, IL-1β, or IL-1β + TNF-α as described in Experimental Procedures. Tension generation was assessed in rings contracted with 10 μM MCh followed by addition of increasing concentrations (1 nM to 10 μM) of ISO (A and B) or PGE_2 (C). Mean maximal MCh-induced tension development (mg tension/mg tissue) did not differ among groups: EP2+/+ (vehicle, IL-1β, IL-1β + TNF-α-treated), 1830 ± 300, 1560 ± 140, 1650 ± 100; EP2-/- (vehicle, IL-1β, IL-1β + TNF-α-treated), 1930 ± 190, 1660 ± 210, 1870 ± 250. Calculated mean IC_{50} values for vehicle treated groups: EP2+/+ (panel A data, 140 nM; panel B data, 170 nM); EP2-/- (panel A data, 130 nM; panel B data, 200 nM). IC_{50} values for IL-1β- and IL-1β + TNF-α-treated groups could not be accurately determined due to poor goodness of fit. Data represent mean ± SE values ($n = 8$).

(*31*). Relaxation elicited by 100 μM FSK ranged from 77 to 83% and did not differ as a function of genotype or treatment condition (data not shown).

DISCUSSION

Results from the present study provide a novel insight into the role of PKA in mediating both agonist-specific β_2AR desensitization, as well as heterologous desensitization that occurs with cytokine treatment, in physiologically relevant primary cells. Multiple elements of PKA-dependent regulation of the β_2AR are identified using a combination of molecular and genetic approaches to target either PKA, its upstream activators, or downstream effectors.

To date, the β_2AR is the only Gs-coupled receptor whose role in asthma has received significant attention, and the preponderance of evidence suggests that β_2ARs on ASM are most responsible for the effect of β-agonists on airway tone (*49*). Whether β_2AR dysfunction, and specifically β_2AR dysfunction in ASM, plays a prominent role in asthma has been a hotly debated topic for over 30 years. Asthma triggers, such as viral infections, can diminish β_2AR function (*50*), and numerous animal models of airway inflammation, ex vivo analyses of ASM strips treated with cytokines or asthmatic serum (*8*−*10*, *51*), and limited data from ASM tissue from severe asthmatics (*52*, *53*) have all provided evidence that β_2AR-mediated relaxant effect and signaling are depressed in asthma. Examining the specific effect of cytokines, studies employing in vivo (*51*), ex vivo (*9*), and cell culture models (*5*, *6*) of ASM contraction have reported that chronic treatment with IL-1β diminishes the ability of β-agonists to reduce cholinergic-induced increases in lung resistance, smooth muscle strip tension development, or cell stiffness.

The mechanisms by which such β_2AR hyporesponsiveness occurs have not been established. Using cell-free systems (*54*), human astrocytoma 1321N1 (*55*), and S49 lymphoma (*56*) cells, early studies examining mechanisms of β_2AR desensitization demonstrated that PKA has the capacity to phosphorylate β_2ARs and that this phosphorylation inhibits coupling of the β_2AR with the heterotrimeric G protein subunit Gαs, resulting in a reduction in cAMP generation via the β_2AR−Gαs-adenylyl cyclase pathway. Short-term pretreatment of cells with nonhydrolyzable cAMP or cAMP-generating agents such as PGE_2 or FSK resulted in reduced levels of β-agonist-stimulated adenylyl cyclase activity in plasma membrane preparations and in cellular cAMP generation when cells were subsequently challenged with β-agonist (*55*, *57*, *58*). Similarly, low concentrations of β-agonists, resulting in a low percentage of receptor occupancy yet significant PKA activation, resulted in β_2AR desensitization largely attributable to PKA (*59*−*61*). Selective inhibition of GRK2 or PKA employing an antisense strategy suggests that the role of PKA in agonist-specific desensitization of the β_2AR can be significant in certain cell types (*62*).

Although the above mentioned studies clearly demonstrate the capacity of PKA to promote heterologous desensitization of the β_2AR, directly establishing the role of PKA in primary cell types has been problematic, given the difficulty in employing genetic and molecular approaches in these cells. Although pharmacologic agents known to inhibit PKA activity in vitro are frequently used as a means to inhibit PKA activity in intact cells, effective inhibition cannot be assumed and will depend on numerous factors including the permeability of the inhibitor and the relative stoichiometry of inhibitor, PKA, and any competing (nonspecific) target enzymes (*29*). Results from the present study demonstrate the limited utility of pharmacologic approaches for PKA inhibition in ASM cells, prompting the development of alternative, molecular approaches. PKA inhibition by expression of either PKI-GFP or RevAB-GFP was evidenced by inhibition of agonist- or cytokine-induced phosphorylation of the intracellular PKA substrate VASP and was associated with significant inhibition of the loss of β_2AR responsiveness conferred by acute PGE_2 or FSK pretreatment as well as chronic IL-1β or IL-1β + TNF-α treatment. Interestingly, under control (vehicle pretreatment) conditions, ISO-stimulated cAMP production was also significantly higher in the PKI-GFP and RevAB-GFP lines compared to that in

the GFP lines, suggesting that PKA, in addition to GRKs, suppresses β-agonist-stimulated cAMP accumulation (during a 10 min challenge in our protocol) and thereby contributes to agonist-specific β_2AR desensitization in ASM that occurs during challenge. Such a prominent role for PKA could possibly reflect low levels of GRKs or arrestins, which could limit their role in agonist-specific β_2AR desensitization in ASM (*31*, *63*).

Despite a clear effect on β_2AR desensitization, PKI-GFP and RevAB-GFP expression in ASM did not fully reverse the loss of ISO-stimulated cAMP production induced by cytokine treatment. The most likely explanation is that we were unable to achieve full inhibition of PKA in all cells in the culture, suggested by the inability to completely inhibit the cytokine-induced VASP shift. The lack of complete inhibition may result from heterogeneity of expression in cells in culture or a competitive, as opposed to dominant-negative, nature of the inhibitory effect of these constructs.

To clarify the role of PGE_2 and EP receptor subtypes in mediating the effects of cytokines on βAR desensitization and functional consequences in ASM, we employed a genetic approach. We previously utilized transgenic knockout mice to establish that the Gs-coupled EP2 receptor is the predominant EP receptor subtype mediating acute effects of PGE_2 treatment on ASM contraction (*38*). In the present study, we observed a significant loss of the β-agonist-mediated relaxant effect following chronic treatment of rings from EP2+/+ mice with IL-1β or IL-1β + TNF-α. In rings from EP2-/- mice, IL-1β treatment caused only a small loss of the relaxant effect of ISO. Treatment with IL-1β + TNF-α caused a greater loss of ISO-mediated relaxation in rings from EP2+/+ mice, and this effect was again attenuated in rings from EP2-/- mice. This residual desensitization observed in EP2-/- mice suggests a role for another GPCR (e.g., EP4 or IP prostanoid receptor) activated by a COX-2 product whose induction is significant when COX-2 is robustly induced by IL-1β + TNF-α.

Although our data suggest a prominent role of PKA-mediated phosphorylation and uncoupling of the β_2AR in mediating cytokine effects on β_2AR responsiveness, other PKA-dependent mechanisms, acting upstream or in concert with this mechanism, likely contribute to the full effect of cytokines in promoting β_2AR desensitization. COX-2 itself is an important PKA target, as COX-2/PGE_2-dependent PKA activation participates in a positive feedback loop via EP2 receptor/PKA-mediated augmentation of COX-2 induction. A small degree of β_2AR downregulation, possibly mediated by a PKA-mediated inhibition of β_2AR gene transcription (*64*), is also associated with the loss of β_2AR responsiveness. Although such downregulation could be redundant in light of the loss of β_2AR coupling, it would predictably affect the rate of recovery from desensitization (*1*).

An effect of PKA not enhanced by cytokine treatment per se is an apparent inhibition of adenylyl cyclase activity. The capacity of PKA to inhibit the adenylyl cyclase isoforms V and VI via modification by phosphorylation has been previously reported in other systems (*65*−*68*) and is consistent with the increased FSK-stimulated cAMP production observed in PKI-GFP- and RevAB-expressing lines. Under physiologic conditions, this effect would serve to complement the quenching effects of PKA- and GRK-mediated desensitization at the receptor locus.

Additional targets of PKA not examined in the current study are phosphodiesterases (PDEs). PDE4 isoforms are known to be phosphorylated and activated by PKA (*69*). Ablation of the PDE45D gene has a profound influence on ASM contractile state (*70*), and PDE4D5 expression can be upregulated in human ASM via a cAMP-dependent mechanism (*71*). Our preliminary studies demonstrate that inclusion of inhibitor RO-20-1724 increases (10 min) ISO-stimulated cAMP accumulation 2−3-fold in GFP-expressing ASM cells. Interestingly, the effect of PDE inhibition is not as great in PKI-GFP and RevAB cells (data not shown), suggesting an important role for PKA-activated PDE activity in physiologic regulation of β_2AR function in ASM. Future studies will clarify this contribution of PKA-regulated PDE4 activity under conditions promoting homologous and heterologous β_2AR desensitization.

In summary, we have employed molecular and genetic approaches to establish roles for PKA and EP2 receptors in the desensitization of β-agonist-mediated signaling and function in ASM. Effective inhibition of PKA results in attenuation of both agonist-specific as well as cytokine-induced heterologous desensitization of the β_2AR in ASM cells, through modulation of multiple targets, β_2AR, adenylyl cyclase, and COX-2. Ablation of the EP2 receptor significantly inhibits the loss of β-agonist-mediated ASM relaxation induced by cytokine treatment. These findings suggest mechanisms contributing to effects of airway inflammation on ASM β_2AR responsiveness, and that selective inhibition of these mechanisms may impact asthma pathogenesis or the effectiveness of β-agonist therapy.

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