Prolonged heterologous β2-adrenoceptor desensitization promotes proasthmatic airway smooth muscle function via PKA/ERK1/2-mediated phosphodiesterase-4 induction

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Hu A, Nino G, Grunstein JS, Fatma S, Grunstein MM. Prolonged heterologous β2-adrenoceptor desensitization promotes proasthmatic airway smooth muscle function via PKA/ERK1/2-mediated phosphodiesterase-4 induction. Am J Physiol Lung Cell Mol Physiol 294: L1055–L1067, 2008. First published March 21, 2008; doi:10.1152/ajplung.00021.2008.—β2-Adrenergic receptor (β2AR) agonists acutely relieve bronchospasm via cAMP-mediated relaxation of airway smooth muscle (ASM). Airway constrictor responsiveness may be significantly heightened, however, following protracted exposure to these agents, presumably reflecting the effects of β2AR desensitization in ASM accompanying prolonged cAMP signaling. Because cAMP phosphodiesterase (PDE) activity can significantly modulate ASM contractility, we investigated the mechanism regulating PDE expression and its potential role in mediating changes in agonist-induced constrictor and relaxation responsiveness in ASM following its heterologous β2AR desensitization by prolonged exposure to cAMP-elevating agents. Isolated rabbit ASM tissues and cultured human ASM cells treated for 24 h with the receptor- or nonreceptor-coupled cAMP-stimulating agent, prostaglandin E₂ (PGE₂) or forskolin, respectively, exhibited constrictor hyperresponsiveness to acetylcholine and impaired β2AR-mediated relaxation and cAMP accumulation. These proasthmatic-like changes in ASM function were associated with upregulated PDE4 activity, reflective of increased transcription of the PDE4D5 isomeric, and were prevented by pretreatment of the ASM with a PDE4 inhibitor. Extended studies using gene silencing and pharmacological approaches to inhibit specific intracellular signaling molecules demonstrated that the mechanism underlying PGE₂-induced transcriptional upregulation of PDE4D5 involves PKA-dependent activation of Gₛ protein signaling via the β3-subunits, the latter eliciting downstream activation of ERK1/2 and its consequent induction of PDE4D5 transcription. Collectively, these findings identify that β2AR desensitization in ASM following prolonged exposure to cAMP-elevating agents is associated with proasthmatic-like changes in ASM responsiveness that are mediated by upregulated PDE4 expression induced by activated cross talk between the PKA and ERK1/2 signaling pathways.

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signaling exhibited in ASM under conditions of prolonged homologous or heterologous β2AR desensitization.

Among the PDE superfamily, isoforms of PDE4 account for most of the cAMP hydrolyzing activity in smooth muscle cells, and PDE4 activity has been importantly implicated in regulating ASM contractility (37). The PDE4 family is encoded by four distinct genes (PDE4A–D) that generate multiple PDE4 enzyme variants through activation of different promoters or alternative splicing (9, 24). The dominant PDE4 type expressed in both vascular smooth muscle and ASM cells is PDE4D, and, via alternate promoters, PDE4D can encode six “long” isoforms (PDE4D3–5 and PDE4D7–9) and two PDE4D “short” isoforms (PDE4D1–2) (9, 24). Cellular expression of PDE4D is regulated by PKA, and the promoter driving transcription of the functionally dominant long isoform, PDE4D5, in ASM cells contains a cAMP response element (CRE) (5, 32). In light of this, the present study was undertaken to identify the mechanism regulating PDE4 expression and its role in mediating the changes in ASM constrictor and relaxation responsiveness associated with long-term heterologous β2AR desensitization. The results provide new evidence demonstrating that 1) desensitization of ASM to β2AR agonists, resulting from its prolonged exposure to either receptor- or nonreceptor-coupled cAMP elevating agents, evokes increased ASM constrictor responsiveness together with impaired β2AR-mediated ASM relaxation and attenuated cAMP accumulation; 2) these proatherosclerotic-like changes in ASM function are associated with ERK1/2/activation and its induced transcriptional upregulation of PDE4D5 expression via phosphorylation of the CREB and ATF1 transcription factors; and 3) the activation of ERK1/2 is attributed to PKA-dependent activation of G1 protein signaling via its βγ-subunits, which leads to downstream activation of the Ras/Raf1/MEK pathway in the β2AR-desensitized state. Collectively, these findings are the first to identify a mechanism that potentially underlies the association between airway tolerance to β2AR agonists and heightened expression of the asthmatic phenotype accompanying prolonged exposure of the airways to cAMP-elevating agents.

**MATERIALS AND METHODS**

**Materials.** All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. The human ASM cells were obtained from BioWhittaker.

**Animals.** Nineteen adult New Zealand White rabbits were used in this study, which was approved by the Biosafety and Animal Research Committee of the Joseph Stokes Research Institute at Children’s Hospital of Philadelphia. The animals had no signs of respiratory disease for several weeks before the study, and their cure and use were in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council.

**Culture and treatment of AS M cells.** Human ASM cells were grown in SmBm media supplemented with 10% FBS (BioWhittaker) and maintained throughout in a humidified incubator containing 5% CO2 in air at 37°C. The experimental protocols involved growing the cells to ~95% confluence in the above medium. Thereafter, in separate experiments, the cells were starved in unsupplemented Ham’s F-12 media for 24 h, treated with different concentrations and with varying durations with prostaglandin E2 (PGE2) or forskolin, and then examined for induced changes in PDE activity, ERK1/2 and CREB/ATF1 phosphorylation, and PDE4D5 mRNA expression in the absence and presence of specific inhibitors, as described.

**Assay of cAMP accumulation.** In initial studies, intracellular cAMP levels were determined in near confluent ASM cell cultures at varying times following treatment of ASM cells with varying concentrations of isoproterenol (0.1–100 µM). In all subsequent experiments, cells were pretreated for 24 h with either vehicle alone or PGE2 (100 µM), both in the absence and presence of rolipram (10 µM). Thereafter, in the continued presence of these treatments, cells were exposed for 5 min to isoproterenol (10 µM) at 37°C, and intracellular cAMP accumulation was quantified by radioimmunoassay, as previously described (21). The cAMP measurements were expressed in units of picomoles per milligram of tissue membrane protein.

**Assay of cAMP PDE activity.** Levels of cAMP PDE activity were measured in ASM cell lysates using a colorimetric, nonradioactive enzymatic assay kit from Biomol per the manufacturer’s protocol. PDE activity was determined following exposure of ASM cells for 24 h to either vehicle alone (control) or PGE2 (100 µM) or forskolin (100 µM) in the absence and presence of pretreatment with rolipram (10 µM), cycloheximide (100 µM), actinomycin D (4 µM), or H89 (10 µM). The measured levels of PDE activity were standardized to protein content in the cell extracts.

**Detection of PDE4D5 mRNA transcripts.** Total RNA was extracted from the cultured ASM cells using the TRIzol method (Invitrogen), and cDNAs were isolated by RT-PCR using the SuperScript First Strand Synthesis System kit from Invitrogen, with the following oligonucleotide primer sets (Integrated DNA Technologies): for PDE4D5, 5'-TGGCACGTGTACAAAGTTGACCA-3' (forward) and 5'-TCTCCAGAGATGACTTGAGATT-3' (reverse); and for β-actin, 5'-GAGAAGACGTCGAGCTTCCTGAGCA-3' (forward) and 5'-CGGAGCTTGGCCTAGGAGGAGG-3' (reverse). The reaction volume was 20 µl, and cycling conditions used were 35 cycles of 30-s denaturation at 95°C, followed by 30-s annealing at 60°C and elongation at 72°C for 30 s. Ex-Tag (Takara Biotechnology) was used as DNA polymerase.

**Immunoblot analysis of CREB/ATF1 and ERK1/2 activation.** Levels of phosphorylated CREB, ATF1, and ERK1/2 protein were detected by Western blot analysis of lysates isolated from ASM cells before and at various times after treatment with PGE2 in the absence and presence of specific inhibitors, as described. Following protein extraction and the addition of gel-loading buffer, the extracts were loaded on a 10% SDS-PAGE gel for immunoblotting after transfer to a polyvinylidene difluoride membrane. The membranes were then incubated overnight with monoclonal mouse anti-human primary antibodies directed against phospho-CREB and -ATF1 or phospho-ERK1/2, ERK2, or α-actin, and levels were detected by enhanced chemiluminescence after a 1-h incubation with a 1:2,000 dilution of horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody, followed by exposure to autoradiography film. The protein band intensities were quantified by densitometry.

**siRNA-mediated knockdown of PKA.** ASM cells were seeded into six-well plates, and, at ~40% confluency, the medium was replaced with the reduced serum-containing medium, Opti-MEM (Invitrogen). The cells were then transfected twice during a 24-h interval with two pools of three siRNA duplexes, each pool targeted against the human PKA catalytic subunits (Santa Cruz Biotechnology; sc-36240 and sc-36236, respectively) or with a nontargeted control (scrambled) siRNA duplex, using Oligofectamine (Invitrogen) as the transfection agent. The pools of siRNAs were applied to each well at 100 nM each for siRNA preparation. On the basis of preliminary studies, this double-transfection approach was associated with high transfection efficiency and, as detected by Western blot analysis, markedly inhibited PKAα expression by the targeted siRNA duplexes, with maximal inhibition detected at 72 h following siRNA transfection.
Transfection of ASM cells with adenovirus-βARK-ct. Adenovirus (adeno)-βARK-ct, an adenovirus vector encoding the βARK1 carboxy-terminal domain that blocks Gβγ signaling (27, 28), and adenovirus-β-gal, an adenovirus vector expressing lacZ as a negative control, were constructed using the AdenoX adenovirus construction kit (BD-Clontech). Recombinant plaques were isolated and propagated in HEK293 cells (Invitrogen) with viral purification using the cesium chloride gradient method, and viral titer was detected by plaque assay. The ASM cells were transfected with one of the adenoviral vectors at a multiplicity of infection (MOI) of 100, and experiments were conducted at 24 h following adenoviral transfections.

Pharmacodynamic studies of constrictor and relaxation responsiveness in rabbit ASM tissues. Following initial sedation and subsequent general anesthesia with intramuscular injections of xylazine (10 mg/kg) and ketamine (50 mg/kg), respectively, rabbits were killed with an intravenously administered overdose of pentobarbital sodium (100 mg/kg). As described previously (18), the tracheae were excised via open thoracotomy, the loose connective tissue and epithelium were scraped and removed, and the tracheae were divided into eight-ring segments, each of 6–8 mm in length. The airway segments were then placed in modified Krebs-Ringer solution containing indo-methacin (10 μM), and each alternate ring was incubated for 24 h at room temperature in the presence of either vehicle alone (control) or varying concentrations of PGE2, both in the absence and presence of either rolipram (10 μM), the PKA inhibitor, H89 (10 μM), the MEK-ERK1/2 inhibitors, U0126 (5 μM), or pertussis toxin (100 ng/ml). Thereafter, the tissues were promptly placed into organ baths containing the same concentrations of their respective pharmacological treatments in modified Krebs-Ringer solution and aerated with 5% CO2 in oxygen (pH 7.35–7.40). The tissues were then attached to force transducers to continuously monitor isometric tension, and their cholinergic contractility was assessed in response to cumulative administration of acetylcholine (ACh) in final bath concentrations ranging from 10^-5 to 10^-3 M. The tissues were then rinsed three to four times over ~15 min with fresh buffer, the pharmacological agents were immediately reintroduced into the organ baths, and, ~20 min thereafter, relaxation dose-response curves to isoproterenol (10^-8–10^-4 M) were generated after the tissues were half-maximally contracted with their respective ED50 doses of ACh. The constrictor and relaxation dose-response curves were analyzed with respect to each tissue’s maximal isometric contractile force (Tmax) to ACh and maximal relaxation response (Rmax) to isoproterenol from the initial level of active cholinergic contraction.

Statistical analyses. Results are expressed as mean ± SE values. Comparisons between groups were made with Student’s t-test (two-tailed) or ANOVA with Tukey’s posttest analysis, where appropriate. A probability of <0.05 was considered statistically significant. Statistical analyses were conducted using the Prism computer program by GraphPad software.

RESULTS

Role of PDE4 in regulating constrictor and relaxation responsiveness in β2AR-desensitized ASM. To assess the role of PDE4 in mediating the effects of heterologous β2AR desensitization on ASM function, constrictor responses to ACh and relaxation responses to isoproterenol, which acts via β2AR activation in ASM, were compared in isolated rabbit ASM tissues exposed for 24 h to either vehicle alone (control) or to a predetermined maximally effective concentration of PGE2 (100 nM), both in the absence and the presence of pretreatment of the tissues with the PDE4-selective inhibitor, rolipram (10 μM). Relative to their respective vehicle-treated controls, ASM tissues exposed to PGE2 exhibited significantly increased constrictor responsiveness to exogenously administered ACh (Fig. 1A), yielding a mean ± SE maximal constrictor response (Tmax) value of 119.3 ± 5.9 g/g ASM wt vs. the value of 101.2 ± 6.1 g/g ASM wt obtained in the control tissues (P < 0.05). This enhanced constrictor responsiveness to ACh was completely abrogated in PGE2-exposed tissues that were pretreated with rolipram. Under the same treatment conditions, during subsequent sustained half-maximal contraction of the tissues with ACh, administration of isoproterenol produced cumulative dose-dependent relaxation of the preconstricted ASM segments. Relative to control tissues, the relaxation responses to isoproterenol were significantly attenuated in the PGE2-exposed ASM segments (Fig. 1B), consistent with their development of heterologous β2AR desensitization. Accordingly, the mean ± SE maximal relaxation (Rmax) response in the PGE2-exposed tissues amounted to 31.4 ± 5.4% vs. the average Rmax value of 51.5 ± 5.5% obtained in the control ASM segments (P < 0.01). This impaired relaxant responsiveness to isoproterenol was also completely ablated in PGE2-exposed tissues that were pretreated with rolipram. As further
depicted in Fig. 1, relative to the control preparations, ASM tissues that were treated with rolipram alone showed somewhat decreased constrictor responses to ACh and increased relaxation responses to isoproterenol, but neither of these changes attained statistical significance.

As with PGE$_2$, heterologous β2AR desensitization conferred by prolonged exposure to forskolin (100 μM × 24 h), an agent that stimulates cAMP accumulation via direct (nonreceptor mediated) activation of adenylyl cyclase, also evoked significant increases in ASM constrictor responsiveness to ACh and impaired relaxation responsiveness to isoproterenol. Accordingly, the $T_{\text{max}}$ values for ACh in the forskolin vs. control tissues averaged 115.4 ± 4.9 vs. 97.5 ± 5.4 g/g ASM wt, respectively ($P < 0.05$), and the corresponding $R_{\text{max}}$ values averaged 34.3 ± 5.2 vs. 47.8 ± 6.1%, respectively ($P < 0.01$). Moreover, these effects were also completely abrogated in forskolin-exposed ASM tissues that were pretreated with rolipram, yielding mean $T_{\text{max}}$ and $R_{\text{max}}$ values of 104.2 ± 7.4 g/g ASM wt and 52.4 ± 6.3%, respectively, which were not significantly different from those obtained in the control tissues.

**Role of PDE4 in regulating altered cAMP responses in β2AR-desensitized ASM cells.** We next examined whether the changes in ASM constrictor and relaxation responsiveness obtained in the β2AR-desensitized state are reflective of rolipram-sensitive changes in β2AR agonist-induced cAMP accumulation. In these studies, acute changes in intracellular cAMP accumulation detected at 5 min following administration of a near half-maximal effective concentration of isoproterenol (1.0 μM) were compared in confluent cultures of ASM cells that were pretreated for 24 h either with vehicle alone or PGE$_2$ (100 nM), both in the absence and the presence of pretreatment with rolipram (10 μM). As shown in Fig. 2, in the absence of rolipram, the PGE$_2$-exposed ASM cells exhibited heterologous β2AR desensitization, as evidenced by significantly reduced cAMP responses to isoproterenol relative to those detected in cells that were not exposed to the prostanoid. In contrast to these observations, ASM cells pretreated with rolipram exhibited increased isoproterenol-induced cAMP accumulation, and this response was preserved in the PGE$_2$-exposed cells. Thus, in concert with the results obtained in rabbit ASM tissues, these observations implicate PDE4 activity in mediation of the impaired isoproterenol-induced accumulation of cAMP accompanying heterologous β2AR desensitization in human ASM cells.

**Regulation of cAMP PDE activity in β2AR-desensitized ASM cells.** To elucidate the mechanism underlying the above rolipram-sensitive changes in ASM function, total cAMP PDE activity and the effects of selective inhibitors on this activity were examined in cultured human ASM cells following their prolonged exposure to PGE$_2$ or forskolin. Relative to the mean (±SE) basal level of PDE activity detected in vehicle-treated (control) cells (i.e., 69.5 ± 4.8 pmol·min$^{-1}$·mg protein$^{-1}$), ASM cells incubated for 24 h with 100 μM PGE$_2$ or forskolin exhibited significantly increased levels of PDE activity that averaged 3.74- and 4.01-fold above control, respectively (Fig. 3A). These stimulatory effects on PDE activity were completely abrogated in PGE$_2$- and forskolin-exposed cells that were pretreated with rolipram (10 μM), implying that the augmented PDE activity exhibited by the β2AR-desensitized cells was attributed to PDE4. In separate experiments, inclusion of the transcriptional inhibitor actinomycin D (4 μM) or the protein synthesis inhibitor cycloheximide (100 μM) in the ASM culture medium also completely ablated both the PGE$_2$- and forskolin-induced increases in PDE activity (Fig. 3B), whereas neither actinomycin D nor cycloheximide alone significantly affected basal PDE activity (data not shown). Finally, extended studies demonstrated that the stimulatory effects of PGE$_2$ and forskolin on PDE activity were also abrogated by coincubation of the ASM cells with the putative selective PKA inhibitor H89 (10 μM), whereas cells treated with H89 alone showed no significant change in basal PDE activity (Fig. 3C). Taken together, these data are consistent with the notion that heterologous β2AR desensitization in ASM cells, resulting from prolonged exposure to PGE$_2$ or forskolin, evokes upregulated PDE4 activity that is due to PKA-dependent de novo mRNA and protein synthesis.

**Role of PKA in regulating PDE4D5 expression in PGE$_2$-exposed ASM cells.** Given recent evidence demonstrating that PDE4D5 is the functionally dominant cAMP-regulating PDE4 isofrom in human ASM cells (5), we next examined whether heterologous β2AR desensitization evokes altered expression of PDE4D5 transcripts. Confluent cultures of ASM cells exposed to PGE$_2$ (100 nM × 24 h) exhibited time-dependent increases in PDE4D5 mRNA expression, with peak induction detected at 6 h and sustained upregulated expression observed for up to 24 h (Fig. 4A). Densitometric analysis of the temporal changes in PDE4D5 mRNA expression examined in three separate experiments demonstrated that peak expression averaged 5.9-fold above that detected in unstimulated cells. Because PDE4D5 expression in ASM cells is regulated by a CRE-containing promoter (32), we subsequently examined the effects of PGE$_2$ on cAMP/PKA-dependent downstream signaling events coupled to CRE activation. As shown in Fig. 4B, ASM cells treated with PGE$_2$ (100 nM) exhibited transiently increased phosphorylation of the CRE-binding transcription co-factors CREB and ATF1, which peaked at 30 min and was subsequently ablated by 180 min. To determine the role of PKA in mediating the latter response to PGE$_2$, we next assessed the effects of knockdown of PKA expression using siRNA duplexes directed against the human PKAα and PKAγ catalytic subunits. Preliminary experiments (n = 3) demon-
strated that Lipofectamine transfection of the PKA siRNA duplexes produced maximal knockdown of PKA protein levels at 72 h posttransfection that ranged between 70 and 90%, as exemplified in Fig. 4C. Accordingly, confluent cultures of ASM cells were initially treated for 72 h with either vehicle alone, a scrambled siRNA sequence serving as control, or the siRNA duplexes directed against the PKA catalytic subunits. Cells were then examined for induced expression of phosphorylated CREB/ATF1 proteins at 30 min following exposure to PGE2 (100 nM). As depicted in Fig. 4D, in the absence of PGE2, basal levels of phosphorylated CREB/ATF1 protein expression were not much affected by transfection with either the scrambled RNA (scRNA; Fig. 4D, lane 3) or PKA siRNA (Fig. 4D, lane 5) preparations. Conversely, the PGE2-induced upregulated expression of phosphorylated CREB/ATF1 (Fig. 4D, lane 2) was distinctly inhibited in cells transfected with siRNAs directed against PKA (Fig. 4D, lane 6), whereas the scrambled siRNA sequence had no effect (Fig. 4D, lane 4). Thus, in concert with above observations implicating PKA activation in mediating the upregulated PDE4 activity in ASM cells exposed to PGE2 and forskolin. Relative to vehicle-treated (control) ASM cells, levels of PDE4 activity are significantly increased in cells exposed for 24 h to 100 nM PGE2 or forskolin (FSK). The stimulated PDE activity is ablated in PGE2- and forskolin-exposed ASM cells by pretreatment with either the PDE4 inhibitor rolipram (Rol; A), the transcription or protein synthesis inhibitor actinomycin D (Act D) or cycloheximide (CHX), respectively (B), or the PKA inhibitor H89 (C). Data represent mean ± SE values based on 3–4 measurements obtained under each treatment condition. *P < 0.05; **P < 0.01.

Fig. 3. Regulation of cAMP PDE4 activity in cultured human ASM cells exposed to PGE2 and forskolin. Relative to vehicle-treated (control) ASM cells, levels of PDE4 activity are significantly increased in cells exposed for 24 h to 100 nM PGE2 or forskolin (FSK). The stimulated PDE activity is ablated in PGE2- and forskolin-exposed ASM cells by pretreatment with either the PDE4 inhibitor rolipram (Rol; A), the transcription or protein synthesis inhibitor actinomycin D (Act D) or cycloheximide (CHX), respectively (B), or the PKA inhibitor H89 (C). Data represent mean ± SE values based on 3–4 measurements obtained under each treatment condition. *P < 0.05; **P < 0.01.

Fig. 4. PGE2-exposed ASM cells exhibit upregulated PDE4D5 mRNA expression associated with PKA-dependent activation of CREB/ATF1. A: as detected by RT-PCR, PGE2 (100 nM) elicits temporal increases in PDE4D5 mRNA expression in ASM cells, with peak expression of transcripts detected at 6 h. B: Western blot depicting that PGE2 elicits transient phosphorylation of CREB and ATF1 proteins in ASM cells, with peak phosphorylation detected at 30 min. C: Western blot depicting knockdown of PKAα and PKAβ catalytic subunits. D: PGE2-induced phosphorylation of CREB/ATF1 is prevented in ASM cells transfected with the PKA siRNA duplexes, whereas transfection with a scrambled (control) RNA duplex (scRNA) has no effect.

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PKA activates CREB/ATF1 phosphorylation associated with upregulated ERK1/2 at 30 min following exposure to PGE2. As shown in subunits, and then examined for induced phosphorylation of the CREB kinases, p90RSK or MSK-1 (31), which, in turn, can mediate CREB phosphorylation via activation of the CREB kinases, p90RSK or MSK-1 (25). Given this evidence, together with recent reports implicating a critical role for ERK1/2 activation in mediating the hyporesponsiveness of ASM to β2AR stimulation under different proasthmatic conditions (16, 30, 42), we next examined the potential regulatory interplay between PKA and ERK1/2 signaling in PGE2-exposed ASM cells. Initial studies demonstrated that, relative to unstimulated control cells, ASM cells exposed to PGE2 exhibited increased expression of phosphorylated ERK1/2, with peak phosphorylation detected at 30 min, followed by sustained enhanced phosphorylation for at least up to 180 min (Fig. 5A, left). As with PGE2, forskolin (100 nM) was also found to elicit ERK1/2 phosphorylation (Fig. 5A, right). In subsequent evaluation of the potential role of PKA in mediating the PGE2-induced activation of ERK1/2, ASM cells were first treated for 72 h with vehicle alone or either scrambled RNA or siRNAs directed against the PKA catalytic subunits, and then examined for induced phosphorylation of ERK1/2 at 30 min following exposure to PGE2. As shown in Fig. 5B, relative to vehicle-treated control cells (Fig. 5B, lane 1), ERK1/2 phosphorylation was markedly increased in PGE2-treated cells (Fig. 5B, lane 2), and, while cells pretreated with either the scrambled or PKA siRNAs alone showed little change in ERK1/2 phosphorylation (Fig. 5B, lanes 3 and 5, respectively), the PGE2-induced upregulation of ERK1/2 phosphorylation was distinctly inhibited in ASM cells that were pretreated with the PKA siRNAs (Fig. 5B, lane 6), whereas pretreatment of the PGE-exposed cells with scrambled RNA had no appreciable effect (Fig. 5B, lane 4). Analysis of the results obtained in four experiments is depicted in Fig. 5C, wherein the levels of ERK1/2 phosphorylation detected under the different experimental conditions are displayed as the mean ± SE values of the fold changes in the densitometric ratios of phosphorylated ERK1/2 to α-actin. It will be noted that the PGE2-induced increase in ERK1/2 phosphorylation amounted to 3.76 ± 0.64-fold above that detected in control (vehicle treated) cells (P < 0.05), and, in contrast to the lack of effect of scRNA, the PGE2-induced phosphorylation of ERK1/2 was inhibited in cells transfected with PKA siRNA. Thus these data demonstrate that the induction of ERK1/2 activation in PGE2-exposed ASM cells is regulated by PKA, as further examined below.

Role of PKA- and ERK1/2-coupled signaling in regulating CREB/ATF1 activation and PDE4D5 expression in PGE2-exposed ASM cells. Small molecule inhibitors were next employed to further identify the signaling mechanisms involved in regulating ERK1/2 and CREB/ATF1 activation as well as PDE4D5 mRNA expression in PGE2-exposed ASM cells. In these studies, induced changes in ERK1/2 and CREB/ATF1 protein phosphorylation were examined by Western blot analysis following treatment of ASM cells for 30 min with vehicle alone (control) or 100 nM PGE2, both in the absence and the presence of pretreatment with previously reported maximal effective concentrations of specific inhibitors. As depicted by a representative experiment in Fig. 6A, and the corresponding results based on densitometric analysis of the data pertaining to ERK1/2 phosphorylation in four such experiments displayed in Fig. 6B, relative to controls (lane 1), cells treated with PGE2 exhibited significantly increased ERK1/2 and CREB/ATF phosphorylation (lane 2), and these effects were abrogated in PGE2-exposed cells that were pretreated with either the PKA inhibitor H89 (10 μM; lane 3) or the MEK-ERK1/2 inhibitor U0126 (5 μM; lane 4). Conversely, neither PGE2-induced phosphorylation of ERK1/2 nor CREB/ATF was affected in cells pretreated either with the p38 MAPK inhibitor SB202190 (10 μM; lane 5). These data demonstrate that the phosphorylation of ERK1/2 and CREB/ATF1 is prevented in cells pretreated with either the PKA siRNA duplexes. Data are mean ± SE values from 4 experiments (*P < 0.05).
inhibited in cells pretreated with either H89, U1026, SU6656, or PTX. Data are
ADP ribosylation of Gi protein with pertussis toxin (PTX).

CREB/ATF1 proteins is ablated in ASM cells pretreated with inhibitors of

C

phorylated ERK1/2 to total ERK2 are significantly increased in PGE2-exposed

ASM cells. The stimulatory effects of PGE2 on ERK1/2 phosphorylation are

respectively) had an attenuating effect, the PGE2-induced up-

neither the p38 MAPK nor JNK inhibitor (lane 5, lane 6), whereas both ERK1/2 and CREB/ATF phosphorylations were prevented in PGE2-exposed cells that were pretreated with pertussis toxin (PTX; 100 ng/ml) (lane 7), which ADP ribosylates Gi protein, or with the Src family tyrosine kinase inhibitor SU6656 (10 μM; lane 8). As further depicted in Fig. 6C, in concert with their effects on PGE2-induced ERK1/2 and CREB/ATF phosphorylation, pretreatment with the above inhibitors exerted comparable modulatory effects on the expression of PDE4D5 transcripts in ASM cells at 6 h following exposure to 100 nM PGE2. Accordingly, relative to control cells (lane 1), the induced upregulated expression of PDE4D5 mRNA in PGE2-exposed ASM cells (lane 2) was largely prevented by pretreatment of the cells with either H89 (lane 3) or the MEK/ERK1/2 inhibitor (lane 4). Moreover, whereas neither the p38 MAPK nor JNK inhibitor (lanes 5 and 6, respectively) had an attenuating effect, the PGE2-induced up-

regulated expression of PDE4D5 transcripts was markedly inhibited by pretreatment of the cells with either PTX (lane 7) or the Src inhibitor (lane 8). Thus, when taken together, these observations support the notion that PGE2-induced expression of PDE4D5 transcripts in ASM cells is regulated by PKA-dependent activation of ERK1/2, which, in turn, serves to activate CREB/ATF proteins and thereby initiate PDE4D5 transcription.

Mechanism of PKA-dependent activation of ERK1/2 in PGE2-exposed ASM cells: role of altered G protein-coupled signaling. It is well documented that PKA can activate ERK1/2 either via Gs-coupled or Gβγ-subunit-mediated stimulation of Src-induced signaling via the Rap1/B-Raf/MEK1/2 or the Ras/c-Raf1/MEK1/2 pathway, respectively (31). Moreover, cAMP-elevating agents can also activate ERK1/2 via a direct stimulatory effect of cAMP on exchange protein directly activated by cAMP (EPAC) proteins, which, in turn, can initiate sequential downstream signaling via the Rap1/B-Raf/MEK1/2 pathway (31). To distinguish the relative contributions of these downstream signaling pathways, we examined the effects of selective inhibitors of c-Raf1 and Rap1 on PGE2-induced ERK1/2 phosphorylation in ASM cells. As shown in Fig. 7A, relative to control (untreated) cells (lane 1), ASM cells exposed for 30 min to PGE2 exhibited increased expression of phosphorylated ERK1/2 (lane 2), and, as expected, this effect was completely abrogated by pretreatment of the cells with the selective MEK1/2 inhibitor U0126 (lane 3). Comparably, pretreatment with the selective c-Raf1 inhibitor GW5074 (20 μM) also completely ablated the stimulatory effect of PGE2 on phosphorylated ERK1/2 expression (lane 4), whereas cells pretreated with GGTI-298 (25 μM), a potent and selective inhibitor of Rap1 activation (29), did not exhibit attenuated PGE2-induced ERK1/2 phosphorylation (lane 5). Thus these
data implicate downstream Raf1-coupled signaling in mediating ERK1/2 activation in PGE2-exposed ASM cells, supporting the notion that our observed PKA-dependent activation of ERK1/2 is likely attributed to G\textsubscript{i}-\beta\gamma-mediated Src signaling via the Ras/c-Raf1/MEK1/2 pathway. The latter possibility was directly addressed in extended experiments, wherein we compared the effects of PGE2 on ERK1/2 activation in ASM cells at 24 h following their transfection either with an adenovirus vector expressing lacZ (adeno-LacZ), serving as a negative control, or with adeno-\betaARK-ct, which encodes the \betaARK1 carboxy-terminal domain that blocks G\beta\gamma signaling (27, 28), both at a multiplicity of infection (MOI) of 100. As shown in Fig. 7B, cells transfected with adeno-LacZ exhibited distinct PGE2-induced ERK1/2 phosphorylation (lane 2), whereas this response to PGE2 was completely ablated in cells transfected with adeno-\betaARK-ct (lane 4). Thus, together with the above observations, these data support the concept that activation of ERK1/2 in PGE2-exposed ASM cells is attributed to PKA-dependent activation of G\textsubscript{i}-\beta\gamma-mediated signaling via the Ras/c-Raf1/MEK1/2 pathway.

PKA-dependent ERK1/2 signaling regulates PDE4 activity and constrictor and relaxation responsiveness in \beta2AR-desensitized ASM. To ascertain the physiological implications of the above mechanism of interplay between the PKA and ERK1/2 signaling pathways in the \beta2AR-desensitized state, we next examined the independent effects of inhibition of PKA, G\textsubscript{i} protein, and ERK1/2 function on the changes in PDE activity and constrictor and relaxation responsiveness accompanying heterologous \beta2AR desensitization in human ASM cells and rabbit tissues, respectively. As demonstrated in Fig. 8, relative to control (vehicle treated) ASM cells, cAMP PDE activity was significantly increased in cells that were incubated for 24 h with 100 \mu M PGE2 or forskolin, and this induced stimulation of PDE4 activity was completely ablated in both PGE2- and forskolin-exposed cells that were pretreated with either the MEK1/2 inhibitor U1026 (5 \mu M) or PTX (100 ng/ml). Com- comparably, as depicted in Fig. 9, relative to the responses obtained in control rabbit ASM, the significantly increased constrictor responses to ACh (Fig. 9A) and impaired relaxation responses to isoproterenol (Fig. 9B) detected in PGE2-exposed ASM tissues were abrogated by pretreatment of these tissues with either PTX or U1026, or by ADP ribosylation of G\textsubscript{i} protein with H89. In relation to these observations, it should be noted that in separate studies wherein vehicle-exposed control ASM tissues were comparably pretreated with each of the latter inhibitors, there was no significant effect of either agent on the constrictor or relaxation responses of the tissues (data not shown). Thus these data provide physiological evidence supporting the notion that the above mechanism of PGE2-induced cross talk between the cAMP/PKA and ERK1/2 signaling pathways mediates the rolipram-sensitive changes in PDE activity and ASM constrictor and relaxation responsiveness exhibited in the \beta2AR-desensitized state.

**DISCUSSION**

Increased bronchoconstrictor responsiveness to airway spasmogens and impaired \beta2AR-mediated bronchial relaxation are
characteristic features of asthma that may be significantly aggravated following chronic use of long-acting \beta_2AR agonists (4, 33, 41). The phenotypic features of airways are exhibited in ASM tissues isolated from asthmatic individuals (2, 3, 15) and in isolated naive ASM tissues that are exposed to a variety of proinflammatory stimuli (22, 43). Previous studies have attributed the loss of ASM sensitivity to \beta_2AR agonists to heterologous \beta_2AR desensitization that is initiated by proinflammatory cytokine-induced expression of cyclooxygenase (COX)-2, which promotes release of cAMP-elevating prostanoids (notably, PGE_2) that then elicit PKA-dependent phosphorylation of the \beta_2AR and, hence, its uncoupling from G_s protein-mediated cAMP production (14, 17, 39, 43). Other studies have reported that the impaired relaxant responsiveness to \beta_2AR stimulation in ASM exposed to various proinflammatory conditions is attributed to cytokine-induced upregulated expression and action of G_s protein, which attenuates cAMP accumulation and, hence, the bronchodilatory action of \beta_2AR agonists (18, 19, 21). Moreover, recent reports have implicated a critical role for ERK1/2 signaling in mediating both the impaired \beta_2AR-mediated relaxation and increased constrictor responsiveness in ASM exposed to different proinflammatory stimuli (16, 30, 42). We now provide new evidence demonstrating that J) the proinflammatory-like changes in ASM constrictor and relaxation responsiveness accompanying heterologous \beta_2AR desensitization resulting from prolonged exposure to either the receptor-coupled or nonreceptor-coupled cAMP-elevating agent, PGE_2 or forskolin, respectively, are mediated by upregulated PDE4 activity; and 2) the latter is attributed to PKA-dependent induction of G_s-Beta-generated signaling that involves Src-mediated downstream activation of ERK1/2, which, in turn, leads to transcriptional upregulation of PDE4 expression and its consequent action. These novel findings highlight a heretofore unidentified mechanism that potentially underlies the well-established association between prolonged airway desensitization to \beta_2AR agonists and aggravation of the asthmatic phenotype (4, 33, 41).

Our studies examining the role of cAMP PDE activity in regulating the changes in ASM function accompanying heterologous \beta_2AR desensitization demonstrated that pretreatment with the PDE4 inhibitor rolipram ablated both the increased constrictor responsiveness to Ach and impaired relaxation responses to isoproterenol exhibited by rabbit ASM tissues following their prolonged exposure to PGE_2 (Fig. 1). The latter finding is similar to that reported in a previous study wherein human myometrial tissues exposed for 18 h to 10 \mu M PGE_2 were also found to exhibit impaired relaxation responses to the \beta_2AR agonist salbutamol that were prevented by pretreatment of the tissues with a PDE4 inhibitor (36). Our observations in rabbit ASM tissues, together with those demonstrating rolipram-sensitive increases in PDE activity in PGE_2- and forskolin-exposed cultured human ASM cells (Fig. 3A), implicated upregulated PDE4 activity in mediating both the enhanced constrictor responsiveness and attenuated relaxation exhibited by the \beta_2AR-desensitized ASM tissues. The impaired ASM relaxant responsiveness to isoproterenol is readily explained by the inherent role of PDE4 in catalyzing cAMP hydrolysis, which was reflected by the ability of rolipram to ablate the impaired isoproterenol-induced accumulation of cAMP in \beta_2AR-desensitized ASM cells (Fig. 2). The observation that PDE4 activity also serves to regulate ASM constrictor responsiveness to Ach is consistent with the findings in a recent study that demonstrated a critical role for PDE4D in regulating muscarinic cholinergic contractile responsiveness in isolated mouse airway segments (37). The latter study reported that airway tissues isolated from PDE4D-deficient mice, as well as rolipram-pretreated tissues isolated from wild-type mice, display markedly reduced constrictor responses to carbachol in association with an increased sensitivity of the tissues to various cAMP-elevating agents (37). Our present observations, however, concur only in part with these previous findings, since pretreatment of our rabbit ASM tissues with rolipram alone was found to produce relatively modest changes in ASM constrictor and relaxation responsiveness (see Fig. 1). Thus, unlike in mice, it appears that basal PDE4 activity plays a relatively lesser role in regulating rabbit ASM tissue responsiveness under resting conditions. Conversely, our data demonstrate that upregulated PDE4 activity plays a decisive role in mediating the proinflammatory-like changes in both ASM constrictor and relaxation responsiveness that accompany PGE_2-induced heterologous \beta_2AR desensitization.

In accordance with earlier studies that demonstrated stimulatory effects of cAMP-elevating agents on PDE4 expression in various cell types (6, 9, 24, 46), the present results demonstrated that the induction of PDE4 activity in PGE_2- and forskolin-exposed ASM cells was completely abrogated by pretreatment of the cells with either actinomycin D or cycloheximide (Fig. 3B), as well as by pretreatment with H89 (Fig. 3C). These data suggested that the increased PDE4 activity reflected synthesis of new mRNA and protein, and that the latter phenomenon was PKA dependent. This notion was supported by the observation that ASM cells exposed to PGE_2 exhibited upregulated mRNA expression of PDE4D5 (Fig. 4A), which was recently identified as the most functionally relevant PDE4D isoform in human ASM cells (5). Moreover, PKA dependence of the induction of PDE4D5 transcripts was substantiated by the complementary observations that PGE_2-induced phosphorylation of the CRE-regulating transcription co-factors, CREB/ATF1, was prevented by siRNA knockdown of PKA expression (Fig. 4D) or by PKA inhibition with H89 (Fig. 6A), which also prevented the upregulated expression of PDE4D5 transcripts in the PGE_2-exposed ASM cells (Fig. 6C). In relation to these observations, it is noteworthy that, apart from upregulating PDE4D5 transcription, PKA is also known to directly stimulate PDE4D5 activity, as well as that of other long PDE4D isoforms, by phosphorylating a regulatory site within the amino-terminal region of these isoforms (9, 24). Moreover, in this context, recent studies have demonstrated that the acute cAMP responses detected in subcellular microdomains of HEK293 cells exposed to a \beta_2AR or prostanoid receptor (EP2 and EP4) agonist are rapidly attenuated by PKA-dependent PDE4 activity (notably involving PDE4D5), which also mediates acute homologous desensitization of these receptors to subsequent agonist administration, as well as acute heterologous desensitization of the \beta_2AR following exposure to an EP2/EP4 agonist (48, 49). Of significance, this rapid PKA-dependent/PDE4D5-mediated attenuation of cAMP signaling and desensitization to \beta_2AR and EP2/EP4 agonists was not detected in the presence of receptor-independent activation of adenylyl cyclase in forskolin-exposed HEK293 cells (38). Recent evidence supports the notion that PDE4D5 represents the key regulator of acute \beta_2AR-stimulated cAMP signaling in.
human ASM cells (5). The above findings in HEK293 cells, however, contrast with those in earlier reports wherein PKA-dependent heterologous β2AR desensitization in cultured human ASM cells was observed following more prolonged exposure of the cells to either PGE2 or forskolin, as well as following pretreatment of the cells with the proinflammatory cytokines IL-1β and tumor necrosis factor-α (TNF-α) (17, 39). Our present observations concur with those of the latter studies insofar as the effects of heterologous β2AR desensitization on ASM function were detected in both the rabbit and human ASM preparations following prolonged exposure to either PGE2 or forskolin. Moreover, because we found that the enhanced PDE4 activity exhibited in β2AR-desensitized ASM cells is completely ablated by inhibiting de novo mRNA or protein synthesis (Fig. 3B), it is unlikely that the above posttranslational mechanism of acute PKA-dependent activation of PDE4 activity significantly contributed to the upregulated PDE4 activity detected herein following prolonged exposure to PGE2 or forskolin.

Our studies addressing the mechanism underlying the PKA-dependent increase in PDE4 expression demonstrated that the PGE2-induced phosphorylation of CREB/ATF1 proteins and upregulated expression of PDE4D5 transcripts were inhibited by pretreatment of the ASM cells with either PTX, the Src inhibitor SU6656, or the MEK1/2 inhibitor U0126 (Fig. 6), implicating key intermediate regulatory roles for Src, protein, Src, and ERK signaling in mediating the PKA-dependent increase in PDE4 expression in the β2AR-desensitized state. These observations are consistent with the prevailing concept that GPCR-dependent and receptor-independent stimulation of Ras-mediated ERK1/2 activation uses signals generated by the βγ-subunits of the heterotrimeric G proteins (10, 27, 35, 44). In this context, it is noteworthy that PKA-dependent phosphorylation of specific Gα-coupled receptors, including the β2AR, prostacyclin, and β1AR receptor, was found to provoke a “switch” in receptor coupling from Gα to Gβγ, a phenomenon that enables the βγ-subunits of Gα to activate Src, which, in turn, elicits downstream MEK/ERK1/2 activation (11, 31). Moreover, both receptor/Gα-coupled and nonreceptor-mediated PKA activation by cAMP-elevating agents were reported to stimulate ERK via βγ-generated signaling (13). Our observations based on a series of complementary experiments provided evidence in support of this mechanism, given that J) PGE2 and forskolin were found to acutely elicit ERK1/2 phosphorylation (Fig. 5A); 2) the PGE2-induced activation of ERK1/2 was prevented by siRNA knockdown of PKA expression (Fig. 5, B and C) or by PKA inhibition with H89 (Fig. 6A); and 3) as in previous studies that identified PKA-dependent Gαβγ signaling coupled to ERK activation (13, 27, 28), ADP-ribosylation of Gα with PTX (Fig. 6A) and transfection of ASM cells with the Gαβγ scavenger βARK-ct (Fig. 7B) prevented the PKA-dependent phosphorylation of ERK1/2 in PGE2-exposed ASM cells. Moreover, the results from our experiments examining the effects of Raf1 and Rap1 inhibition on PGE2-induced ERK1/2 phosphorylation provided additional evidence implicating Gαβγ-mediated ERK1/2 activation, as these studies examined whether the PKA-dependent activation of ERK1/2 was attributed to either Gαβγ-coupled or Gαβγ-coupled stimulation of Src-induced signaling via the Rap1/B-Raf/MEK1/2 or the Ras/c-Raf1/MEK1/2 cascade, respectively (31). Accordingly, in demonstrating that PGE2-induced ERK1/2 phosphorylation was inhibited in ASM cells pretreated with the c-Raf1 inhibitor GW5074, whereas pretreatment with a Rap1 inhibitor (GOKI1-298) had no effect (Fig. 7A), these data further implicated Gαβγ-generated signaling in transducing the PKA dependence of ERK1/2 activation in the PGE2-exposed ASM cells. In relation to this collection of findings, it is important to note that previous studies have demonstrated that the β2AR represents the site of action of PKA in regulating both acute homologous and heterologous β2AR desensitization in ASM induced by isoproterenol and PGE2 or forskolin, respectively (17, 39). Thus it is conceivable that our observations implicating Gαβγ signaling in the heterologous β2AR desensitized state may involve a PKA-dependent switch in coupling of the β2AR from Gα to Gβγ acutely following PGE2 or forskolin administration. However, an alternative explanation seems more likely given that J) the phenomenon of PKA-induced β2AR switching from Gα to Gβγ has been demonstrated only in the presence of a β2AR agonist (12, 31), whereas our observations implicating PKA-dependent activation of Gαβγ were obtained in the absence of β2AR occupancy on the initial exposure of ASM to either PGE2 or forskolin; 2) there is no evidence to support the presence of Gαβγ switching at an EP receptor(s) in response to PGE2 administration; and 3) as with PGE2, our data demonstrated that forskolin also elicits ERK1/2 activation and PTX-sensitive changes in PDE activity, suggesting that stimulation of Gαβγ signaling can result from either receptor/Gαβγ-coupled or nonreceptor-mediated PKA activation by cAMP. This notion is supported by the findings in an earlier study wherein treatment of COS-7 cells with either agonists acting via Gα or Gβγ-coupled receptors, a constitutively activated αβγ mutant, a cAMP analog, or forskolin was found to acutely activate ERK, and this effect was inhibited by sequestering the βγ-subunits (13). In another study using COS-7 cells, treatment with isoproterenol was also found to elicit βγ-dependent MAPK activation, and, while MAPK activation was also exhibited in forskolin-treated cells, this effect was not observed in cells expressing an activated Gαs mutant or by treating the cells with a cell-permeable cAMP analog (11). Interestingly, the latter study also reported that isoproterenol-induced MAPK activation was inhibited in the presence of the cAMP analog, and the authors concluded that Gβγ-coupled receptors might simultaneously elicit both βγ-dependent activating and inhibitory MAPK signals (11). Our present observations, together with those of the above previous studies, highlight the important need to further elucidate the mechanism(s) by which receptor/Gαβγ-coupled and nonreceptor-mediated cAMP signaling can elicit βγ-dependent MAPK activation.

The physiological relevance of the above interplay between PKA activation and Gαβγ-mediated ERK1/2 signaling in regulating the rolipram-sensitive changes in ASM function in the β2AR-desensitized state was substantiated by the observations that the upregulated PDE activity in human ASM cells exposed to PGE2 or forskolin (Fig. 8) and the heightened constrictor responses to ACh and impaired relaxation responses to isoproterenol in PGE2-exposed rabbit ASM tissues (Fig. 9) were both prevented by pretreatment of the ASM preparations with inhibitors of either PKA, Gα protein function, or ERK1/2 activation. These findings are in general agreement with those in previous studies that reported key regulatory roles for PKA and ERK1/2 in mediating heterologous β2AR desensitization in ASM cells (17, 30), as well as the findings in other studies
The impaired relaxation responses to β2AR stimulation in ASM tissues exposed to various proasthmatic stimuli were found to be PTX sensitive and associated with increased ASM expression of Gαi protein (18, 19, 21). More recently, Liggett and co-workers (38) provided evidence supporting a direct causal relationship between changes in Gαi expression and altered airway responsiveness by demonstrating that transgenic mice that overexpress the Gαi2 subunit in ASM exhibit decreased β2AR-mediated airway relaxation, whereas β2AR function is increased in mice expressing a Gαi2 inhibitory peptide in ASM. Unexpectedly, these authors also reported that wild-type mice pretreated with PTX exhibited increased airway constrictor responses to methacholine, as did mice expressing the Gαi2 inhibitory peptide, whereas the bronchoconstrictor responses were decreased in mice overexpressing Gαi2 in ASM (38). The latter findings appear contradictory to those of the present study, as we found that pretreatment with PTX inhibited the heightened ASM constrictor responses to ACh accompanying heterologous β2AR desensitization while having no effect on ASM contractility under control conditions (Fig. 9). Although these discordant observations are not readily explained, apart from potential species differences, consideration should be given to other possibilities, including differences in the relative contributions of the regulatory actions of the α- vs. βγ-subunits of Gαi implicated under the different study conditions. In this respect, whereas Liggett and co-workers attributed the attenuating effect of Gαi2 on airway constrictor responsiveness to an observed downregulated expression of phospholipase C (PLC)β3 in the Gαi2-overexpressing ASM cells (38), it is well established that the βγ-subunit of Gαi can activate PLCβ isoforms, including PLCβ3 (7), thereby conferring PTX sensitivity to increases in agonist-induced inositol phospholipid signaling (20). Accordingly, the latter mechanism may account for the reported PTX-sensitive increase in ASM contractility associated with heightened PLC-mediated inositol phospholipid signaling in ASM isolated from allergic rabbits (1), as well as in rabbit ASM passively sensitized with atopic asthmatic serum (20, 22). Conversely, recent evidence demonstrates that the increased histamine-induced inositol phosphate responses detected in human ASM cells following β2AR desensitization by prolonged exposure to β2AR agonists is not associated with changes in either PLC activity or PLCβ1 expression (40). Comparably, our present observations provide no evidence to suggest that the increased ASM constrictor responsiveness exhibited in the β2AR-desensitized state was due to Gαi-βγ-induced upregulated PLC signaling. Rather, our data implicating PKA-dependent Gαi-βγ signaling demonstrated that this phenomenon is coupled to ERK1/2-mediated upregulated expression of PDE4 activity, and that the latter is fundamentally responsible for the changes in ASM constrictor and relaxation responsiveness accompanying heterologous β2AR desensitization resulting from prolonged exposure to cAMP-elevating agents. Thus, when considering our present findings in relation to those in previous reports, it appears that different signaling events may be responsible for the altered ASM constrictor responsiveness exhibited under different experimental conditions used to simulate the asthmatic phenotype.

In evaluating the implications of the present observations, it must be emphasized that our observations on the regulation of ASM constrictor and relaxation responsiveness pertain to studies conducted in isolated rabbit ASM tissues. Thus certain fundamental issues are raised, including the extent to which these observations relate to the in vivo state and the applicability of the findings to the human condition. Regarding these issues, it is noteworthy that our observations are consistent with a body of recent evidence that implicates upregulated PDE4 activity in mediating the airway responses to allergen challenge in asthmatic subjects (47) and the in vivo changes in airway responsiveness in animal models of allergic asthma (8, 23, 26, 45). Moreover, our data generated in the rabbit ASM tissues are compatible with those obtained in the cultured human ASM cells, as both these experimental preparations exhibited complementary changes in ASM function accompanying heterologous β2AR desensitization, including rolipram-sensitive changes in β2AR-induced ASM relaxation and cAMP accumulation, as well as comparable effects of specific inhibitors on the signaling events implicated in mediating the changes in ASM function. Finally, it should be noted that our observed changes in constrictor and relaxant responsiveness in the β2AR-desensitized rabbit ASM tissues mimicked the perturbations in airway function that characterize the asthmatic ASM phenotype, including enhanced constrictor responsiveness to cholinergic stimulation and impaired β2AR-mediated airway relaxation (4–6). Thus, in light of these considerations, we believe that the findings of the present study are likely applicable to the human condition.

In conclusion, the present study examined the regulation and role of PDE activity in mediating the changes in ASM constrictor and relaxation responsiveness associated with heterologous β2AR desensitization. The results provide new evidence demonstrating that 1) heterologous β2AR desensitization in ASM resulting from its prolonged exposure to either the receptor- or nonreceptor-coupled cAMP-elevating agent, PGE2 or forskolin, respectively, confers increased cholinergic agonist-mediated ASM constrictor responsiveness together with impaired β2AR-mediated ASM relaxation and cAMP accumulation; 2) these changes in ASM function are mediated by transcriptional upregulation of PDE4 activity, reflected by increased expression of the PDE4D5 isoform; 3) the upregulated expression of PDE4D5 is attributed to PKA-dependent activation of Gαi protein signaling, resulting in Gαi-βγ-subunit-mediated downstream activation of ERK1/2 via the Src/Ras/Raf1/MEK1/2 signaling pathway; and 4) the latter activation of ERK1/2, in turn, leads to phosphorylation of the transcription factors CREB and ATF1, which mediate CRE-driven PDE4D5 gene transcription. Taken together, these new findings identify that the proasthmatic changes in ASM function that accompany prolonged airway exposure to cAMP-elevating agents are mediated by upregulated PDE4 activity that is attributed to an induced cross talk between the cAMP/PKA and ERK1/2 signaling pathways. Thus interventions targeted at regulatory sites within this cross talk mechanism may provide novel therapeutic approaches to mitigate the aggravated asthmatic condition associated with prolonged β2AR desensitization of the airways.

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