Mechanism regulating proasthmatic effects of prolonged homologous β2-adrenergic receptor desensitization in airway smooth muscle

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Nino G, Hu A, Grunstein JS, Grunstein MM. Mechanism regulating proasthmatic effects of prolonged homologous β2-adrenergic receptor desensitization in airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 297: L746–L757, 2009. First published August 7, 2009; doi:10.1152/ajplung.00079.2009.—Use of long-acting β2-adrenergic receptor (β2AR) agonists to treat asthma incurs an increased risk of asthma morbidity with impaired bronchodilation and heightened bronchoconstriction, reflecting the adverse effects of prolonged homologous β2AR desensitization on airway smooth muscle (ASM) function. Since phosphodiesterase 4 (PDE4) regulates ASM relaxation and contractility, we examined whether the changes in ASM function induced by prolonged homologous β2AR desensitization are attributed to altered expression and action of PDE4. Cultured human ASM cells and isolated rabbit ASM tissues exposed for 24 h to the long-acting β2AR agonist salmeterol exhibited impaired acute β2AR-mediated cAMP accumulation and relaxation, respectively, together with ASM constrictor hyperresponsiveness. These proasthmatic-like changes in ASM function were associated with upregulated PDE4 activity due to enhanced expression of the PDE4D5 isoform and were prevented by pretreating the ASM preparations with the PDE4 inhibitor rolipram or with inhibitors of either PKA or ERK1/2 signaling. Extended studies using gene silencing and pharmacological approaches demonstrated that: 1) the mechanism underlying upregulated PDE4D5 expression following prolonged β2AR agonist exposure involves PKA-dependent activation of G protein signaling via its βγ-subunits, which elicits downstream activation of ERK1/2 and its induction of PDE4D5 transcription; and 2) the induction of PDE4 activity and consequent changes in ASM responsiveness are prevented by pretreating the β2AR agonist-exposed ASM preparations with inhibitors of Gi-βγ signaling. Collectively, these findings identify that the proasthmatic changes in ASM function resulting from prolonged homologous β2AR desensitization are attributed to upregulated PDE4 expression induced by Gi-βγ-mediated cross-talk between the PKA and ERK1/2 signaling pathways.

asthma; long-acting β2-agonists; salmeterol; cAMP signaling; G proteins; phosphodiesterase-4

CHRONIC USE OF LONG-ACTING β2-ADRENERGIC RECEPTOR (β2AR) agonists to treat asthma has been associated with loss of bronchodilator effect, worsening of airway hyperreactivity, and an increased incidence of asthma-related morbidity and mortality (1, 24, 28). These adverse effects on the asthmatic phenotype are thought to result from the development of airway tolerance to β2AR stimulation due, in large part, to prolonged homologous (agonist-specific) β2AR desensitization of the airway smooth muscle (ASM) (10). Investigations into the etiology of homologous β2AR desensitization have largely focused on the roles played by G protein-coupled receptor (GPCR) kinases (GRKs) and cAMP-dependent PKA in mediating phosphorylation of the β2AR. While these studies have provided valuable insight into the mechanisms involved in acute uncoupling of the β2AR from its associated G protein-mediated accumulation of cAMP, leading to impaired ASM relaxation in the β2AR-desensitized state (10, 23), these mechanisms may not fully explain the clinical worsening of the asthmatic condition that is characteristically observed following prolonged use of β2AR agonists (5, 37, 38). Heterologous (nonagonist-specific) β2AR desensitization, also mediated by PKA activation, has been demonstrated following relatively prolonged exposure of ASM to other receptor-coupled or nonreceptor-coupled cAMP-elevating agents (10, 12, 29, 31, 34), and we recently reported that the proasthmatic changes in ASM constrictor and relaxation responsiveness evoked by prolonged heterologous β2AR desensitization are attributed to upregulated phosphodiesterase 4 (PDE4) activity (16). The latter finding concurs with those in previous studies on different cell types wherein the attenuated physiological responses to cAMP-elevating agents detected under conditions associated with either heterologous or homologous β2AR desensitization were found to be critically regulated by PDE activity (3, 6, 15, 34). Furthermore, it is well documented that PDE4 activity plays a crucial role in regulating ASM contractility (27) and in mediating the constrictor hyperresponsiveness of the airways accompanying allergen challenge in asthmatic subjects (35) and in animal models of allergic asthma (4, 14, 18, 32, 33). Little is known, however, regarding the mechanism regulating PDE4 expression and its role in contributing to the impaired cAMP signaling and altered responsiveness of ASM under conditions of prolonged homologous β2AR desensitization.

Most of the cAMP hydrolyzing activity in smooth muscle cells is attributed to isoforms of the PDE4 family, which is encoded by four distinct genes (PDE4A-D) that generate multiple PDE4 enzyme variants through activation of different promoters or alternative splicing (6, 15). 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) (6, 15). 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) (2, 22). In vascular smooth muscle cells, PDE4D expression was also found to be translationally regulated by PKA as well as by the MAPK ERK1/2 (25). Moreover, PKA and ERK1/2 were shown to directly regulate PDE4D catalytic activity (6, 15). In light of this information, the present study was undertaken to identify the mechanism regulating PDE4 expression and its...
potential role in mediating the changes in ASM constrictor and relaxation responsiveness associated with prolonged homologous β2AR desensitization. The data provide new evidence demonstrating that: 1) homologous β2AR desensitization of ASM following its prolonged exposure to β2AR agonists evokes increased ASM constrictor responsiveness and impaired β2AR-mediated ASM relaxation associated with attenuated cAMP accumulation; 2) these proatherosclerotic-like changes in ASM function are mediated by increased PDE4 activity, which reflects transcriptional upregulation of the PDE4D5 isoform that is due to ERK1/2 activation; and 3) the latter is attributed to PKA-dependent activation of Gβ protein signaling via its βγ-subunits, which leads to downstream activation of the Ras/Raf1/MEK-ERK pathway in the homologous β2AR-desensitized state. Together, these findings are the first to identify a mechanism that potentially underlies the heightened expression of the asthmatic phenotype following prolonged exposure of the airways to long-acting β2AR agonists.

MATERIALS AND METHODS

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

Animals. Seventeen 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 care 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 ASM 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 in air at 37°C. The experimental protocols involved growing the cells in SmBm media supplemented with 10% FBS (BioWhittaker) and in accordance with the “Guide for the Care and Use of Laboratory Committee of the Joseph Stokes Research Institute at Children’s exposure of the airways to long-acting expression of the asthmatic phenotype following prolonged desensitized state. Together, these findings are the first to MATERIALS AND METHODS demonstrate that:

1. isoproterenol (10−5 M) both in the absence and presence of rolipram (10−5 M), H89 (10−5 M), U0126 (5×10−5 M), and the latter treatments were maintained in the lysates used to assay cAMP PDE activity. PDE activity was also assessed in salmeterol- and isoproterenol-exposed ASM cells that were pretreated with either an anti-α3 inhibitory peptide coupled to a membrane-permeable sequence (MPS, 1 μM) or an anti-βγ inhibitory peptide (phosphducin-like protein) coupled to MPS (1 μM), both from AnaSpec (San Jose, CA). 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′-TGGCAGCTGTACAAAGTGACC-3′ (forward) and 5′-TTCTCGGAGGATCATCTGGAGA-3′ (reverse); and for β-actin, 5′-GAGAAGAGCTACAGCTCGCTGAC-3′ (forward) and 5′-CGGATCTTGCCTCAAGGGAG-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 PDE4D5 expression and CREB/ATF1 and ERK1/2 phosphorylation. Levels of PDE4D5 and β-actin proteins, as well as phosphorylated CREB, ATF1, and ERK1/2 proteins, were detected by Western blot analysis of lysates isolated from ASM cells before and at various times after treatment with isoproterenol or salmeterol 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 PVDF membrane. The membranes were then incubated overnight with monoclonal mouse anti-human primary antibodies directed against PDE4D5 (FabGennix), phospho-CREB/ATF1, phospho-ERK1/2, or β-actin (Cell Signaling Technology), and levels were detected by ECL after a 1-h incubation with a 1:2,000 dilution of HRP-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% confluence, 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 PKAα or PKAγ catalytic subunits (Santa Cruz Biotechnology; sc-36240 and sc-36236, respectively) or with a nontargeted negative control (scrambled) siRNA duplex, using Oligofectamine (Invitrogen) as the transfection agent. The siRNAs were applied to each well at a final concentration of 100 nM for each siRNA preparation. As previously described (16), administration of these siRNA preparations using this double-transfection approach greatly enhanced transfection efficiency, and, as detected by Western blot analysis, markedly inhibited PKAα expression by the targeted siRNA duplexes, with maximal inhibition ranging between ~70 and 90% detected at 72 h following siRNA transfection (16).

Transfection of ASM cells with adeno-βARK-ct. Adenovirus (adeno)-βARK-ct, an adenosine vector encoding the βARK1 COOH-terminal domain which blocks Gβγ signaling (19, 20), and adeno-β-γ, an adenosine vector expressing lacZ as a negative control, were constructed using the AdenoX adenovirus construction kit (BD-Clontech). Recombinant plaque were isolated and propagated in HEK-293 cells (Invitrogen), with viral purification using the cesium chloride gradient method, and viral titer determined by plaque assay. The ASM cells were transfected with either of the adenoviral vectors at a final concentration of 100 nM for each adenovirus preparation. As previously described (16), adenoviral vectors were selected at 24 h following adenoviral transfections, as previously described (16).

Pharmacodynamic studies of constrictor and relaxation responsiveness in rabbit ASM tissues. Following initial sedation and subsequent general anesthesia with intramuscular injections of xylazine (10

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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 (11, 13), 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 6–8 mm in length. Each alternate ring was incubated for 24 h at room temperature in the presence of either vehicle alone (control) or salmeterol (10 μM), both in the absence and presence of either rolipram (10 μM), the PKA inhibitor H89 (10 μM), the ERK1/2 inhibitor U0126 (10 μM), or the anti-Goα or anti-βγ peptide coupled to MPS (30 μM). Thereafter, the tissues were placed in organ baths containing modified Krebs-Ringer solution aerated with 5% CO2 in oxygen (pH 7.35–7.40) and attached to force transducers to continuously monitor isometric tension. Cholinergic contractility was then assessed in the tissues following cumulative administration of ACh in final bath concentrations ranging from 10−9 to 10−3 M. The tissues were then repeatedly rinsed with fresh buffer, and relaxation dose-response curves to isoproterenol (10−9 to 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 means ± SE. Comparisons between groups were made using the Student’s t-test (two-tailed) or ANOVA with the Tukey posttest analysis, where appropriate. P <0.05 was considered statistically significant. The statistical analyses were performed using the Prism computer program by GraphPad Software.

RESULTS

Role of PDE4 in regulating cAMP responses in ASM cells following prolonged homologous β2AR desensitization. The role of PDE4 activity in mediating homologous β2AR desensitization in human ASM cells was examined by comparing the acute changes in intracellular cAMP accumulation detected at 5 min following administration of a near half-maximal effective concentration of isoproterenol (ISO; 1.0 μM) in cells that were initially exposed to either vehicle alone or to a maximally effective concentration of the long-acting β2AR agonist, salmeterol (10 μM × 24 h), both in the absence and presence of cotreatment with the PDE4-selective inhibitor rolipram (10 μM). As shown in Fig. 1, relative to vehicle-exposed (control) cells, in the continued presence of salmeterol, ASM cells exhibited homologous β2AR desensitization, as evidenced by significantly reduced cAMP responses to ISO. By comparison, ASM cells pretreated with rolipram exhibited slightly increased ISO-induced cAMP accumulation, and the response to ISO was largely preserved in salmeterol-exposed cells that were cotreated with rolipram. Thus, these observations implicate PDE4 activity in mediating the impaired isoproterenol-induced accumulation of cAMP in human ASM cells following their prolonged homologous β2AR desensitization.

Regulation of cAMP PDE activity in homologous β2AR-desensitized ASM cells. To elucidate the mechanism underlying the above rolipram-sensitive effects of homologous β2AR desensitization on cAMP accumulation, the changes in total cAMP PDE activity and the effects of pretreatment with selective small-molecule inhibitors on this activity were examined in cultured ASM cells following their prolonged exposure to a β2AR agonist. Relative to the mean (± SE) basal level of PDE activity detected in vehicle-treated (control) cells (i.e., 150.2 ± 11.1 pmol·min⁻¹·mg⁻¹ protein), ASM cells incubated for 24 h with a maximally effective concentration (10 μM) of either salmeterol or isoproterenol exhibited significantly increased levels of PDE activity that averaged approximately twofold above control (Fig. 2A). The stimulatory effect of prolonged exposure to salmeterol or isoproterenol on PDE activity was completely abrogated in cells that were pretreated with rolipram (10 μM), implying that the upregulated cAMP-PDE activity exhibited by the β2AR-desensitized cells was attributed to PDE4. In separate experiments, pretreatment of cells with the transcriptional inhibitor actinomycin D (4 μM) or with the protein synthesis inhibitor cycloheximide (100 μM) also completely ablated the increase in PDE activity elicited by prolonged exposure to the β2AR agonists (Fig. 2B), whereas neither actinomycin D nor cycloheximide alone significantly affected basal PDE activity (data not shown). Finally, extended studies demonstrated that pretreatment of cells with either the PKA inhibitor H89 (10 μM) or the MEK-ERK1/2 inhibitor U0126 (5 μM) prevented the stimulatory effects of salmeterol and isoproterenol on PDE activity (Fig. 2C), whereas neither H89 nor U0126 alone had a significant effect on basal PDE activity (data not shown). Collectively, these data are consistent with the notion that homologous β2AR desensitization in ASM cells resulting from prolonged exposure to a β2AR agonist elicits upregulated PDE4 activity that is attributed to PKA- and ERK1/2-dependent de novo mRNA and protein synthesis.

Role of PDE4 in regulating constrictor and relaxation responsiveness in homologous β2AR-desensitized ASM tissues. In light of the above observations, we next examined the regulatory role of PDE4 activity and the contributions of PKA and ERK1/2 signaling in mediating the effects of prolonged homologous β2AR desensitization on ASM function. In these studies, constrictor responses to ACh and relaxation responses to isoproterenol were compared in isolated rabbit ASM tissues that were exposed for 24 h to either vehicle alone (control) or a maximally effective concentration of salmeterol (SAL; 10 μM), both in the absence and presence of pretreatment with...
either rolipram (10 μM), H89 (10 μM), or U0126 (5 μM). Relative to vehicle-treated controls, SAL-exposed ASM tissues exhibited significantly increased constrictor responsiveness to exogenously administered ACh (Fig. 3A), yielding a mean ± SE maximal constrictor response (T_{max}) value of 106.2 ± 8.9 g/g ASM weight (wt) vs. the value of 84.3 ± 7.1 g/g ASM wt obtained in the control tissues (P < 0.05). This enhanced constrictor responsiveness to ACh was completely abrogated in SAL-exposed tissues that were pretreated either with rolipram, H89, or U0126. Under the same treatment conditions, during subsequent sustained half-maximal contraction of the tissues with ACh, cumulative administration of isoproterenol produced acute dose-dependent relaxation of the preconstricted ASM segments. Relative to control tissues, the relaxation responses to isoproterenol were significantly attenuated in the SAL-exposed ASM segments (Fig. 3B), consistent with the development of homologous β2AR desensitization. Accordingly, the means ± SE maximal relaxation (R_{max}) response in the SAL-exposed tissues amounted to 33.4 ± 5.1% vs. the average R_{max} value of 52.3 ± 4.9% obtained in the control ASM segments (P < 0.01). This impaired relaxant responsiveness to isoproterenol was also completely ablated in SAL-

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**Fig. 2.** Regulation of cAMP PDE activity in cultured human ASM cells following prolonged exposure to β2AR agonists. Relative to vehicle-treated (control) ASM cells, levels of PDE activity are significantly increased in cells exposed for 24 h to 10 μM of either salmeterol or isoproterenol. The stimulated PDE activity is ablated in β2AR agonist-exposed ASM cells by pretreatment either with the PDE4 inhibitor rolipram (A), the transcription or protein synthesis inhibitors actinomycin D (Act D) or cycloheximide (CHX), respectively (B), the PKA inhibitor H89, or the ERK1/2 inhibitor U0126 (C). Data represent means ± SE values based on 3–4 determinations made under each treatment condition. **P < 0.01.

**Fig. 3.** Inhibition of PDE4 and either the PKA and ERK signaling pathways prevents the induction of altered ASM tissue constrictor and relaxation responsiveness accompanying prolonged homologous β2AR desensitization. Relative to vehicle-treated controls, rabbit ASM tissues exposed for 24 h to salmeterol (10 μM) exhibit significantly increased constrictor responses to ACh (A) and impaired relaxation responses to isoproterenol (B). Pretreatment with either the PDE4-selective inhibitor rolipram (10 μM), the PKA inhibitor H89 (10 μM), or the ERK1/2 inhibitor U0126 (5 μM) prevents the salmeterol-induced changes in ASM constrictor and relaxation responsiveness. Data represent means ± SE values from 6–8 paired experiments.
exposed tissues that were pretreated either with rolipram, H89, or U0126. Contrasting these observations in SAL-exposed tissues, relative to controls, ASM tissues that were comparably treated for 24 h with rolipram, H89, or U0126 alone did not exhibit any significant changes in either constrictor or relaxation responsiveness (data not shown). Together, these observations implicate a key regulatory role for PDE4 activity that is coupled to PKA and ERK1/2 activation in mediating the proasthmatic-like changes in ASM constrictor and relaxation responsiveness that accompany prolonged homologous β2AR desensitization.

Regulation of PDE4D5 expression by PKA and ERK1/2 in homologous β2AR-desensitized ASM. Based on the above observations, together with recent evidence that identifies PDE4D5 as the functionally dominant cAMP-regulating PDE4 isoform in human ASM cells (2), we next conducted a series of experiments to identify the effects of prolonged β2AR agonist exposure on ASM expression of PDE4D5 and the potential roles played by the PKA and MAPK signaling pathways in regulating any induced changes in PDE4D5 expression. Initial studies demonstrated that ASM cell cultures exposed for 24 h to 10 μM of either salmeterol (Fig. 4A) or isoproterenol (Fig. 4B) exhibited time-dependent increases in PDE4D5 mRNA expression, with peak induction of PDE4D5 transcripts by either agonist detected at 6 h and sustained upregulated expression observed for up to 24 h. Densitometric analysis of the temporal changes in mRNA expression examined in three paired experiments demonstrated that peak induction of PDE4D5 transcripts with salmeterol and isoproterenol averaged 7.3- and 5.7-fold above the levels detected in unstimulated cells, respectively. Small-molecule inhibitors were subsequently employed to identify the roles of PKA and MAPK signaling in regulating the induction of PDE4D5 expression. As demonstrated in one of four representative experiments in Fig. 4C, relative to vehicle-exposed (control) cells, the induced upregulated expression of PDE4D5 mRNA transcripts detected at 6 h following exposure of ASM cells to 10 μM isoproterenol was largely prevented by pretreating the cells with either H89 or the MEK/ERK1/2 inhibitor U0126. Conversely, isoproterenol-induced upregulation of PDE4D5 mRNA was unaffected in cells pretreated with previously reported maximal effective concentrations of either the specific JNK inhibitor SP-600125 (10 μM) or the p38 MAPK inhibitor SB-202190 (10 μM), whereas the induction of PDE4D5 transcripts was prevented in isoproterenol-exposed cells that were pretreated with the Src family tyrosine kinase inhibitor SU-6656 (10 μM) or pertussis toxin (PTX; 100 ng/ml), which ADP ribosylates Gt protein. Of note, similar suppression of isoproterenol-induced PDE4D5 expression was also detected in ASM cells that were pretreated with PP2 (10 μM), another Src tyrosine kinase inhibitor (data not shown). The latter observations are consistent with the known role of Gt protein-mediated activation of Src in eliciting ERK1/2 activation in other cell types, and, when taken together, the above results support the concept that β2AR agonist-induced expression of PDE4D5 transcripts in ASM cells is regulated by Gt protein-coupled interaction between PKA and Src-mediated ERK1/2 signaling. Studies were next pursued to examine this suspected interplay between these signaling pathways.

PKA-dependent regulation of CREB/ATF1 and ERK1/2 activation in β2AR agonist-exposed ASM. Because PDE4D5 transcription in ASM cells is regulated by a CRE-containing promoter (22), we investigated the effects of β2AR stimulation on cAMP/PKA-dependent downstream signaling events coupled to CRE activation. As exemplified by a representative immunoblot (i.e., 1 of 3 experiments) in Fig. 5A, ASM cells treated with isoproterenol (10 μM) exhibited transiently increased phosphorylation of the CRE-binding transcription cofactors, CREB and ATF1, which peaked at 30 min and was subsequently largely abrogated by 180 min. Qualitatively similar temporal changes in CREB/ATF1 phosphorylation were also detected in ASM cells that were exposed to 10 μM salmeterol (data not shown). To ascertain whether the stimulatory effect of β2AR agonist exposure on CREB/ATF1 phosphorylation was due to PKA activation, we next compared the effects of isoproterenol on CREB/ATF1 phosphorylation in vehicle-exposed ASM cells and in cells wherein PKA expression was suppressed by transfection with a pool of three siRNA duplexes directed against the human PKAα and PKAγ catalytic subunits. We recently demonstrated that Lipofectamine transfection of ASM cells with this pool of siRNAs against PKA produced maximal knockdown of PKAα protein levels at 72 h posttransfection that ranged between ~70 and 90% (16). Confluent cultures of ASM cells were initially treated for 72 h with either vehicle alone, a scrambled siRNA sequence serving as a negative control, or the siRNA duplexes directed against the PKA catalytic subunits. Cells were then examined for
transfected with siRNA duplexes directed against the PKA agonist-induced phosphorylation of CREB/ATF1 is prevented in ASM cells. A of the CRE-regulating transcription cofactors CREB and ATF1 and upregulation with scrambled siRNA has no effect. 

Finally, because H89 is a nonselective PKA inhibitor, we also used these siRNA preparations to further substantiate the role of PKA in regulating β2AR agonist-induced PDE4 expression. Accordingly, we compared the effects of salmeterol (10 μM × 24 h) on PDE4D5 protein expression in control ASM cells vs. cells that were initially transfected with either the siRNAs directed against PKA or the scrambled siRNA sequence. As shown by a representative immunoblot in Fig. 5C (i.e., 1 of 3 experiments), relative to control cells, PDE4D5 protein expression was upregulated in salmeterol-exposed ASM cells, and the latter induction of PDE4D5 by the β2AR agonist was suppressed in cells that were transfected with PKA siRNA, whereas transfection with the scrambled siRNA sequence had no effect on salmeterol-induced upregulation of PDE5D5 protein. Thus, in concert with the above observations implicating PKA activation in mediating upregulated PDE4 activity in isoproterenol-exposed ASM cells, these data demonstrate that PKA activation is intimately involved in regulating isoproterenol-induced CREB/ATF1 phosphorylation associated with upregulated PDE4D5 protein expression.

Apart from the cAMP/PKA pathway, CREB can also be activated via other signaling events, notably including ERK1/2 activation (17). Moreover, in this context, cross-talk between the cAMP/PKA and ERK1/2 signaling pathways has also been demonstrated wherein PKA can activate the Ras/c-Raf1/MEK1/2 and/or the Rap1/B-Raf/MEK1/2 signaling pathway, leading to downstream activation of ERK1/2 (15, 21), which, in turn, can mediate CREB phosphorylation via activation of the CREB kinases p90RSK or MSK-1 (17). This evidence, together with the above observations implicating a critical role for ERK1/2 activation in mediating the induced changes in PDE4 expression and in ASM constrictor and relaxation responsiveness in the homologous β2AR-desensitized state, we next examined whether β2AR agonist exposure elicits a regulatory interplay between PKA and ERK1/2 signaling in ASM cells. As depicted by representative immunoblots in Fig. 6A, treatment of ASM cells with 10 μM of either isoproterenol or salmeterol acutely elicited increased phosphorylation of ERK1/2 proteins that peaked at 20 min and was sustained for at least up to 80 min. To subsequently assess the role of PKA in mediating this β2AR agonist-induced activation of ERK1/2, ASM cells were first treated for 72 h with vehicle alone or with either scrambled RNA or the siRNAs directed against the PKA catalytic subunits, and then examined for induced phosphorylation of ERK1/2 at 20 min following exposure to isoproterenol. As shown in Fig. 6B, relative to vehicle-treated control cells, ERK1/2 phosphorylation was markedly increased in isoproterenol-treated cells, and, while cells pretreated with either the scrambled or PKA siRNAs preparations alone showed little change in basal ERK1/2 phosphorylation, the isoproterenol-induced increase in ERK1/2 phosphorylation was distinctly inhibited in ASM cells that were pretreated with the PKA siRNAs, whereas pretreatment of isoproterenol-exposed cells with the scrambled RNA had no appreciable effect. Analysis of the results obtained in four experiments is depicted in Fig. 6C, wherein the levels of ERK1/2 phosphorylation detected under the corresponding different experimental conditions are displayed as means ± SE values of the fold-changes in the densitometric ratios of phosphorylated ERK1/2-to-total ERK1/2. It can be seen that isoproterenol-induced phosphorylation of ERK1/2 amounted to 2.30 ± 0.18-fold above that detected in control (vehicle-treated) cells (P < 0.01) and that in contrast to the lack of effect of scRNA, the β2AR agonist-induced phosphorylation of ERK1/2 was inhibited in cells transfected with the PKA siRNA duplexes. Thus, these data demonstrate that the induction of ERK1/2 activation in β2AR agonist-exposed ASM cells is regulated by PKA.

$G_\beta\gamma$ signaling mediates PKA-dependent activation of ERK1/2 in β2AR agonist-exposed ASM. The above observations in β2AR agonist-exposed ASM are consistent with those in a recent study wherein we found that PKA-dependent ERK1/2 activation mediates heterologous β2AR desensitization in ASM evoked by its prolonged exposure to PGE2 or to non-receptor-mediated stimulation of cAMP production with forskolin (16). The findings in the latter study further demon-
stratified that PKA-dependent activation of ERK1/2 in PGE2-exposed ASM cells was attributed to G$_i$-βγ-subunit-mediated activation of Src signaling (16). Given this recent evidence, together with our observations herein that implicate Src and G$_i$ protein activation in mediating β2AR agonist-induced PDE4D5 expression (Fig. 4C), we examined whether G$_i$βγ signaling also mediates ERK1/2 activation in β2AR agonist-exposed ASM cells. This issue was addressed in experiments that compared the effects of isoproterenol administration (10 μM × 20 min) 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 adenov-βARK-ct, which encodes the βARK1 COOH-terminal domain that blocks G$_i$βγ signaling (19, 20). As shown in Fig. 7A, cells transfected with aden-LacZ exhibited distinct isoproterenol-induced ERK1/2 phosphorylation, whereas this response to isoproterenol was completely ablated in cells transfected with aden-βARK-ct, implicating G$_i$βγ signaling in mediating ERK1/2 activation by the β2AR agonist. In light of these data, together with earlier reports demonstrating that PKA-dependent activation of ERK1/2 can occur via G$_i$-βγ-subunit-mediated stimulation of Src-induced signaling via either the Rap1/B-Raf/MEK1/2 or the Ras/c-Raf1/MEK1/2 pathway (21), we next examined whether our observed G$_i$βγ-mediated activation of ERK1/2 in β2AR agonist-exposed ASM is attributed to induced downstream signaling via the Rap1/B-Raf/MEK1/2 and/or the Ras/c-Raf1/MEK1/2 pathway. Accordingly, to ascertain the relative contributions of these downstream signaling pathways, we compared the effects of selective inhibitors of c-Raf1 and Rap1 activation on β2AR agonist-induced ERK1/2 phosphorylation. As depicted in Fig. 7B, relative to control (vehicle-exposed) cells, ASM cells exposed to isoproterenol (10 μM × 20 min) exhibited increased expression of phosphorylated ERK1/2, and, as expected, activation of ERK1/2 was ablated by pretreating the cells with the selective MEK-ERK1/2 inhibitor U0126. By comparison, pretreatment with the selective c-Raf1 inhibitor GW5074 (20 μM) completely abrogated the stimulatory effect of isoproterenol on ERK1/2 phosphorylation, whereas cells pretreated with GGTI-298 (25 μM), a potent selective inhibitor of Rap1 activation (21), did not exhibit attenuated isoproterenol-induced ERK1/2 phosphorylation. Thus, together with the above results, these observations support the concept that activation of ERK1/2 in β2AR agonist-exposed ASM cells is attributed to PKA-dependent activation of G$_i$βγ signaling, which, in turn, mediates Src-induced stimulation of the Ras/c-Raf1/MEK-ERK1/2 pathway.

Fig. 6. β2AR stimulation elicits PKA-dependent activation of ERK1/2 in ASM cells. A: Western blot depicting that isoproterenol and salmeterol acutely evoke enhanced ERK1/2 phosphorylation. B: Isoproterenol-induced phosphorylation of ERK1/2 is prevented in ASM cells that are transfected with the PKA siRNA duplexes, whereas transfection with a scrambled (control) siRNA duplex (scRNA) has no effect. C: Corresponding densitometric analysis of the changes in ERK1/2 phosphorylation demonstrates that, relative to control cells (lane 1), β2AR-stimulated cells exhibit a mean 2.3-fold increase in ERK1/2 phosphorylation (lane 2). The latter β2AR agonist-induced phosphorylation of ERK1/2 is prevented in cells pretreated with the PKA siRNA duplexes (lane 6), whereas pretreatment with the scRNA duplex has no effect (lane 4). Data represent means ± SE values based on 4 measurements obtained under each treatment condition. *P < 0.05.

Fig. 7. β2AR agonist-induced phosphorylation of ERK1/2 in ASM cells is mediated by G protein βγ-subunit-mediated activation of the Ras signaling cascade. A: Western blot showing that, in contrast to ASM cells transfected with aden-LacZ (i.e., negative control), β2AR agonist-induced ERK1/2 phosphorylation is prevented in ASM cells wherein Gβγ signaling is inhibited by transfection with aden-βARK-ct. B: Western blot depicting that pretreatment with the c-Raf1 inhibitor GW5074 prevents isoproterenol-induced phosphorylation of ERK1/2 in ASM cells, whereas inhibition of Rap1 with GGTI-298 has no effect.
$G_{	ext{i}}$-$\beta_{\gamma}$ signaling regulates PDE4 activity and constrictor and relaxation responsiveness in homologous $\beta$2AR-desensitized ASM. To ascertain the physiological implications of the above mechanism of $G_{	ext{i}}$-$\beta_{\gamma}$-coupled interplay between the PKA and ERK1/2 signaling pathways in the homologous $\beta$2AR-desensitized state, we next examined the effects of inhibition of $G_{	ext{i}}$ protein function and $G_{	ext{i}}$-$\beta_{\gamma}$-specific signaling on the changes in PDE activity and constrictor and relaxation responsiveness induced by prolonged homologous $\beta$2AR desensitization in human ASM cells and rabbit ASM tissues, respectively. As shown in Fig. 8, relative to untreated (vehicle-exposed) control ASM cells, cAMP PDE activity was significantly increased in cells that were incubated for 24 h with 10 $\mu$M salmeterol or isoproterenol, and this stimulation of PDE4 by either $\beta$2AR agonist was completely abrogated in cells wherein $G_{	ext{i}}$ protein signaling was interrupted by pretreatment with PTX (100 ng/ml) or with 1 $\mu$M of a $G_{	ext{i}}$-$\beta_{\gamma}$-specific sequestering peptide (anti-$G_{	ext{i}}$-$\beta_{\gamma}$ peptide) coupled to a cell membrane-permeable carrier peptide sequence (MPS). By comparison, stimulation of PDE activity by either $\beta$2AR agonist was unaffected in cells pretreated with a MPS-coupled peptide directed at inhibiting $G_{\alpha}3$ signaling (anti-$G_{\alpha}3$ peptide; 1 $\mu$M). Comparably, as depicted in Fig. 9, relative to the responses obtained in control rabbit ASM tissues, the significantly increased constrictor responses to ACh (Fig. 9A) and impaired relaxation responses to isoproterenol (Fig. 9B) detected in SAL-exposed ASM tissues were prevented by pretreating these tissues with the anti-$G_{	ext{i}}$-$\beta_{\gamma}$ peptide (30 $\mu$M), whereas pretreatment with the anti-$G_{\alpha}3$ peptide (30 $\mu$M) had no effect. In relation to these observations, it should be noted that, in separate experiments, we found that: 1) the induced changes in constrictor and relaxation responsiveness in SAL-exposed rabbit ASM tissues were also completely abrogated by pretreating these tissues with 100 ng/ml PTX (data not shown); and 2) in contrast to SAL-exposed ASM tissues, neither constrictor nor relaxation responsiveness was appreciably affected by pretreating vehicle-exposed (control) tissues with either the peptide inhibitors or PTX (data not shown). Thus, these data provide physiological evidence supporting the notion that the above $G_{	ext{i}}$-$\beta_{\gamma}$-coupled mechanism of $\beta$2AR agonist-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 (see Figs. 2 and 3, respectively) that are exhibited in the homologous $\beta$2AR-desensitized state.

**DISCUSSION**

While inhaled $\beta$2AR agonists are highly effective in acutely relieving bronchospasm, chronic use of long-acting $\beta$2AR agonists has been associated with an increase in asthma morbidity and mortality (1, 24, 28), which is thought to result, at least in part, from the development of airway tolerance to $\beta$2AR agonists due to homologous $\beta$2AR desensitization of the ASM (10). In examining the mechanism underlying the induction of altered ASM function under conditions of prolonged $\beta$2AR agonist exposure, the present findings demonstrate that:
1) homologous β2AR desensitization of ASM following its prolonged exposure to β2AR agonists evokes proaesthetic-like changes in ASM constrictor and relaxation responsiveness that are mediated by upregulated PDE4 activity; and 2) the latter is attributed to PKA-dependent induction of G_{i}βγ signaling that stimulates Src-mediated downstream activation of ERK1/2 via the Ras/c-Raf1/MEK-ERK1/2 pathway which, in turn, leads to transcriptional upregulation of PDE4 expression and its consequent proaesthetic actions. Thus, these novel findings identify a mechanism that potentially underlies the well-established association between prolonged airway exposure to β2AR agonists and aggravation of the asthmatic phenotype (1, 24, 28).

Our results demonstrated that pretreatment of human ASM cells with the PDE4 inhibitor rolipram prevented the impaired acute isoproterenol-induced accumulation of cAMP induced by initial prolonged exposure of the cells to the long-acting β2AR agonist salmeterol (Fig. 1). Moreover, the induction of cAMP PDE activity in ASM cells following prolonged exposure to salmeterol or isoproterenol was aborted by pretreating the cells either with rolipram, actinomycin D, or cycloheximide (Fig. 2, A and B), and also by pretreatment with either a PKA or ERK1/2 inhibitor (Fig. 2C). Comparably, the induction of proaesthetic-like changes in constrictor and relaxation responsiveness in rabbit ASM tissues exposed for 24 h to salmeterol was also prevented by pretreatment with rolipram or either the PKA or ERK1/2 inhibitor (Fig. 3). Collectively, these data suggested that the changes in ASM function accompanying prolonged homologous β2AR desensitization are mediated by upregulated PDE4 activity that is attributed to both PKA- and ERK1/2-dependent de novo mRNA and protein synthesis. This notion was substantiated by the extended observations demonstrating that β2AR agonist-exposed ASM cells exhibit increased mRNA and protein expression of PDE4D5, the functionally dominant PDE4 isoform in ASM cells (22), and that this induction of PDE4D5 is prevented by inhibiting either PKA or ERK1/2 activation (Figs. 4C and 5C).

Since PDE4D5 transcription in ASM cells is regulated by a CRE-containing promoter (22), we examined the role of PKA activation in mediating the effects of β2AR stimulation on signaling events coupled to CRE activation. The results demonstrated that stimulation of ASM cells with isoproterenol evokes acute phosphorylation of the CRE-regulating transcription cofactors CREB/ATF1 (Fig. 5A) and that this effect is inhibited by siRNA knockdown of PKA expression (Fig. 5B). Moreover, β2AR stimulation elicits acute phosphorylation of ERK1/2 (Fig. 6A), which is also known to evoke CREB phosphorylation via activation of the CREB kinases p90RSK or MSK-1 (17), and this effect is also prevented in ASM cells transfected with siRNAs directed against PKA (Fig. 6, B and C). Thus, these data suggested a regulatory interplay between PKA and ERK1/2 signaling in the β2AR agonist-exposed ASM, wherein PKA activation elicits ERK1/2 activation that, in turn, leads to CRE-driven PDE4D5 expression. In further investigating this suspected sequence of signaling events, the results demonstrated that, in addition to inhibitors of PKA and ERK1/2, β2AR agonist-induced PDE4D5 expression is also prevented in ASM cells that are pretreated with PTX or the Src inhibitor SU-6656 (Fig. 4C), implicating key intermediate regulatory roles for G_{i} protein and Src signaling that are coupled to PKA-dependent ERK1/2 activation and its consequent induction of PDE4D5. In this connection, it is noteworthy that previous studies in other cell types have demonstrated that phosphorylation of the β2AR by PKA can provoke a “switch” in receptor coupling from G_{i} to G_{s} protein signaling, a phenomenon that enables the βγ-subunits of G_{i} to activate Src, which, in turn, leads to downstream MEK/ERK1/2 activation (8, 23). In support of this mechanism, our extended observations demonstrated that PKA-dependent phosphorylation of ERK1/2 is prevented in β2AR agonist-exposed ASM cells that are transfected with the G_{i}βγ scavenger βARK-ct (Fig. 7A). Moreover, in demonstrating that β2AR agonist-induced ERK1/2 phosphorylation is inhibited in ASM cells pretreated with the c-Raf1 inhibitor GW5074, whereas pretreatment with a Rap1 inhibitor (GGTI-298) has no effect (Fig. 7B), our observations further implicated G_{i}βγ-generated signaling via the Ras/c-Raf1/MEK1/2 cascade in transducing the PKA dependence of ERK1/2 activation in the β2AR agonist-exposed ASM cells. Finally, the physiological relevance of G_{i}βγ signaling in mediating the PKA- and ERK1/2-dependent changes in ASM function in the β2AR-desensitized state (i.e., Fig. 3) was substantiated by the observations demonstrating that the upregulated PDE activity in human ASM cells exposed to β2AR agonists (Fig. 8) as well as the altered constrictor and relaxation responsiveness in β2AR agonist-exposed rabbit ASM tissues (Fig. 9) are prevented by pretreating the ASM preparations with inhibitors of G_{i} protein function, including PTX, which ribosylates G_{i}, or a cell-permeable anti-G_{i}βγ signaling peptide. Together, these data support the concept that, in concert with PKA and ERK1/2 activation, the rolipram-sensitive changes in ASM function in the homologous β2AR-desensitized state are attributed to G_{i} signaling via its βγ-subunits.

The present findings raise certain noteworthy considerations. In this regard, while our observations may be viewed as in concert with the above concept that G_{i}βγ signaling in the homologous β2AR-desensitized state is due a PKA-dependent switch in coupling of the β2AR from G_{i} to G_{s}, the latter leading to G_{i}βγ-mediated ERK1/2 activation, it should be noted that PKA-dependent activation of G_{i}βγ signaling has also been reported in the absence of β2AR agonist exposure. Accordingly, it has been demonstrated that COS-7 cells treated either with agonists acting via G_{s} or G_{i}-coupled receptors, a constitutively activated α_{i} mutant, a cAMP analog, or forskolin can acutely activate ERK, and that the latter effect is inhibited by sequestering the βγ-subunits (7, 9). Moreover, we recently demonstrated that PKA activation in ASM cells induced by receptor/G_{i}-coupled or nonreceptor-mediated stimulation of cAMP production with PGE_{2} or forskolin, respectively, also evokes G_{i}βγ-induced ERK1/2 activation (16). These latter findings do not support the concept of PKA-induced β2AR switching from G_{i} to G_{s}, since the latter phenomenon has been demonstrated only in the presence of a β2AR agonist (8, 23). Thus, although not inconsistent with a mechanism involving PKA-dependent switching of the β2AR from G_{i} to G_{s} activation, it is also possible that our observations implicating PKA-dependent activation of G_{i}βγ signaling leading to ERK1/2 activation in β2AR agonist-exposed ASM cells may be attributed to another mechanism that remains to be identified.

Another consideration relates to the extended known role of PKA in regulating PDE4 activity, including that PKA can directly stimulate PDE4D5 activity, as well as that of other
PDE4D long isoforms, by phosphorylating a regulatory site within the NH2-terminal region of these isoforms (6, 15). Interestingly, in this context, it has been reported that PKA activation can acutely reverse the reported inhibitory phosphorylation of PDE4D5 by ERK1/2 (15). Moreover, recent studies have demonstrated that the acute cAMP responses detected in subcellular microdomains of HEK-293 cells exposed to a β2AR or prostanoid receptor agonist are rapidly attenuated by PKA-dependent PDE4 activity (notably involving PDE4D5), which mediates homologous desensitization of these receptors to subsequent agonist administration (36, 37). While potentially also important in ASM under conditions associated with acute homologous β2AR desensitization, it is unlikely that the latter posttranslational mechanisms involving acute PKA-dependent activation of PDE4 activity significantly contributed to the upregulated PDE4 activity detected herein following prolonged β2AR agonist exposure, given that the enhanced PDE4 activity was completely ablated by inhibiting either de novo mRNA or protein synthesis with actinomycin D or cycloheximide, respectively (Fig. 2B).

Finally, it is noteworthy that our data showed similar effects of prolonged exposure to either salmeterol or isoproterenol on the induction and regulation of PDE4 activity and its associated changes in ASM function. Salmeterol is a long-acting β2AR agonist that exhibits relatively low intrinsic efficacy and high β2AR selectivity, whereas isoproterenol is a shorter-acting agonist that has high intrinsic efficacy and low β2AR selectivity (26). These differences notwithstanding, there is ample clinical evidence demonstrating that chronic use of long-acting β2AR agonists with low intrinsic efficacy (24, 38, 39) and overuse of short-acting β2AR agonists with high intrinsic efficacy (30, 38) are both associated with worsening of the asthmatic condition. In light of this evidence, it is likely that the herein described mechanism underlying the proasthmatic effects of prolonged homologous β2AR desensitization in ASM is responsible for mediating the adverse effects on airway function associated with prolonged or frequent exposure of asthmatic patients to β2AR agonists, independently of their intrinsic efficacy or β2AR selectivity.

In conclusion, the present study examined the regulation and role of PDE4 activity in mediating the changes in ASM function that accompany its prolonged homologous β2AR desensitization. The results provide new evidence demonstrating that: 1) prolonged exposure of ASM to β2AR agonists results in attenuated cAMP accumulation associated with increased cholinergic agonist-mediated ASM constriction and impaired β2AR-mediated ASM relaxation; 2) these changes in ASM function are attributed to upregulated PDE4 activity, reflective of increased transcription of the PDE4D5 isoform; 3) the upregulated expression of PDE4D5 is due to PKA-dependent activation of G, protein signaling, consistent with a potential switch in coupling of the

Fig. 10. Schematic representation of the proposed mechanism of induction of proasthmatic changes in ASM responsiveness following prolonged homologous β2AR desensitization. During early β2AR signaling initiated by ASM exposure to a β2AR agonist (e.g., salmeterol or isoproterenol), activation of PKA leads to acute uncoupling of the phosphorylated β2AR from G, protein (10, 23) and activation of G,-βγ signaling, a phenomenon that may be reflective of PKA-induced switching of β2AR coupling from G, to G, (8, 23). Activation of the G,-βγ-subunit then initiates Src-mediated stimulation of the Ras/Raf1/MEK1/2 signaling pathway, resulting in downstream ERK1/2 activation which, in turn, leads to phosphorylation of the transcription factors CREB and ATF1 and their induction of CRE-driven PDE4D5 gene transcription. The latter induction of PDE4 expression and action results in homologous β2AR desensitization (i.e., late β2AR hyporesponsiveness) that is evidenced by attenuated cAMP accumulation in response to subsequent β2AR agonist exposure, which is associated with impaired ASM relaxation and heightened ASM contractility (16). The inhibitors used herein to identify the above signaling pathways are indicated within the boxes shown.

It is important to note that this schematic representation of the proposed mechanism underlying prolonged homologous β2AR desensitization in ASM does not depict the reported acute interactions between PKA and PDE4 activity and between ERK and PDE4 activity and its acute feedback modulation by PKA activation that have been implicated in other cell types (15). AC, adenosyl cyclase.
β2AR from Gα to Gi, resulting in Gβγ-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 initiates CRE-driven PDE4D5 gene transcription. Collectively, these novel findings identify that the proasthmatic changes in ASM function that accompany its homologous β2AR desensitization due to prolonged exposure to β2AR agonists are mediated by upregulated PDE4 activity that is attributed to Gβγ-coupled cross-talk between the cAMP/PKA and ERK1/2 signaling pathways. A schematic representation of this identified mechanism of induction of proasthmatic changes in ASM responsiveness following prolonged homologous β2AR desensitization is provided in Fig. 10. Based on this new evidence, interventions targeted at regulatory sites within this cross-talk mechanism may provide novel therapeutic approaches to mitigate the adverse consequences of chronic or frequent use of β2AR agonists in the treatment of asthma.

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REFERENCES


