

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
13 August 2009 (13.08.2009)

PCT

(10) International Publication Number
WO 2009/100445 A2

(51) International Patent Classification:
A61K 38/10 (2006.01)

(21) International Application Number:
PCT/US2009/033581

(22) International Filing Date:
9 February 2009 (09.02.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/026,915 7 February 2008 (07.02.2008) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report (Rule 48.2(g))

(54) Title: COMPOSITIONS AND METHODS WHICH MODULATE G-PROTEIN SIGNALING FOR THE TREATMENT OF ASTHMA

(57) Abstract: Compositions and methods for the treatment of asthma are disclosed.



WO 2009/100445 A2

**COMPOSITIONS AND METHODS WHICH MODULATE
G-PROTEIN SIGNALING
FOR THE TREATMENT OF ASTHMA**

By Michael M. Grunstein

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This application claims priority to US Provisional Application 61/026,915, filed February 7, 2008, the entire disclosure of which is incorporated by reference herein.

10 This work was supported in part with grant money from the National Institutes of Health, Grant Numbers, HL-31467 and HL-61038. Accordingly, the United States government may have certain rights in the invention described herein.

FIELD OF THE INVENTION

15 This invention relates to the fields of signal transduction, respiratory physiology and the treatment or alleviation of the asthmatic condition. More specifically, the invention provides molecules which modulate G protein signaling which, upon delivery to the airway, alleviate the symptoms of asthma.

20 **BACKGROUND OF THE INVENTION**

Several publications and patent documents are cited throughout the specification in order to describe the state of the art to which this invention pertains. Each of these citations is incorporated herein by reference as though set forth in full.

25 Inhalation of β_2 -adrenergic receptor (β_2 AR) agonists in the treatment of asthma is the most effective approach to acutely relieve bronchospasm, reflecting the ability of these agents to dilate the airways by relaxing the surrounding airway smooth muscle (ASM). Chronic use of long-acting β_2 AR agonists, however, has been associated with heightened bronchoconstrictor responsiveness to airway spasmogens, exacerbation of
30 asthma symptoms, and an increased incidence of asthma-related morbidity and mortality (4,31,37). This aggravation of the asthmatic condition is thought to result from

heightened desensitization of the airways to the bronchodilatory action of β 2AR agonists, a phenomenon that is exhibited in isolated asthmatic ASM tissues, together with an increased contractile responsiveness of the tissues to bronchoconstrictor agents (2,3,13). Similarly, naïve ASM tissues and cultured ASM cells exposed to a variety of pro-asthmatic stimuli including atopic asthmatic serum, inflammatory cytokines, and inoculation with rhinovirus, also display attenuated β 2AR-mediated relaxation accompanying reduced cAMP generation and increased ASM constrictor responsiveness (20,39). To date, investigations into the etiology of the tolerance of asthmatic airways to β 2AR stimulation have largely focused on the potential contributions by mechanisms implicated in mediating homologous (agonist-specific) and/or heterologous (non-agonist-specific) desensitization of the β 2AR in ASM (12,39). Accordingly, the role played by phosphorylation of the β 2AR by G protein-coupled receptor (GPCR) kinases (GRKs) and cAMP-dependent protein kinase A (PKA) in mediating homologous β 2AR desensitization has been demonstrated acutely following exposure of ASM cells to β 2AR agonists, and that played by PKA activation in mediating heterologous β 2AR desensitization has been demonstrated following more prolonged exposure of ASM to other receptor-coupled or non-receptor-coupled cAMP-elevating agents (12,15,35,39). Collectively, these studies have provided important information regarding the mechanisms involved in uncoupling of the β 2AR from its associated Gs protein-mediated accumulation of cAMP, a process resulting in impaired ASM relaxation in the β 2AR-desensitized state. These mechanisms notwithstanding, a host of studies conducted on different cell types have demonstrated that the attenuated physiological responses to cAMP-elevating agents detected under conditions associated with homologous or heterologous β 2AR desensitization are critically regulated by cAMP phosphodiesterase (PDE) activity (6,9,22,42). Moreover, there is compelling recent evidence that PDE activity plays a crucial role in regulating ASM contractility (33), and in mediating the constrictor hyperresponsiveness of the airways accompanying allergen challenge in asthmatic subjects (43) and in animal models of allergic asthma (8,21,24,41). Little is known, however, regarding the mechanism regulating PDE expression and its role in

contributing to the impaired cAMP signaling exhibited in ASM under conditions of prolonged homologous or heterologous β 2AR 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 (33). 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. (9,22). 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) (9,22). 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) (5,30). In vascular smooth muscle cells, PDE4D expression was also found to be translationally regulated by PKA, as well as by the MAPK, ERK1/2 (32). Moreover, PKA and ERK1/2 were shown to directly regulate PDE4D catalytic activity (9,22).

SUMMARY OF THE INVENTION

The results presented herein demonstrate that Gi- β y signaling serves to mediate upregulated PDE4 activity in β 2AR-desensitized airway tissues and the associated pro-asthmatic state. Thus, in accordance with the present invention, compositions and methods are provided for delivering G β y inhibitors into the airway of patients with asthma, particularly, those patients experiencing symptoms associated with β 2-adrenergic receptor desensitization. The compositions of the invention can be directly delivered to the lung, preferably in aerosolized form. They may optionally be combined with one or more conventional agents employed in the treatment of asthma, including without limitation, corticosteroids, sodium cromolyn, methylxanthines, phosphodiesterase inhibitors, beta 2 adrenergic agents, and leukotriene modifiers.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Inhibition of PDE4 prevents the induction of altered ASM tissue constrictor and relaxation responsiveness accompanying heterologous β 2AR desensitization.

- 5 Relative to vehicle-treated controls, rabbit ASM tissues exposed for 24 hr to prostaglandin (PG)E2 (100 nM) exhibit significantly increased constrictor responses to acetylcholine (ACh) (A) and impaired relaxation responses to isoproterenol (B). Pretreatment with the PDE4 inhibitor, rolipram (10 μ M), prevents these PGE2-induced changes in ASM constrictor and relaxation responsiveness, while having no significant
10 effect on ASM responsiveness in vehicle-exposed tissues. Data represent mean $\pm$ SE values from 5 paired experiments.

- Figure 2.** Inhibition of PDE4 prevents the induction of impaired β 2AR-mediated cAMP accumulation accompanying heterologous β 2AR desensitization in cultured human ASM
15 cells. In vehicle-exposed ASM cells (*open bars*), isoproterenol (ISO; 1.0 μ M) acutely elicits cAMP accumulation, and the magnitude of this response is significantly attenuated in PGE2-exposed cells ($p < 0.05$). By comparison, in ASM cells pretreated with rolipram (10 μ M; *hatched bars*), the cAMP responses to isoproterenol are increased, and unaltered by pre-exposing the cells to PGE2. Data represent mean $\pm$ SE values from 3 experiments.

- 20 **Figure 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 hr to 100 nM of PGE2 or forskolin. The stimulated PDE activity is ablated in PGE2- and forskolin-exposed ASM
25 cells by pretreatment either with the PDE4 inhibitor, rolipram (A), the transcription or protein synthesis inhibitors, actinomycin D (Act D) and cycloheximide (CHX), respectively (B), or the PKA inhibitor, H89 (C). Data represent mean $\pm$ SE values based on 3-4 measurements obtained under each treatment condition. * $p < 0.05$; ** $p < 0.01$.

- 30 **Figure 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 hr. (B) Western blot depicting that PGE2 elicits transiently upregulated expression of phosphorylated CREB and ATF1 proteins in ASM cells, with peak phosphorylation detected at 30 min. (C) PGE2-induced phosphorylation of CREB/ATF1 is prevented in ASM cells transfected with siRNA
 5 duplexes directed against the PKA α and PKA β catalytic subunits, whereas transfection with a scrambled (control) siRNA duplex (scRNA) has no effect.

Figure 5. PGE2 elicits PKA-dependent activation of ERK1/2 in ASM cells. (A) Western blot depicting that PGE2 acutely elicits enhanced ERK1/2 phosphorylation. (B) PGE2-
 10 induced phosphorylation of ERK1/2 is prevented in ASM cells that are transfected with siRNA duplexes directed against the PKA α and PKA γ catalytic subunits, whereas transfection with a scrambled (control) siRNA duplex (scRNA) has no effect.

Figure 6. PGE2-induced activation of the PKA and ERK1/2 signaling pathways mediates
 15 upregulated expression of PDE4D5 transcripts in ASM cells. (A) Western blots depicting that PGE2-induced phosphorylation of ERK1/2 and CREB/ATF1 proteins is ablated in ASM cells pretreated with inhibitors of either PKA (H89), MEK-ERK1/2 (U1026), or Src tyrosine kinase (SU6656), or by ADP ribosylation of Gi protein with pertussis toxin (PTX). (B) PGE2-induced upregulated expression of PDE4D5 mRNA transcripts is also
 20 ablated in ASM cells pretreated with either of the latter inhibitors.

Figure 7. PGE2-induced phosphorylation of ERK1/2 in ASM cells is mediated by G protein $\beta\gamma$ -subunit-mediated activation of the Ras signaling cascade. (A) Western blot depicting that pretreatment with the c-Raf1 inhibitor, GW5074, prevents PGE2-induced
 25 phosphorylation of ERK1/2 in ASM cells, whereas inhibition of Rap1 with GGTI-298 has no effect. (B) Western blot showing that, in contrast to ASM cells transfected with adeno-LacZ (i.e., negative control), PGE2-induced ERK1/2 phosphorylation is prevented in ASM cells wherein G $\beta\gamma$ signaling is inhibited by transfection with adeno- β ARK-ct.

Figure 8. Gi protein and ERK1/2 activation mediate upregulated PDE activity in PGE2-
 30 and forskolin-treated ASM cells. PDE4 activity is significantly increased in ASM cells

exposed for 24 hr to 100 nM of PGE2 or forskolin. Stimulation of PDE activity is ablated in PGE2- and forskolin-exposed ASM cells by pretreatment with the ERK1/2 inhibitor, U0126, or by ADP ribosylation of Gi protein with PTX. Data represent mean $\pm$ SE values based on 4 measurements obtained under each treatment condition. ** $p < 0.01$.

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Figure 9. Changes in ASM constrictor and relaxation responsiveness accompanying PGE2-induced heterologous β 2AR desensitization are mediated by activation of the PKA and ERK1/2 signaling pathways. Relative to vehicle-treated controls, rabbit ASM tissues exposed for 24 hr to PGE2 (100 nM) exhibit significantly increased constrictor responses to ACh (A) and impaired relaxation responses to isoproterenol (B). The PGE2-induced changes in ASM constrictor and relaxation responsiveness are abrogated in tissues that are pretreated with inhibitors of either PKA (H89) or MEK1/2 (U1026), or by ADP ribosylation of Gi protein with PTX. Data represent mean $\pm$ SE values from 4 paired experiments.

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Figure 10. Inhibition of PDE4 prevents the induction of impaired β 2AR-mediated cAMP accumulation accompanying homologous β 2AR desensitization with the long-acting β 2AR agonist, salmeterol, in cultured human ASM cells. In contrast to vehicle-exposed (control) ASM cells, acute isoproterenol (ISO; 1.0 μ M)-induced cAMP accumulation is markedly attenuated in salmeterol-exposed cells ($p < 0.01$). By comparison, in ASM cells pretreated with rolipram (10 μ M), the cAMP responses to isoproterenol are increased and unaltered by pre-exposing the cells to salmeterol. Data represent mean $\pm$ SE values from paired 3 experiments.

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Figure 11. Regulation of cAMP PDE4 activity in cultured human ASM cells following prolonged exposure to β 2AR agonists. Relative to vehicle-treated (control) ASM cells, levels of cAMP activity are significantly increased in cells exposed for 24 hr to 10 μ M of either salmeterol or isoproterenol. The stimulated PDE activity is ablated in β 2AR agonist-exposed ASM cells by pre-treatment either with the PDE4 inhibitor, rolipram (A), the transcription or protein synthesis inhibitors, actinomycin D (Act D) and

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cycloheximide (CHX), respectively (**B**), the PKA inhibitor, H89, or the ERK1/2 inhibitor, U0126 (**C**). Data represent mean $\pm$ SE values based on 3-4 determinations made under each treatment condition. ** $p < 0.01$.

5 **Figure 12.** Inhibition of PDE4 or the PKA and ERK signaling pathways prevents the induction of altered ASM tissue constrictor and relaxation responsiveness accompanying prolonged homologous β 2AR desensitization. Relative to vehicle-treated controls, rabbit ASM tissues exposed for 24 hr to salmeterol (10 μ M) exhibit significantly increased constrictor responses to ACh (**A**) and impaired relaxation responses to isoproterenol (**B**).

10 Pre-treatment with the PDE4-selective inhibitor, rolipram (10 μ M), the PKA inhibitor, H89 (10 μ M) or the ERK1/2 inhibitor, U0126 (5 μ M) prevents the salmeterol-induced changes in ASM constrictor and relaxation responsiveness. Data represent mean $\pm$ SE values from 6-8 paired experiments.

15 **Figure 13.** Regulation of PDE4D5 mRNA expression in β 2AR agonist-exposed ASM cells. ASM cells treated with salmeterol (10 μ M) (**A**) or isoproterenol (10 μ M) (**B**) exhibit temporal increases in PDE4D5 mRNA expression, with peak expression of transcripts detected at 6 hr. (**C**) β 2AR agonist-induced upregulated expression of PDE4D5 mRNA transcripts is ablated in ASM cells pretreated with inhibitors of PKA
20 (H89), MEK-ERK1/2 (U0126), Src tyrosine kinase (SU6656), or by ADP ribosylation of Gi protein with PTX, whereas pre-treatment with the JNK inhibitor (SP600125) or the p38 MAPK inhibitor (SB202190) has no effect.

Figure 14. β 2AR agonist-exposed ASM cells exhibit PKA-dependent activation of the
25 CRE-regulating transcription co-factors, CREB and ATF1. (**A**) Western blot depicting that β 2AR stimulation elicits transiently upregulated expression of phosphorylated CREB and ATF1 proteins in ASM cells, with peak phosphorylation detected at 30 min. (**B**) β 2AR agonist-induced phosphorylation of CREB/ATF1 is prevented in ASM cells transfected with siRNA duplexes directed against the PKA α and PKA γ catalytic subunits,
30 whereas transfection with a scrambled (control) siRNA duplex (scRNA) has no effect.

Figure 15. β 2AR stimulation elicits PKA-dependent activation of ERK1/2 in ASM cells.

(A) Western blot depicting that isoproterenol or 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
 5 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*), β 2AR-stimulated cells exhibit a mean 2.3-fold increase in ERK1/2 phosphorylation (*lane 2*). The latter β 2AR agonist-induced phosphorylation of ERK1/2 is prevented in cells pretreated with the PKA siRNA
 10 duplexes (*lane 6*), whereas pretreatment with the scRNA duplex has no effect (*lane 4*). Data represent mean $\pm$ SE values based on 3 measurements obtained under each treatment condition. * $p < 0.05$.

Figure 16. β 2AR agonist-induced phosphorylation of ERK1/2 in ASM cells is mediated

15 by G protein $\beta\gamma$ -subunit-mediated activation of the Ras signaling cascade. (A) Western blot showing that, in contrast to ASM cells transfected with adeno-LacZ (i.e., negative control), β 2AR agonist-induced ERK1/2 phosphorylation is prevented in ASM cells wherein G $\beta\gamma$ signaling is inhibited by transfection with adeno- β ARK-ct. (B) Western blot depicting that pretreatment with the c-Raf1 inhibitor, GW5074, prevents isoproterenol-
 20 induced phosphorylation of ERK1/2 in ASM cells, whereas inhibition of Rap1 with GGTI-298 has no effect.

Figure 17. Pretreatment with the G $\beta\gamma$ inhibitory peptide prevents the induction of altered ASM tissue constrictor and relaxation responsiveness accompanying heterologous β 2AR
 25 desensitization. Relative to vehicle-treated controls, rabbit ASM tissues exposed for 24 hr to PGE2 (100 nM) exhibit significantly increased constrictor responses to ACh (A) and impaired relaxation responses to isoproterenol (B). Pretreatment with the G $\beta\gamma$ inhibitory peptide (30 μ M), prevents these PGE2-induced changes in ASM constrictor and relaxation responsiveness, while having no effect on ASM responsiveness in vehicle-
 30 exposed tissues. Data represent mean $\pm$ SE values from 4 paired experiments.

Figure 18. Pretreatment with the anti-G $\beta\gamma$ inhibitory peptide prevents the induction of cAMP PDE4 activity in cultured human ASM cells exposed to PGE2. Relative to vehicle-treated (control) ASM cells, levels of PDE4 activity are significantly increased in cells exposed for 24 hr to 100 nM of PGE2. The stimulated PDE activity is completely ablated in PGE2-exposed ASM cells that are pretreated either with the G $\beta\gamma$ inhibitory peptide or with PTX, whereas pretreatment with the anti-G α i2 or anti-G α i3 inhibitory peptide only exerts a partial inhibitory effect. Data represent mean $\pm$ SE values from 3 measurements made under each treatment condition. *p<0.05; **p<0.01.

Figure 19. Pretreatment with the G $\beta\gamma$ inhibitory peptide prevents the induction of altered relaxation responsiveness in ASM tissues passively sensitized with allergic asthmatic (AS) serum. Relative to tissues exposed to control (non-asthmatic) serum, rabbit ASM tissues exposed for 24 hr to AS serum exhibit significantly impaired relaxation responses to isoproterenol. The induction of impaired relaxation is prevented in AS serum-exposed ASM tissues by pretreating the tissues with the G $\beta\gamma$ inhibitory peptide, and the latter also enhances the relaxation responses to isoproterenol in control serum-exposed tissues. Data represent mean $\pm$ SE values from 3 paired experiments.

Figure 20. Gi- $\beta\gamma$ signaling mediates upregulated PDE activity in prolonged β 2AR-stimulated ASM cells. PDE4 activity is significantly increased in ASM cells exposed for 24 hr to 10 μ M of isoproterenol or salmeterol. Stimulation of cAMP PDE activity is ablated in β 2AR-exposed ASM cells by ADP ribosylation of Gi protein with PTX or by blockage of the $\beta\gamma$ subunit of Gi protein with the membrane permeable anti- $\beta\gamma$ MPS-coupled peptide, whereas pretreatment with the membrane permeable anti-G α i3 peptide has no effect. Data represent mean $\pm$ SE values based on 4 measurements obtained under each treatment condition. **p<0.01.

Figure 21. Changes in ASM constrictor and relaxation responsiveness accompanying salmeterol-induced homologous β 2AR desensitization are mediated by Gi- $\beta\gamma$ signaling. Relative to vehicle-treated controls, rabbit ASM tissues exposed for 24 hr to salmeterol exhibit significantly increased constrictor responses to ACh (A) and impaired relaxation

responses to isoproterenol (**B**). The salmeterol-induced changes in ASM constrictor and relaxation responsiveness are ablated in tissues that are pre-treated with the membrane permeable anti- $\beta\gamma$ MPS-coupled peptide, whereas pretreatment with the membrane permeable anti- $G_i\alpha_3$ peptide has no effect. Data represent mean $\pm$ SE values from 4
5 paired experiments.

DETAILED DESCRIPTION OF THE INVENTION

β_2 -adrenergic receptor (β_2 AR) agonists acutely relieve bronchoconstriction via
10 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 β_2 AR-desensitization in ASM accompanying prolonged cAMP signaling. Since cAMP phosphodiesterase (PDE) activity can significantly modulate ASM contractility, we investigated the mechanism
15 regulating PDE expression and its potential role in mediating changes in agonist-induced constrictor and relaxation responsiveness in ASM following heterologous desensitization of its β_2 AR by prolonged exposure to cAMP-elevating agents.

In accordance with the present invention, the mechanism regulating PDE4 expression and its role in mediating these changes has been elucidated and means of
20 modulating this desensitization are disclosed. The results provide new evidence demonstrating that: 1) desensitization of ASM to β_2 AR agonists, resulting from its prolonged exposure to either receptor- or non-receptor-coupled cAMP elevating agents, evokes increased ASM constrictor responsiveness together with impaired β_2 AR-mediated ASM relaxation and attenuated cAMP accumulation; 2) these pro-asthmatic-
25 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 a PKA-dependent activation of Gi protein-generated signaling via the $\beta\gamma$ -subunit of Gi, which leads to downstream activation of the Ras/Raf1/MEK pathway in the β_2 AR-desensitized
30 state. Collectively, these findings are the first to identify a mechanism that underlies the association between airway tolerance to β_2 AR agonists and heightened expression of the

asthmatic phenotype accompanying prolonged exposure of the airways to cAMP-elevating agents, thereby providing new avenues for the development of efficacious agent(s) for the treatment of asthma.

Thus, the present invention relates to the use of $G\beta\gamma$ inhibitors for the treatment of asthma. In a preferred embodiment localized, aerosolized administration of the $G\beta\gamma\beta\gamma$ blocking peptide to the airway and lung provides a higher effective dose than that obtainable via systemic administration. Localized administration of the inhibitor directly to the lung of the patient avoids systemic exposure to the peptide which may result in undesirable secondary side effects. Another advantage of localized aerosolized delivery is that this approach enables administration of lower doses of inhibitor which avoids the inefficient targeting of the $G\beta\gamma$ blocking peptide associated with systemic administration due to clearance by the liver or incomplete absorption in the intestine from oral administration. The compositions and methods are also advantageous in that they provide non-steroidal agents that are effective at alleviating the asthmatic condition.

Aerosolization provides an excellent method for delivering $G\beta\gamma$ blocking peptides. Utilizing this approach, $G\beta\gamma$ peptide inhibitors can be delivered directly in the local environment of the airway as an aerosol, thereby directly targeting airway smooth muscle cells. Inclusion of a membrane permeant peptide sequence from the signal sequence of Kaposi fibroblast growth factor to the $G\beta\gamma$ blocking peptide facilitates entry of the inhibitor into the cells in the airway.

In the present invention, G protein signaling is modulated using a $G\beta\gamma$ blocking peptide. An exemplary blocking peptide attached to a membrane permeable carrier peptide has the following letter code sequence:

AAVALLPAVLLALLAVTDQLGEDFFAVDLEAFLQEFGLLPEKE; (SEQ ID NO: 1)

Exemplary methods entail delivering $G\beta\gamma$ blocking peptides into patients with asthma, particularly, those patients experiencing symptoms associated with $\beta 2$ -adrenergic receptor desensitization. The compositions of the invention can be directly delivered to the lung, as opposed to previous studies which have not investigated delivery to the pulmonary system.

Thus, a new approach for treating asthma is described herein. The peptides of the invention may be used alone or combined with other agents used to treat asthma or genes encoding proteins to augment the anti-asthmatic/anti-inflammatory efficacy of the peptides.

5 The following description sets forth the general procedures involved in practicing the present invention. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the invention. Unless otherwise specified, general biochemical and molecular biological procedures, such as those set forth in Sambrook et al., Molecular Cloning, Cold Spring Harbor Laboratory (1989)
10 (hereinafter "Sambrook et al.") or Ausubel et al. (eds) Current Protocols in Molecular Biology, John Wiley & Sons (1997) (hereinafter "Ausubel et al.") are used.

I. Definitions:

15 The following definitions are provided to facilitate an understanding of the present invention:

 As used herein, the term "G $\beta\gamma$ inhibitor" refers to any molecule or compound which is able to disrupt G protein $\beta\gamma$ interactions and thereby impede downstream signaling. For example, a "G $\beta\gamma$ blocking peptide" refers to a peptide which targets G $\beta\gamma$ subunits, mimics the protein binding domains thereof and blocks their interaction. Such
20 "blocking peptides" also include a membrane permeant peptide sequence. Additional $\beta\gamma$ inhibitors include, without limitation, the carboxy terminus of the BARK protein (BARK-ct as shown in Figure 7 herein), $\beta\gamma$ peptide inhibitors comprising fragments of phosducin e.g. amino acids 217-246 and amino acids 213-233 such as those described in
25 EP 0 896 965 A1 and the alpha subunit of transducin protein. Ideally each of the aforementioned inhibitors will be operably linked to a membrane permeable peptide sequence to facilitate entry into cells surrounding the airway, including the airway smooth muscle cells.

 A "membrane permeant peptide sequence" refers to a peptide sequence which is able to facilitate penetration and entry of the $\beta\gamma$ inhibitor across the cell membrane.
30 Exemplary peptides include with out limitation, the signal sequence from Karposi fibroblast growth factor exemplified herein, the HIV tat peptide (Vives et al., J Biol.

Chem., 272:16010-16017, 1997), Nontoxic membrane translocation peptide from protamine (Park et al. FASEB J. 19(11):1555-7, 2005), CHARIOT® delivery reagent (Active Motif; US Patent 6,841,535) and the antimicrobial peptide Buforin 2.

By the term "asthmatic state" as used herein, is meant the proasthmatic phenotype which is observed in airway smooth muscle cells. This phenotype is characterized by increased contraction and decreased relaxation of the airway tissue when it has been exposed for extended time periods to cAMP-elevating agents such as beta2-adrenergic agonists, pro-asthmatic stimuli such as specific cytokines, high IgE-containing atopic asthmatic serum or exogenous IgE, compared with airway tissue which has not been exposed to these agents or stimuli. By the term "treating asthma" is meant curing asthma, causing the symptoms of asthma to diminish, ablating or otherwise alleviating the disease.

The term "aerosol formulation" refers to a pharmaceutical composition suitable for administration through the respiratory system or nasal passages. Examples of aerosol formulations are described below. Similarly, the term "aerosol administration" is intended to refer to a mode of administering an aerosol formulation to the respiratory system or nasal passages.

The invention provides a composition of matter comprising an aerosol formulation of the inhibitor where the G β γ blocking peptides is present at a concentration ranging from 0.001 mg to 1000 mg. Peptides of the invention can be functional fragments of proteins.

"Peptide" and "polypeptide" are used interchangeably herein and refer to a compound made up of a chain of amino acid residues linked by peptide bonds. The sequence for peptides is given in the order from the amino terminus to the carboxyl terminus. A peptide or peptide fragment is "derived from" a parent peptide or polypeptide if it has the amino acid sequence that is identical or homologous to the amino acid sequence of the parent peptide or polypeptide.

The term "substantially pure" refers to a preparation comprising at least 50-60% by weight of a given material (e.g., peptide, protein, etc.). More preferably, the preparation comprises at least 75% by weight, and most preferably 90-95% by weight of the given compound. Purity is measured by methods appropriate for the given compound

(e.g. chromatographic methods, agarose or polyacrylamide gel electrophoresis, HPLC analysis, and the like).

The term “functional” as used herein implies that the nucleic or amino acid sequence is functional for the recited assay or purpose.

5 The phrase “consisting essentially of” when referring to a particular amino acid means a sequence having the properties of a given SEQ ID NO. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the essential and novel characteristics of the sequence.

10 As disclosed herein, Gβγ blocking peptides are effective at reducing a sign or symptom of asthma and thus are useful for the treatment thereof. The compositions of the invention are effective at inhibiting pro-asthmatic changes in airway smooth muscle tissue.

II. Pharmaceutical Compositions:

15 Methods of the invention directed to treating asthma involve the administration of a Gβγ inhibitor in a pharmaceutical composition. A Gβγ inhibitor is administered to an individual as a pharmaceutical composition comprising a Gβγ inhibitor and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known in the art and include aqueous solutions such as physiologically buffered saline,
20 other solvents or vehicles such as glycols, glycerol, oils such as olive oil or injectable organic esters.

A pharmaceutically acceptable carrier can contain physiologically acceptable compounds that act, for example, to stabilize the Gβγ blocking peptide or increase the absorption of the agent. Such physiologically acceptable compounds include, for
25 example, carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. One skilled in the art would know that the choice of a pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the Gβγ blocking peptide and on
30 the particular physico-chemical characteristics of the peptide.

One skilled in the art appreciates that a pharmaceutical composition comprising a

G β y blocking peptide can be administered to a subject by various routes including, for example, orally or parenterally, such as intravenously (i.v.), intramuscularly, subcutaneously, intraorbitally, intranasally, intracapsularly, intraperitoneally (i.p.), intracisternally, intra-tracheally (i.t), or intra-articularly or by passive or facilitated
5 absorption, and most preferably, using a nasal spray or inhalant.

Administration of a G β y inhibitor by inhalation is a particularly preferred means of treating an individual having asthma. One skilled in the art would recognize that a G β y blocking peptide can be suspended or dissolved in an appropriate pharmaceutically acceptable carrier and administered, for example, directly into the lungs using a nasal
10 spray or inhalant.

A pharmaceutical composition comprising a G β y peptide inhibitor can be administered as an aerosol formulation which contains the inhibitor in dissolved, suspended or emulsified form in a propellant or a mixture of solvent and propellant. The aerosolized formulation is then administered through the respiratory system or nasal
15 passages.

An aerosol formulation used for nasal administration is generally an aqueous solution designed to be administered to the nasal passages in drops or sprays. Nasal solutions are generally prepared to be similar to nasal secretions and are generally isotonic and slightly buffered to maintain a pH of about 5.5 to about 6.5, although pH
20 values outside of this range can additionally be used. Antimicrobial agents or preservatives can also be included in the formulation.

An aerosol formulation used for inhalations and inhalants is designed so that the G β y blocking peptide is carried into the respiratory tree of the patient administered by the nasal or oral respiratory route. Inhalation solutions can be administered, for example, by a
25 nebulizer. Inhalations or insufflations, comprising finely powdered or liquid drugs, are delivered to the respiratory system as a pharmaceutical aerosol of a solution or suspension of the drug in a propellant.

An aerosol formulation generally contains a propellant to aid in disbursement of the G β y blocking peptide. Propellants can be liquefied gases, including halocarbons, for
30 example, fluorocarbons such as fluorinated chlorinated hydrocarbons, hydrochlorofluorocarbons, and hydrochlorocarbons as well as hydrocarbons and

hydrocarbon ethers (Remington's Pharmaceutical Sciences 18th ed., Gennaro, A.R., ed., Mack Publishing Company, Easton, Pa. (1990)).

Halocarbon propellants useful in the invention include fluorocarbon propellants in which all hydrogens are replaced with fluorine, hydrogen-containing fluorocarbon propellants, and hydrogen-containing chlorofluorocarbon propellants. Halocarbon propellants are described in Johnson, U.S. Pat. No. 5,376,359, and Purewal et al., U.S. Pat. No. 5,776,434.

Hydrocarbon propellants useful in the invention include, for example, propane, isobutane, n-butane, pentane, isopentane and neopentane. A blend of hydrocarbons can also be used as a propellant. Ether propellants include, for example, dimethyl ether as well as numerous other ethers.

The Gβγ blocking peptide can also be dispensed with a compressed gas. The compressed gas is generally an inert gas such as carbon dioxide, nitrous oxide or nitrogen.

An aerosol formulation of the invention can also contain more than one propellant. For example, the aerosol formulation can contain more than one propellant from the same class such as two or more fluorocarbons. An aerosol formulation can also contain more than one propellant from different classes. An aerosol formulation can contain any combination of two or more propellants from different classes, for example, a fluorohydrocarbon and a hydrocarbon.

Effective aerosol formulations can also include other components, for example, ethanol, isopropanol, propylene glycol, as well as surfactants or other components such as oils and detergents (Remington's Pharmaceutical Sciences, 1990; Purewal et al., U.S. Pat. No. 5,776,434). These aerosol components can serve to stabilize the formulation and lubricate valve components.

The aerosol formulation can be packaged under pressure and can be formulated as an aerosol using solutions, suspensions, emulsions, powders and semisolid preparations. A solution aerosol consists of a solution of an active ingredient such as a Gβγ blocking peptide in pure propellant or as a mixture of propellant and solvent. The solvent is used to dissolve the active ingredient and/or retard the evaporation of the propellant. Solvents useful in the invention include, for example, water, ethanol and glycols. A solution aerosol contains the active ingredient peptide and a propellant and can include any

combination of solvents and preservatives or antioxidants.

An aerosol formulation can also be a dispersion or suspension. A suspension aerosol formulation will generally contain a suspension of an effective amount of the G β γ blocking peptide and a dispersing agent. Dispersing agents useful in the invention
5 include, for example, sorbitan trioleate, oleyl alcohol, oleic acid, lecithin and corn oil. A suspension aerosol formulation can also include lubricants and other aerosol components.

An aerosol formulation can similarly be formulated as an emulsion. An emulsion can include, for example, an alcohol such as ethanol, a surfactant, water and propellant, as well as the active ingredient G β γ blocking peptide. The surfactant can be nonionic,
10 anionic or cationic. One example of an emulsion can include, for example, ethanol, surfactant, water and propellant. Another example of an emulsion can include, for example, vegetable oil, glyceryl monostearate and propane.

An aerosol formulation containing a G β γ blocking peptide will generally have a minimum of 90% of the particles in inhalation products between about 0.5 and about 10
15 μ m to maximize delivery and deposition of the G β γ blocking peptide to respiratory fluids. In particular, the particle size can be from about 3 to about 6 μ m.

A pharmaceutical composition comprising a G β γ blocking peptide inhibitor also can be incorporated, if desired, into liposomes, microspheres, microbubbles, or other polymer matrices (Gregoriadis, Liposome Technology, Vols. I to III, 2nd ed., CRC Press,
20 Boca Raton Fla. (1993)). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

In order to treat an individual having asthma, to alleviate a sign or symptom of the disease, a G β γ blocking peptide should be administered in an effective dose. The total
25 treatment dose can be administered to a subject as a single dose or can be administered using a fractionated treatment protocol, in which multiple doses are administered over a more prolonged period of time, for example, over the period of a day to allow administration of a daily dosage or over a longer period of time to administer a dose over a desired period of time. One skilled in the art would know that the amount of a G β γ
30 blocking peptide required to obtain an effective dose in a subject depends on many factors, including the age, weight and general health of the subject, as well as the route of

administration and the number of treatments to be administered. In view of these factors, the skilled artisan would adjust the particular dose so as to obtain an effective dose for treating an individual having asthma.

5 The effective dose of a G β γ blocking peptide will depend on the mode of administration, and the weight of the individual being treated. The dosages described herein are generally those for an average adult but can be adjusted for the treatment of children. The dose will generally range from about 0.001 mg to about 1000 mg.

10 The concentration of a G β γ blocking peptide in a particular formulation will depend on the mode and frequency of administration. A given daily dosage can be administered in a single dose or in multiple doses so long as the G β γ blocking peptide concentration in the formulation results in the desired daily dosage. One skilled in the art can adjust the amount of G β γ blocking peptide in the formulation to allow administration of a single dose or in multiple doses that provide the desired concentration of G β γ inhibitor over a given period of time.

15 In an individual suffering from asthma, in particular a more severe form of the disease, administration of a G β γ blocking peptide can be particularly useful when administered in combination, for example, with a conventional agent for treating such a disease. The skilled artisan would administer a G β γ blocking peptide, alone or in combination with a second agent, based on the clinical signs and symptoms exhibited by the individual and would monitor the effectiveness of such treatment using routine
20 methods such as pulmonary function determination, radiologic, immunologic or, where indicated, histopathologic methods.

A G β γ blocking peptide can be administered in combination with steroidal anti-inflammatory agents including corticosteroids, for example, dexamethasone, beclomethasone, fluticasone, triamcinolone and budesonide. A G β γ inhibitor can also be
25 administered in combination with non-steroidal anti-inflammatory agents such as, indomethacin, ibuprofen, naproxen, diclofenac, sulindac, oxaprozin, diflunisal, bromfenac, piroxicam, etodolac and fenoprofen. Inhibitor administration can also be combined with short- and long-acting β 2- adrenergic agents such as albuterol and
30 salmeterol, respectively, as the inhibitor alleviates the desensitization to the β 2-adrenoreceptor agent. When a G β γ blocking peptide is used with another anti-

inflammatory agent, the G $\beta\gamma$ inhibitor can generally be administered at a lower dosage. For example, a G $\beta\gamma$ inhibitor can be administered at a dose of less than 10 mg per day in combination with another anti-inflammatory agent.

When a G $\beta\gamma$ blocking peptide is administered in combination with one or more
5 other anti-inflammatory agent, the G $\beta\gamma$ blocking peptide and other anti-inflammatory agent can be co-administered in the same formulation. Alternatively, the G $\beta\gamma$ blocking peptide and other anti-inflammatory agent can be administered simultaneously in separate formulations. In addition, the G $\beta\gamma$ blocking peptide can be administered in separate
10 formulations, where the separate formulations are not administered simultaneously but are administered during the same period of treatment, for example, during a daily or weekly period of treatment. Alternatively, each agent may be given sequentially during a daily or weekly period of treatment.

Administration of the pharmaceutical preparation is preferably in a
“prophylactically effective amount” or a “therapeutically effective amount” (as the case
15 may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. This amount prevents, alleviates, abates, or otherwise reduces the severity of symptoms in a patient.

The pharmaceutical preparation is formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form, as used herein, refers to a
20 physically discrete unit of the pharmaceutical preparation appropriate for the patient undergoing treatment. Each dosage should contain a quantity of active ingredient calculated to produce the desired effect in association with the selected pharmaceutical carrier. Procedures for determining the appropriate dosage unit are well known to those skilled in the art.

25 Dosage units may be proportionately increased or decreased based on the weight of the patient. Appropriate concentrations for alleviation of a particular pathological condition may be determined by dosage concentration curve calculations, as known in the art. As mentioned previously, a preferred embodiment of the invention comprises aerosolized delivery of the G $\beta\gamma$ blocking peptide to the lungs of a patient in need thereof.
30 The G $\beta\gamma$ blocking peptide described herein can also be injected intra-peritoneally (i.p.),

intravenously (i.v.), or intratracheally (i.t.). Formulation, dosages and treatment schedules have also been described hereinabove.

The examples set forth below are provided to illustrate certain embodiments of the invention. They are not intended to limit the invention in any way.

5

The following materials and methods are provided to facilitate the practice of the Examples below.

Materials: All chemicals were purchased from Sigma-Aldrich unless otherwise indicated.

10 The human ASM cells were obtained from BioWhittaker, Inc.,

Animals: 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
15 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: The human ASM cells were grown in SmBm media
20 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 F12 media for 24 hr, treated with different concentrations and for varying durations with PGE₂ or forskolin
25 (heterologous desensitization) or salmeterol or isoproterenol (homologous desensitization), 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.

30 *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 to 100 μ M). In subsequent experiments, cells were pretreated for 24 hr with either vehicle alone or PGE2 (100 μ M), both in the absence and presence of rolipram (10 μ M). Following pretreatment, cells were exposed for 5 min to isoproterenol (10 μ M) at 37 °C, and intracellular cAMP accumulation was quantified by radioimmunoassay, as previously described (19).

In additional experiments, cells were pretreated for 24 h with either vehicle alone or salmeterol (10 μ M), both in the absence and presence of rolipram (10 μ M). Thereafter, in the continued presence of these treatments, cells were exposed for 5 min to isoproterenol (10 μ M) at 37°C, and intracellular cAMP concentrations were determined per manufacturer's protocols using a direct cAMP Enzyme Immunoassay Kit (R&D Systems, Minneapolis, MN). In both cases, the cAMP measurements were expressed in units of picomoles /milligram protein.

Assay of cAMP PDE activity: Levels of cAMP PDE activity were assayed in ASM cell lysates using a colorimetric, non-radioactive enzymatic assay kit from Biomol, as per the manufacture's protocol. PDE activity was determined following exposure of ASM cells for 24 hr to either vehicle alone (control), PGE2 (100 μ M), or forskolin (100 μ M) in the absence and presence of pretreatment with rolipram (10 μ M), cycloheximide (100 μ M), actinomycin D (4 μ M), or H89 (10 μ M).

In additional experiments, PDE activity was determined following exposure of ASM cells for 24 hr to either vehicle alone (control), salmeterol (10 μ M) or isoproterenol (10 μ M), in the absence and presence of pre-treatment with rolipram (10 μ M), cycloheximide (100 μ M), actinomycin D (4 μ M), H89 (10 μ M), U0126 (5 μ M), Pertussis toxin (100ng/ml), anti- α 3 peptide MPS (1 μ M) or anti- β γ peptide MPS-phosducin-like protein (1 μ M) from Ana spec, Inc. (San Jose, USA). In each case, the measured levels of PDE activity were standardized to the 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'-

TGCCAGCTGTACAAAGTTGACC-3' (forward; SEQ ID NO: 2) and 5'-
TTCTCGGAGAGATCACTGGAGA-3' (reverse; SEQ ID NO: 3); and for β -actin, 5'-
GAGAAGAGCTACGAGCTGCCTGAC-3' (forward; SEQ ID NO: 4) and 5'-
CGGAGTACTTGCGCTCAGGAGGAG-3' (reverse; SEQ ID NO: 5). The reaction
5 volume was 20 μ l and cycling conditions used were 35 cycles of 30 sec denaturation at
95°C, followed by 30 sec annealing at 60°C and elongation at 72°C for 30 sec. Ex-Tag
(Takara Biotechnology) was used as DNA polymerase.

Immunoblot analysis of CREB/ATF1 and ERK1/2 activation: Levels of phosphorylated
10 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,
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
15 PVDF membrane. The membranes were then incubated overnight with monoclonal
mouse anti-human primary antibodies directed against phospho-CREB and -ATF1,
phospho-ERK1/2, or α -actin, and levels were detected by ECL after a 1-hr 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
20 quantified by densitometry.

siRNA-mediated knockdown of PKA: ASM cells were seeded into 6- 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-hr interval
25 with two pools of three siRNA duplexes, each pool targeted against the human PKA α or
PKA γ catalytic subunits (Santa Cruz Biotechnology; sc-36240 and sc-36236,
respectively), or with a non-targeted control (scrambled) siRNA duplex, using
Oligofectamine (Invitrogen) as the transfection agent. The pools of siRNAs were applied
to each well at a final concentration of 100 nM for each siRNA preparation. Based on
30 preliminary studies, this double-transfection approach was found to greatly enhance
transfection efficiency and, as detected by Western blot analysis, markedly inhibited

PKA α expression by the targeted siRNA duplexes, with maximal inhibition detected at 72 hr following siRNA transfection.

Transfection of ASM cells with adeno- β ARK-ct: Adenovirus (adeno)- β ARK-ct, an
5 adenovirus vector encoding the β ARK1 carboxyl-terminal domain which blocks G $\beta\gamma$
signaling (25,26), and adeno- 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 detected by
10 plaque assay. The ASM cells were transfected with either of the adenoviral vectors at a
multiplicity of infection (MOI) of 100, and experiments were conducted at 24 hr
following adenoviral transfections.

Pharmacodynamic studies of constrictor and relaxation responsiveness in rabbit ASM
15 *tissues:* Following initial sedation and subsequent general anesthesia with intramuscular
injections of xylazine (10 mg/kg) and ketamine (50 mg/kg), respectively, rabbits were
sacrificed with an intravenously administered overdose of sodium pentobarbital (100
mg/kg). As described previously (16), the tracheae were excised via open thoracotomy,
the loose connective tissue and epithelium were scraped and removed, and the tracheae
20 were divided into 8 ring segments, each of 6-8 mm in length. The airway segments were
then placed in modified Krebs-Ringer solution containing indomethacin (10 μ M), and
each alternate ring was incubated for 24 hr at room temperature in the presence of either
vehicle alone (control) or varying concentrations of PGE2 or salmeterol, each in the
absence and presence of either rolipram (10 μ M), the PKA inhibitor, H89 (10 μ M), the
25 MEK-ERK1/2 inhibitors, U0126 (5 μ M), or pertussis toxin (100 ng/ml). In the
homologous desensitization experiments using salmeterol, the cells were also incubated
in the presence of an anti-Gi α 3 peptide coupled to a membrane-permeable sequence
(MPS) (30 μ M) or an anti- $\beta\gamma$ peptide coupled to MPS (30 μ M) from AnaSpec, Inc. (San
Jose, USA). After these incubations, the tissues were placed in organ baths containing
30 modified Krebs-Ringer solution aerated with 5% CO₂ in oxygen (pH of 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 acetylcholine (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} - 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 $\pm$ SE values. Comparisons between groups were made using the Student's t-test (two-tailed) or ANOVA with Tukey's post-test analysis, where appropriate. A probability of <0.05 was considered statistically significant. Statistical analyses were conducted using the Prism computer program by GraphPad Software Inc.

15

EXAMPLE I

Prolonged heterologous β 2-adrenergic receptor desensitization elicits pro-asthmatic airway smooth muscle function via Gi- β -mediated upregulation of phosphodiesterase-4

Role of PDE4 in regulating constrictor and relaxation responsiveness in β 2AR-desensitized ASM.

To assess the role of PDE4 in mediating the effects of heterologous β 2AR desensitization on ASM function, constrictor responses to ACh and relaxation responses to isoproterenol, which acts via β 2AR activation in ASM, were compared in isolated rabbit ASM tissues exposed for 24 hr to either vehicle alone (control) or to a pre-determined maximally effective concentration of PGE2 (100 nM), both in the absence and presence of pretreatment of the tissues with the PDE4-selective inhibitor, rolipram (10 μ M). 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 $\pm$ 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 pre-constricted 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 β_2 AR desensitization. Accordingly, the mean $\pm$ SE maximal relaxation (R_{max}) response in the PGE2-exposed tissues amounted to 31.4 ± 5.4 % vs. the average R_{max} 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 (filled triangles) showed only modestly decreased constrictor responses to ACh and increased relaxation responses to isoproterenol, with neither of these changes being statistically significant.

As with PGE2, heterologous β_2 AR desensitization conferred by prolonged exposure to forskolin ($100 \mu\text{M} \times 24 \text{ hr}$), an agent that stimulates cAMP accumulation via direct (non-receptor-mediated) activation of adenylyl cyclase, also evoked significant increases in ASM constrictor responsiveness to ACh and impaired relaxation responsiveness to isoproterenol. Moreover, these effects were also completely abrogated by pretreating the forskolin-exposed ASM tissues with rolipram (data not shown).

Role of PDE4 in regulating altered cAMP responses in β_2 AR-desensitized ASM cells.

We next examined whether the changes in ASM constrictor and relaxation responsiveness obtained in the β_2 AR-desensitized state are reflective of rolipram-sensitive changes in β_2 AR 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 ($1.0 \mu\text{M}$) were compared in confluent cultures of ASM cells that were pretreated for 24 hr either with vehicle alone

or PGE2 (100 nM), both in the absence and presence of pretreatment with rolipram (10 μ M). As shown in Fig. 2, in the absence of rolipram (open bars), the PGE2-exposed ASM cells exhibited heterologous β 2AR desensitization, as evidenced by significantly reduced cAMP responses to isoproterenol relative to those detected in cells that were not exposed to the prostanoid. Contrasting these observations, ASM cells pretreated with rolipram (filled bars) exhibited increased isoproterenol-induced cAMP accumulation, and this response was preserved in the PGE2-exposed cells. Thus, in concert with the results obtained in rabbit ASM tissues, these observations implicate PDE4 activity in mediating the impaired isoproterenol-induced accumulation of cAMP accompanying heterologous β 2AR desensitization in human ASM cells.

Regulation of cAMP PDE activity in β 2AR-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 PGE2 or forskolin. Relative to the mean ($\pm$ SE) basal level of PDE activity detected in vehicle-treated (control) cells (i.e., 69.5 ± 4.8 pmol/min/mg protein), ASM cells incubated for 24 hr with 100 μ M of PGE2 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 PGE2- and forskolin-exposed cells that were pretreated with rolipram (10 μ M), implying that the augmented PDE activity exhibited by the β 2AR-desensitized cells was attributed to PDE4. In separate experiments, inclusion of the transcriptional inhibitor, actinomycin D (4 μ M), or the protein synthesis inhibitor, cycloheximide (100 μ M), in the ASM culture medium also completely ablated both the PGE2- 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 PGE2 and forskolin on PDE activity were also abrogated by co-incubating the ASM cells with the putative selective PKA inhibitor, H89 (10 μ M), 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

β 2AR desensitization in ASM cells, resulting from prolonged exposure to PGE2 or forskolin, evokes upregulated PDE4 activity that is due to PKA-dependent *de novo* mRNA and protein synthesis.

5 *Role of PKA in regulating PDE4D5 expression in PGE2-exposed ASM cells.*

Given recent evidence demonstrating that PDE4D5 is the functionally dominant cAMP-regulating PDE4 isoform in cultured human ASM cells (5), we next examined whether heterologous β 2AR desensitization in ASM cells evokes altered expression of *PDE4D5* transcripts. Confluent cultures of ASM cells exposed to PGE2 (100 nM x 24 hr) exhibited time-dependent increases in PDE4D5 mRNA expression, with peak induction detected at 6 hr and sustained upregulated expression observed for up to 24 hr (Fig. 4A). Densitometric analysis of the temporal changes in PDE4D5 mRNA expression examined in 3 separate experiments demonstrated that peak expression averaged 5.9-fold above that detected in unstimulated cells. Since PDE4D5 expression in ASM cells was previously shown to be regulated by a CRE-containing promoter (30), we subsequently examined the effects of PGE2 on cAMP/PKA-dependent downstream signaling events coupled to CRE activation. As shown in Fig. 4B, ASM cells treated with PGE2 (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 PGE2, we next assessed the effects of knockdown of PKA expression using siRNA duplexes directed against the human PKA α and PKA γ catalytic subunits. Preliminary experiments (n=3) demonstrated that Lipofectamine transfection of the PKA siRNA duplexes produced maximal knockdown of PKA α protein levels at 72 hr post-transfection that ranged between ~70-90%. Accordingly, confluent cultures of ASM cells were initially treated for 72 hr 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. 4C, in the absence of PGE2, basal levels of phosphorylated CREB/ATF1 protein expression were little affected by transfection with either the scrambled RNA (scRNA; lane 3) or PKA siRNA (lane 5) preparations.

Conversely, the PGE2-induced upregulated expression of phosphorylated CREB/ATF1 (*lane 2*) was distinctly inhibited in cells transfected with siRNAs directed against PKA (*lane 6*), whereas the scrambled siRNA sequence had no effect (*lane 4*). Thus, in concert with above observations implicating PKA activation in mediating the upregulated PDE4 activity in PGE2-exposed ASM cells, these data demonstrate that PKA activation is intimately involved in regulating PGE2-induced CREB/ATF1 phosphorylation associated with upregulated PDE4D5 expression.

PKA-mediated regulation of ERK1/2 activation in PGE2-exposed ASM cells.

Apart from its activation by the cAMP/PKA pathway, CREB can also be activated via other signaling events, notably including ERK1/2 activation (24). 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 (29) which, in turn, can mediate CREB phosphorylation via activation of the CREB kinases, p90RSK or MSK-1 (23). Given this evidence, together with recent reports implicating a critical role for ERK1/2 activation in mediating the hyporesponsiveness of ASM to β 2AR stimulation under different pro-asthmatic conditions (14,28,38), 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 (100 nM) 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). Densitometric analysis of the results from 3 separate experiments demonstrated that peak expression of the ratio of phosphorylated ERK1/2-to- α -actin averaged 5.3-fold above that detected in unstimulated cells (data not shown). In subsequently evaluating the potential role of PKA in mediating the PGE2-induced activation of ERK1/2, ASM cells were first treated for 72 hr with vehicle alone or with either scrambled RNA or siRNAs directed against the PKA catalytic subunits, and then examined for induced expression of phosphorylated ERK1/2 at 30 min following exposure to PGE2. As shown in Fig. 5B, relative to vehicle-treated control cells (*lane 1*), phosphorylated ERK1/2 expression was markedly increased in

PGE2-treated cells (*lane 2*) and, while ASM cells pretreated with either the scrambled or PKA siRNAs alone (*lanes 3 and 5*, respectively) exhibited slightly enhanced ERK1/2 phosphorylation, the PGE2-induced upregulated expression of phosphorylated ERK1/2 was distinctly inhibited in cells pretreated with the PKA siRNAs (*lane 6*), whereas pretreatment of the PGE-exposed cells with scrambled RNA had no appreciable effect (*lane 4*). Thus, these data demonstrate that the induction of ERK1/2 activation in PGE2-exposed ASM cells is regulated by PKA, as further examined below.

10 *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 expression of phosphorylated ERK1/2 and CREB/ATF proteins were detected 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 presence of pretreatment with previously reported maximal effective concentrations of specific inhibitors. As depicted in Fig. 6A, relative to controls (*lane 1*), cells treated with PGE2 exhibited increased expression of both phosphorylated ERK1/2 and CREB/ATF proteins (*lane 2*), and these effects were abrogated in PGE2-exposed cells that were pretreated with either the PKA inhibitor, H89 (10 μ M; *lane 3*), or the MEK-ERK1/2 inhibitor, U0126 (5 μ M; *lane 4*). By contrast, neither PGE2-induced phosphorylation of ERK1/2 nor CREB/ATF was affected in cells pretreated either with the p38 MAPK inhibitor, SB202190 (10 μ M; *lane 5*), or the JNK inhibitor, SP600125 (10 μ M; *lane 6*). Of significance, PGE2-induced up-regulated expression of phosphorylated ERK1/2 and CREB/ATF proteins was also largely prevented in 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 μ M; *lane 8*).

The above inhibitors were also examined with respect to their modulatory effects on the upregulated expression of PDE4D5 mRNA in PGE2-exposed ASM cells. PDE4D5 transcripts were detected by RT-PCR following exposure of ASM cells for 6 hr to vehicle

alone (control) or 100 nM PGE₂, both in the absence and presence of pretreatment with either inhibitor. In concert with their effects on PGE₂-induced expression of phosphorylated ERK1/2 and CREB/ATF, the inhibitors exerted comparable modulatory effects on PGE₂-induced upregulated expression of PDE4D5 transcripts. Accordingly, as shown in Fig. 6B, relative to control cells (*lane 1*), the induced upregulated expression of PDE4D5 mRNA in PGE₂-exposed ASM cells (*lane 2*) was largely prevented by pretreating 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 upregulated expression of PDE4D5 transcripts was markedly inhibited by pretreating the cells either with 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 that, in turn, serves to activate CREB/ATF proteins and, thereby, initiate PDE4D5 transcription.

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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 G_s-coupled or G_iβγ subunit-mediated stimulation of Src-induced signaling via the Rap1/B-Raf/MEK1/2 or the Ras/c-Raf1/MEK1/2 pathway, respectively (29). Moreover, cAMP-elevating agents can also activate ERK1/2 via a direct stimulatory effect of cAMP on EPAC (exchange protein directly activated by cAMP) proteins which, in turn, can initiate sequential downstream signaling via the Rap1/B-Raf/MEK1/2 pathway (29). 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 pretreating 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

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with GGTI-298 (25 μ M), a potent and selective inhibitor of Rap1 activation (27), 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 Gi- $\beta\gamma$ -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 hr following their transfection either with an adenovirus vector expressing *lacZ* (adeno-LacZ), serving as a negative control, or with adeno- β ARK-ct, which encodes the β ARK1 carboxyl-terminal domain that blocks G $\beta\gamma$ signaling (25,26), 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- β ARK-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 Gi- $\beta\gamma$ -mediated signaling via the Ras/c-Raf1/MEK1/2 pathway.

PKA-dependent ERK1/2 signaling regulates PDE4 activity and constrictor and relaxation responsiveness in β 2AR-desensitized ASM.

To ascertain the physiological implications of the above mechanism of interplay between the cAMP/PKA and c-Raf1/MEK/ERK1/2 signaling pathways in the β 2AR-desensitized state, we next examined the independent effects of inhibition of PKA, Gi protein, and ERK1/2 function on the changes in PDE activity and constrictor and relaxation responsiveness in human ASM cells and rabbit tissues, respectively, accompanying heterologous β 2AR desensitization. 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 hr with 100 μ M of 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 μ M), or PTX (100 ng/ml). 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 PGE2-exposed ASM tissues were abrogated by pretreating these tissues with either PTX or U1026, or with the PKA inhibitor, 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 tissues' constrictor or relaxation responses (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 β 2AR-desensitized state.

Example II

Homologous β 2-Adrenergic Receptor Desensitization in Airway Smooth Muscle: Role of Phosphodiesterase 4

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Role of PDE4 in regulating cAMP responses in ASM cells following prolonged homologous β 2AR desensitization.

Upregulated PDE4 activity has been implicated in mediating heterologous β 2AR desensitization in ASM following its prolonged exposure to receptor- and non-receptor-coupled cAMP elevating agents. See Example I. To determine the role of PDE4 activity in mediating homologous β 2AR desensitization in ASM, we compared 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 μ M) following prolonged exposure of cultured human ASM cells to either vehicle alone or to a maximally effective concentration of the long-acting β 2AR agonist, salmeterol (10 μ M x 24 hr), both in the absence and presence of co-treatment with the PDE4-selective inhibitor, rolipram (10 μ M). As shown in Fig. 10, relative to vehicle-exposed (control) cells, in the absence of rolipram, salmeterol-exposed ASM cells exhibited homologous β 2AR 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 co-treated with rolipram. Thus, these observations implicate PDE4 activity in mediating the impaired isoproterenol-induced accumulation of cAMP that accompanies prolonged homologous β 2AR desensitization in human ASM cells.

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Regulation of cAMP PDE activity in homologous β 2AR-desensitized ASM cells.

To elucidate the mechanism underlying the above rolipram-sensitive effects of homologous β 2AR desensitization, the changes in total cAMP PDE activity and the effects of pre-treatment with selective small molecule inhibitors on this activity were examined in cultured ASM cells following their prolonged exposure to a β 2AR agonist. Relative to the mean ($\pm$ SE) basal level of PDE activity detected in vehicle-treated (control) cells (i.e., 150.2 ± 11.1 pmol/min/mg protein), ASM cells incubated for 24 hr with a maximally effective concentration (10 μ M) of either salmeterol or isoproterenol exhibited significantly increased levels of PDE activity that averaged ~2-fold above control (Fig. 11A). The stimulatory effect of prolonged exposure to salmeterol or isoproterenol on PDE activity was completely abrogated in cells that were pretreated with rolipram (10 μ M), implying that the upregulated cAMP-PDE activity exhibited by the β 2AR-desensitized cells was attributed to PDE4 activity. In separate experiments, pretreatment of cells with the transcriptional inhibitor, actinomycin D (4 μ M), or with the protein synthesis inhibitor, cycloheximide (100 μ M), also completely ablated the increase in PDE activity elicited by prolonged exposure to the β 2AR agonists (Fig. 11B), whereas neither actinomycin D nor cycloheximide alone significantly affected basal PDE activity (data not shown). Finally, extended studies demonstrated that pre-treatment with either the selective PKA inhibitor, H89 (10 μ M), or the MEK-ERK1/2 inhibitor, U0126 (5 μ M), prevented the stimulatory effects of salmeterol and isoproterenol on PDE activity (Fig. 11C), 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 β 2AR desensitization in ASM cells resulting from prolonged exposure to a β 2AR 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 ASM tissues following prolonged homologous β 2AR desensitization.

5 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 β 2AR 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 hr to either vehicle alone (control)
10 or a pre-determined maximally effective concentration of salmeterol (10 μ M), both in the absence and presence of pretreatment with either rolipram (10 μ M), H89 (10 μ M), or U0126 (5 μ M). Relative to their respective vehicle-treated controls, ASM tissues exposed to salmeterol (SAL) exhibited significantly increased constrictor responsiveness to exogenously administered ACh (Fig. 12A), yielding a mean $\pm$ SE maximal constrictor
15 response (Tmax) value of 106.2 ± 8.9 g/g ASM 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
20 isoproterenol produced dose-dependent relaxation of the pre-constricted ASM segments. Relative to control tissues, the relaxation responses to isoproterenol were significantly attenuated in the SAL-exposed ASM segments (Fig. 12B), consistent with their development of homologous β 2AR desensitization. Accordingly, the mean $\pm$ SE maximal relaxation (Rmax) response in the SAL-exposed tissues amounted to 33.39 ± 5.1 % vs.
25 the average Rmax 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-exposed tissues that were pretreated either with rolipram, H89, or U0126. Contrasting these observations in SAL-exposed tissues, ASM tissues that were comparably treated for 24 hr with rolipram, H89, or U0126 alone did not exhibit any
30 significant changes in either their constrictor responses to ACh or relaxation responses to isoproterenol (data not shown). Taken together, these observations implicate a key

regulatory role for PDE4 activity that is coupled to PKA and ERK1/2 activation in mediating the pro-asthmatic-like changes in ASM constrictor and relaxation responsiveness that are elicited by prolonged homologous β 2AR desensitization.

5 *Regulation of PDE4D5 expression by PKA and ERK1/2 in homologous β 2AR-desensitized ASM.*

Based on the above observations, together with recent evidence that identifies PDE4D5 as the functionally dominant cAMP-regulating PDE4 isoform in cultured
10 human ASM cells (6), we next conducted a series of experiments that were aimed at identifying the effects of prolonged β 2AR agonist exposure on ASM expression of *PDE4D5* mRNA transcripts and the roles played by the PKA and MAPK signaling pathways in regulating β 2AR agonist-induced changes in PDE4D5 expression. Initial studies demonstrated that ASM cell cultures exposed for 24 hr to 10 μ M of either
15 salmeterol (Fig. 13A) or isoproterenol (Fig. 13B) exhibited time-dependent increases in *PDE4D5* mRNA expression, with peak induction of *PDE4D5* transcripts by either agonist observed at 6 hr and sustained upregulated expression detected for up to 24 hr. Densitometric analysis of the temporal changes in mRNA expression examined in 3 paired experiments demonstrated that peak induction of *PDE4D5* transcripts elicited by
20 salmeterol and isoproterenol averaged 7.3- and 5.7-fold above that 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 three representative experiments in Fig. 13C, relative to vehicle-exposed (control) cells (*lane 1*), the induced upregulated expression of
25 PDE4D5 mRNA transcripts detected at 6 hr following exposure of ASM cells to 10 μ M isoproterenol (*lane 2*) was largely prevented by pretreating the cells with either the PKA inhibitor, H89 (*lane 3*), or the MEK/ERK1/2 inhibitor, U0126 (*lane 4*). Conversely, isoproterenol-induced upregulation of PDE4D5 mRNA was unaffected in cells pretreated with previously reported maximal effective concentrations of either the specific JNK
30 inhibitor, SP600125 (10 μ M; *lane 5*), or the p38 MAPK inhibitor, SB202190 (10 μ M; *lane 6*), whereas the induction of PDE4D5 transcripts was prevented in isoproterenol-

exposed cells that were pretreated with the Src family tyrosine kinase inhibitor, SU6656 (10 μ M; *lane 7*), or pertussis toxin (PTX; 100 ng/ml) (*lane 8*), which ADP ribosylates Gi protein. The latter observations are consistent with the known role of Gi protein-mediated activation of Src in eliciting ERK1/2 activation in other cell types (see below) and, when taken together, the above results support the concept that β 2AR agonist-induced upexpression of PDE4D5 transcripts in ASM cells is regulated by Gi protein/Src-coupled interaction between the PKA and ERK1/2 signaling pathways. Studies were next pursued to examine the nature of the suspected interplay between these signaling pathways, as described below.

PKA-dependent regulation of CREB and ERK1/2 activation in β 2AR agonist-exposed ASM.

Because *PDE4D5* transcription in ASM cells is regulated by a CRE-containing promoter (Le Jeune et al. (2002) J. Biol. Chem 277:35980-35989), we examined the effects of β 2AR 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. 14A, ASM cells treated with isoproterenol (10 μ M) exhibited transiently increased phosphorylation of the CRE-binding transcription co-factors, 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 nM salmeterol (data not shown). To ascertain whether the stimulatory effect of β 2AR 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 siRNA duplexes directed against the human PKA α and PKA α catalytic subunits. We previously demonstrated that Lipofectamine transfection of ASM cells with these PKA siRNA duplexes produced maximal knockdown of PKA α protein levels at 72 hr post-transfection that ranged between ~70-90% . See Example I. Accordingly, confluent cultures of ASM cells were initially treated for 72 hr 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 induced expression of phosphorylated CREB/ATF1 proteins at 30 min following exposure to isoproterenol (10 μ M). As depicted in Fig. 14B, in the absence of isoproterenol, basal levels of phosphorylated CREB/ATF1 protein expression were little affected by transfection with either the scrambled RNA (scRNA; *lane 3*) or PKA siRNA (*lane 5*) preparations. By comparison, isoproterenol-induced upregulated expression of phosphorylated CREB/ATF1 (*lane 2*) was distinctly inhibited in cells transfected with siRNAs directed against PKA (*lane 6*), whereas the scrambled siRNA sequence had no effect (*lane 4*). 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 expression.

Apart from the cAMP/PKA pathway, CREB can also be activated via other signaling events, notably including ERK1/2 activation (24). 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 (23, 28) which, in turn, can mediate CREB phosphorylation via activation of the CREB kinases, p90RSK or MSK-1. See Example I. Given 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 β 2AR-desensitized state, we next examined whether β 2AR agonist exposure elicits a regulatory interplay between PKA and ERK1/2 signaling in ASM cells. As depicted by representative immunoblots in Fig. 15A, treatment of ASM cells with 10 μ M 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 β 2AR agonist-induced activation of ERK1/2, ASM cells were first treated for 72 hr with vehicle alone or with either scrambled RNA or siRNAs directed against the PKA catalytic subunits, PKA α and PKA γ , and then examined for induced phosphorylation of ERK1/2 at 20 min following

exposure to isoproterenol. As shown in Fig. 15B, relative to vehicle-treated control cells (*lane 1*), ERK1/2 phosphorylation was markedly increased in isoproterenol-treated cells (*lane 2*) and, while cells pretreated with either the scrambled or PKA siRNAs preparations alone showed little change in basal ERK1/2 phosphorylation (*lanes 3* and *5*, respectively), the isoproterenol-induced increase in ERK1/2 phosphorylation was distinctly inhibited in ASM cells that were pretreated with the PKA siRNAs (*lane 6*), whereas pretreatment of isoproterenol-exposed cells with the scrambled RNA had no appreciable effect (*lane 4*). Analysis of the results obtained in 4 experiments is depicted in Fig. 15C, wherein the levels of ERK1/2 phosphorylation detected under the different experimental conditions are displayed as mean $\pm$ SE values of the fold-changes in the densitometric ratios of phosphorylated ERK1/2-to-total ERK1/2. It can be seen that the 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, in contrast to the lack of effect of scRNA, the β 2AR agonist-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 β 2AR agonist-exposed ASM cells is regulated by PKA.

Gi- $\beta\gamma$ protein signaling mediates PKA-dependent activation of ERK1/2 in β 2AR agonist-exposed ASM

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The above observations in β 2AR agonist-exposed ASM are consistent with those in Example I wherein we found that PKA-dependent ERK1/2 activation mediates heterologous β 2AR desensitization in ASM evoked by its prolonged exposure to PGE2 or to non-receptor-coupled stimulation of cAMP production with forskolin. The findings in the latter study further demonstrated that PKA-dependent activation of ERK1/2 in PGE2-exposed ASM cells was attributed to Gi- $\beta\gamma$ -subunit-mediated activation of Src signaling via the Ras/c-Raf1/MEK1/2 pathway. Given this recent evidence, together with our observations herein that Src and Gi protein activation are intimately involved in mediating β 2AR agonist-induced PDE4D5 expression (Fig. 13C), we examined whether G $\beta\gamma$ signaling also mediates ERK1/2 activation in β 2AR agonist-exposed ASM cells. This issue was directly addressed in experiments that compared the effects of

isoproterenol administration (10 μ M x 20 min) on ERK1/2 activation in ASM cells at 24 hr following their transfection either with an adenovirus vector expressing lacZ (adeno-LacZ), serving as a negative control, or with adeno- β ARK-ct, which encodes the β ARK1 carboxyl-terminal domain that blocks G β γ signaling (26, 27), both at a multiplicity of infection (MOI) of 100. As shown in Fig. 16A, cells transfected with adeno-LacZ exhibited distinct isoproterenol-induced ERK1/2 phosphorylation (*lane 2*), whereas this response to isoproterenol was completely ablated in cells transfected with adeno- β ARK-ct (*lane 4*), implicating G β γ signaling in mediating ERK1/2 activation by the β 2AR agonist. In light of these data, together with earlier reports demonstrating that PKA-dependent activation of ERK1/2 can occur via Gi- β γ subunit-mediated stimulation of Src-induced signaling via either the Rap1/B-Raf/MEK1/2 or the Ras/c-Raf1/MEK1/2 pathway (28), we next examined whether our observed G β γ -mediated activation of ERK1/2 in β 2AR 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 β 2AR agonist-induced ERK1/2 phosphorylation. As depicted in Fig. 16B, relative to control (vehicle-exposed) cells (*lane 1*), ASM cells exposed to isoproterenol (10 μ M x 20 min) exhibited increased expression of phosphorylated ERK1/2 (*lane 2*) and, as expected, activation of ERK1/2 was ablated by pretreating the cells with the selective MEK-ERK1/2 inhibitor, U0126 (*lane 3*). By comparison, pretreatment with the selective c-Raf1 inhibitor, GW5074 (20 μ M), completely abrogated the stimulatory effect of isoproterenol on ERK1/2 phosphorylation (*lane 4*), whereas cells pretreated with GGTI-298 (25 μ M), a potent selective inhibitor of Rap1 activation (29), did not exhibit attenuated isoproterenol-induced ERK1/2 phosphorylation (*lane 5*). Thus, together with the above results, these observations support the concept that activation of ERK1/2 in β 2AR agonist-exposed ASM cells is attributed to PKA-dependent activation of Gi- β γ -mediated signaling, the latter leading to Src-induced stimulation of the Ras/c-Raf1/MEK-ERK1/2 pathway.

EXAMPLE III

Agents which inhibit G β protein signaling and methods of use thereof for the treatment asthma

5 As described above in Example I, the mechanism underlying pro-asthmatic changes in airway smooth muscle (ASM) constrictor and relaxation responsiveness accompanying heterologous β 2AR desensitization induced by prolonged exposure of the ASM to either the receptor- or non-receptor-mediated cAMP-elevating agent, PGE₂ or forskolin, respectively has been elucidated. Rabbit ASM tissues and human ASM cells
10 treated for 24 hours with either of these cAMP-stimulating agents exhibited constrictor hyperresponsiveness to acetylcholine and impaired β 2AR-mediated relaxation and cAMP accumulation. These pro-asthmatic-like changes in ASM function were mediated by upregulated PDE4 activity, associated with increased transcription of the PDE4D5 isoform, and this phenomenon was attributed to activation of the ERK1/2 module by
15 protein kinase A (PKA)-dependent activation of G_i protein signaling via the $\beta\gamma$ -subunit. Given this evidence, we hypothesized that signaling via the G_i- $\beta\gamma$ -subunit in ASM mediates pro-asthmatic changes in ASM function. We tested this hypothesis by treating rabbit ASM tissues and human ASM cells with cell membrane-permeable peptides that disrupt specific protein-protein interactions by mimicking protein-binding domains. The
20 blocking peptides are rendered cell-permeable by incorporating a membrane-permeable sequence (MPS) designed from the signal sequence of Kaposi fibroblast growth factor, which was previously shown to translocate covalently attached peptides across the cell membrane (1-6). Previous studies have confirmed that functional peptides attached to the MPS sequence are membrane penetrating and lack short-term cell toxicity (2,3).

25 The results reveal that a MPS-attached blocking peptide directed against free G $\beta\gamma$ subunits inhibits the induction of pro-asthmatic changes in constrictor and relaxation responsiveness in ASM subjected to heterologous β 2AR desensitization by prolonged exposure of the ASM to PGE₂, as well as in ASM passively sensitized with serum isolated from allergic asthmatic rabbits.

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Methods:

The animal preparation and pharmacodynamic methods used in this study are as described in Example I. To test the effects of blockade of specific G protein subunits, we used a MPS-attached blocking peptide directed against free G $\beta\gamma$ subunits, as well as MPS-attached blocking peptides directed against the G α i2 and G α i3 units. The MPS-attached peptides were purchased from AnaSpec, and consisted of the following sequences:

10 Anti-G $\beta\gamma$ (MPS-Phosducin-like protein C terminus):
1-letter code: AAVALLPAVLLALLAVTDQLGEDFFAVDLEAFLQEFGLLPEKE
(SEQ ID NO: 1)

15 MPS- G α i3:
1-letter code: AAVALLPAVLLALLAKNNLKECGLY
(SEQ ID NO: 6)

20 MPS- G α i2:
1-letter code: AAVALLPAVLLALLAKNNLKDCGLF
(SEQ ID NO: 7)

Results:

Effect of MPS-anti-G $\beta\gamma$ peptide on ASM responsiveness in PGE₂-exposed rabbit ASM tissues: In accordance with our previous observations (see Example I), rabbit ASM tissues exposed for 24 hours to PGE₂ (100 nM) exhibited increased constrictor responsiveness to ACh (Fig. 17A) and impaired ASM relaxation to isoproterenol (Fig. 17B). These pro-asthmatic-like changes in ASM responsiveness were prevented by pre-treating the PGE₂-exposed ASM tissues with the anti- $\beta\gamma$ cell-permeable inhibitory peptide (30 μ M), whereas pre-treating the tissues with the anti-G α i3 blocking peptide had no effect.

Effect of MPS- anti-G $\beta\gamma$ peptide on cAMP PDE activity in PGE₂-exposed human ASM cells. As described above in Example I, the induction of pro-asthmatic changes in constrictor and relaxation responsiveness in ASM exposed for 24 hours to PGE₂ is attributed to an induced increase in PDE4 activity. This phenomenon is depicted in Fig.

18 wherein, relative to control (vehicle-exposed) cells, ASM cells exposed for 24 hours to PGE₂ (100 nM) exhibit a significant increase in PDE activity. It will be noted that the stimulatory effect of PGE₂ on PDE activity is completely abrogated by pretreating the PGE₂-exposed ASM cells with the anti- $\beta\gamma$ cell-permeable inhibitory peptide (30 μ M),
5 whereas pre-treating the cells with either the G α i2 or G α i3 blocking peptide had only a partial inhibitory effect. In this regard, it is further noteworthy that the complete inhibitory effect of the anti- $\beta\gamma$ peptide on PGE₂ -induced PDE activity is similar to that obtained when pretreating the PGE₂-exposed ASM cells with pertussis toxin (PTX), as described above.

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Effect of MPS-anti-G $\beta\gamma$ peptide on ASM responsiveness in asthmatic serum-sensitized rabbit ASM tissues. In light of the above evidence that the G $\beta\gamma$ -inhibitory peptide effectively prevents the induction of pro-asthmatic changes in ASM responsiveness in PGE₂-exposed cells, we next examined whether pretreatment with the G $\beta\gamma$ -inhibitory
15 peptide also has a protective effect in preventing the induction of altered ASM responsiveness in rabbit ASM tissues passively sensitized for 24 hours with serum from allergic asthmatic rabbits (e.g., rendered asthmatic by immunizing rabbits with weekly (4-6 weeks) intraperitoneal injections of an allergen (ovalbumin) in adjuvant, and thereafter challenging animals with inhalation of the immunogen). As shown in Fig. 19,
20 relative to ASM tissues exposed to serum isolated from non-asthmatic (control) rabbits, ASM tissues exposed to allergic asthmatic (AS) serum exhibit markedly decreased relaxation responsiveness to isoproterenol. This impaired relaxation to isoproterenol is prevented by pretreating the AS serum-exposed ASM tissues with the G $\beta\gamma$ -inhibitory peptide. Interestingly, the relaxation responses to isoproterenol are also somewhat
25 increased in control serum-exposed ASM tissues that are pretreated with the G $\beta\gamma$ -inhibitory peptide.

G_i-βγ signaling regulates PDE4 activity and constrictor and relaxation responsiveness in homologous β₂AR-desensitized ASM.

To ascertain the physiological implications of the above mechanism of G_i-βγ-mediated interplay between the PKA and ERK1/2 signaling pathways in the homologous β₂AR-desensitized state, we next examined the effects of inhibition of G_i protein function and Gβγ-specific signaling on the changes in PDE activity and constrictor and relaxation responsiveness induced by prolonged homologous β₂AR desensitization in human ASM cells and rabbit tissues, respectively. As shown in Fig. 20, relative to untreated (vehicle-exposed) control ASM cells, cAMP PDE activity was significantly increased in cells that were incubated for 24 hr with 10 μM of salmeterol or isoproterenol, and this stimulation of PDE4 by either β₂AR agonist was completely abrogated in cells wherein G_i protein signaling was interrupted by pretreatment with PTX (100 ng/ml) or with 1 μM of a Gβγ-specific sequestering peptide (anti-Gβγ peptide) coupled to a cell membrane permeable carrier peptide sequence (MPS). By comparison, stimulation of PDE activity by either β₂AR agonist was unaffected in cells pretreated with a MPS-coupled peptide directed at inhibiting G_iα₃ signaling (anti-G_iα₃ peptide; 1 μM). Comparably, as depicted in Fig. 21, relative to the responses obtained in control rabbit ASM tissues, the significantly increased constrictor responses to ACh (Fig. 21A) and impaired relaxation responses to isoproterenol (Fig. 21B) detected in salmeterol-exposed ASM tissues were prevented by pretreating these tissues with the anti-Gβγ peptide (30 μM), whereas pretreatment with the anti-G_iα₃ peptide (30 μM) had no effect. In relation to these observations, it should be noted that in separate studies wherein vehicle-exposed control ASM tissues were comparably pretreated with the latter inhibitory peptides, there was no significant effect of either peptide on the tissues' constrictor or relaxation responses (data not shown). Thus, these data provide physiological evidence supporting the notion that the above G_i-βγ-coupled mechanism of β₂AR 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 (Figures 11 and 12, respectively) that are exhibited in the homologous β₂AR-desensitized state.

Discussion

Despite the fact that inhaled β 2AR agonists are a very effective approach to acutely relieve the airway bronchospasm resulting from the altered ASM constrictor and relaxation responsiveness characteristic of asthma, there is compelling evidence demonstrating that prolonged β 2AR stimulation following chronic use of long acting β 2AR agonists is associated with worsening of the asthmatic condition and subsequent increase in morbidity and mortality (5, 32, 38). These pro-asthmatic-like changes are believed to result from homologous desensitization of the airways to the bronchodilatory action of β 2AR agonists. Previous studies have attributed the generation of β 2AR desensitization to the phosphorylation of the β 2AR and, hence, its uncoupling from Gs protein-mediated cAMP production (13, 16, 36, 40). Accordingly, the role played by phosphorylation of the β 2AR by G protein-coupled receptor (GPCR) kinases (GRKs) and cAMP-dependent protein kinase A (PKA) in mediating homologous β 2AR desensitization has been demonstrated acutely following exposure of ASM cells to β 2AR agonists. This acute loss of β 2AR function contrasts with the clinical worsening of asthma that needs prolonged use of β 2AR agonists to occur (Cheung (1992) N Engl. J. Med. 327:1198-1203; Wong et al. (1997) Eur. Resp. J. 10:330-336; Yates et al. (1997) 156:988-91). In fact, prolonged β 2AR stimulation of ASM seems to be necessary for an intracellular mechanism to underlie the worsening of asthma induced by β 2AR agonists. This is illustrated by the clinical observation that acute β 2AR agonist administration transiently improves the altered ASM responsiveness seen in asthma whereas the chronic use of long acting β 2AR agonists leads to loss of this bronchoprotective effect and to enhanced ASM constriction responsiveness to cholinergic stimulation. As shown in Example I, the pro-asthmatic-like changes in ASM constrictor and relaxation responsiveness evoked by prolonged heterologous β 2AR desensitization are attributed to an induced increase in PDE4 activity mediated by a cAMP/PKA dependent Gi- β y signaling which, in turn, elicits downstream MEK/ERK1/2 activation. In addition, PDE4 activity has been previously reported to play a fundamental role in regulating the altered ASM contractility and in mediating the constrictor hyperresponsiveness of the airways seen in allergic asthma (9, 22, 25, 42, 44). Other studies have documented that the impaired relaxant responsiveness to β 2AR stimulation in ASM exposed to various pro-

asthmatic conditions is also attributed to upregulated expression and action of Gi protein, which attenuates cAMP accumulation and, hence, the bronchodilatory action of β 2AR agonists (18, 19, 20, 35). Moreover, recent reports have implicated a critical role for ERK1/2 signaling in mediating both the impaired β 2AR-mediated relaxation and increased constrictor responsiveness in ASM exposed to different pro-asthmatic stimuli (15, 29, 39). We now provide new evidence demonstrating that: 1) the pro-asthmatic-like changes in ASM constrictor and relaxation responsiveness accompanying homologous β 2AR desensitization resulting from prolonged exposure to β 2AR agonists are mediated by upregulated PDE4 activity; 2) the latter is attributed to PKA-dependent induction of Gi- $\beta\gamma$ -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 mechanism that potentially elucidates the well-established association between prolonged airway desensitization to β 2AR agonists and aggravation of the asthmatic phenotype.

The physiologic relevance of the above interplay between PKA activation and Gi- $\beta\gamma$ -mediated ERK1/2 signaling in regulating the rolipram-sensitive changes in ASM function associated with homologous β 2AR desensitization was supported by the observations that the upregulated cAMP PDE activity in human ASM cells following prolonged β 2AR stimulation by either salmeterol or isoproterenol, was prevented by either ADP ribosylation of Gi protein using PTX or by specific blockage of the $\beta\gamma$ subunit of Gi using the membrane permeable anti- $\beta\gamma$ peptide, MPS-phosducin-like protein (Fig 20). By comparison, stimulation of cAMP PDE activity by either β 2AR agonist was unaffected in cells pretreated with a MPS-coupled peptide directed at inhibiting Gi α 3 signaling (Fig 20). Moreover, the pro-asthmatic-like changes in contraction and relaxation induced by prolonged β 2AR stimulation in the ASM tissues were ablated by pre-treatment with the membrane permeable anti- $\beta\gamma$ peptide, MPS-phosducin-like protein, whereas pretreating the salmeterol-exposed tissues with the anti-Gi α 3 peptide had no effect (Fig 21). These findings are in general agreement with our previous study that reported key regulatory roles for PKA, ERK1/2 and Gi- $\beta\gamma$ signaling in mediating heterologous β 2AR desensitization and overall demonstrate that the above mechanisms of PDE4 upregulation play a decisive role in mediating the pro-asthmatic-

like changes in both ASM constrictor and relaxation responsiveness that accompany prolonged homologous β 2AR desensitization.

To summarize, the present study examined the regulation and role of PDE4 activity in mediating the changes in ASM constrictor and relaxation responsiveness associated with homologous β 2AR desensitization. The results provide new evidence demonstrating that: 1) homologous β 2AR desensitization in ASM elicits attenuated cAMP accumulation and increased cholinergic agonist mediated ASM constrictor responsiveness together with impaired β 2AR-mediated ASM relaxation; 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 Gi protein signaling, resulting in Gi- $\beta\gamma$ -subunit-mediated downstream activation of ERK1/2 via the Src/Ras/Raf1/MEK1/2 signaling pathway) 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.

Collectively, these new findings identify that the pro-asthmatic-like changes in ASM function that accompany prolonged airway exposure to β 2AR agonists 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 chronic use of long acting β 2AR agonist in asthma. Indeed, our data indicate that administration of a MPS-associated anti-G $\beta\gamma$ peptide represents an effective new therapeutic approach for the treatment of asthma.

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While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.

WHAT IS CLAIMED IS:

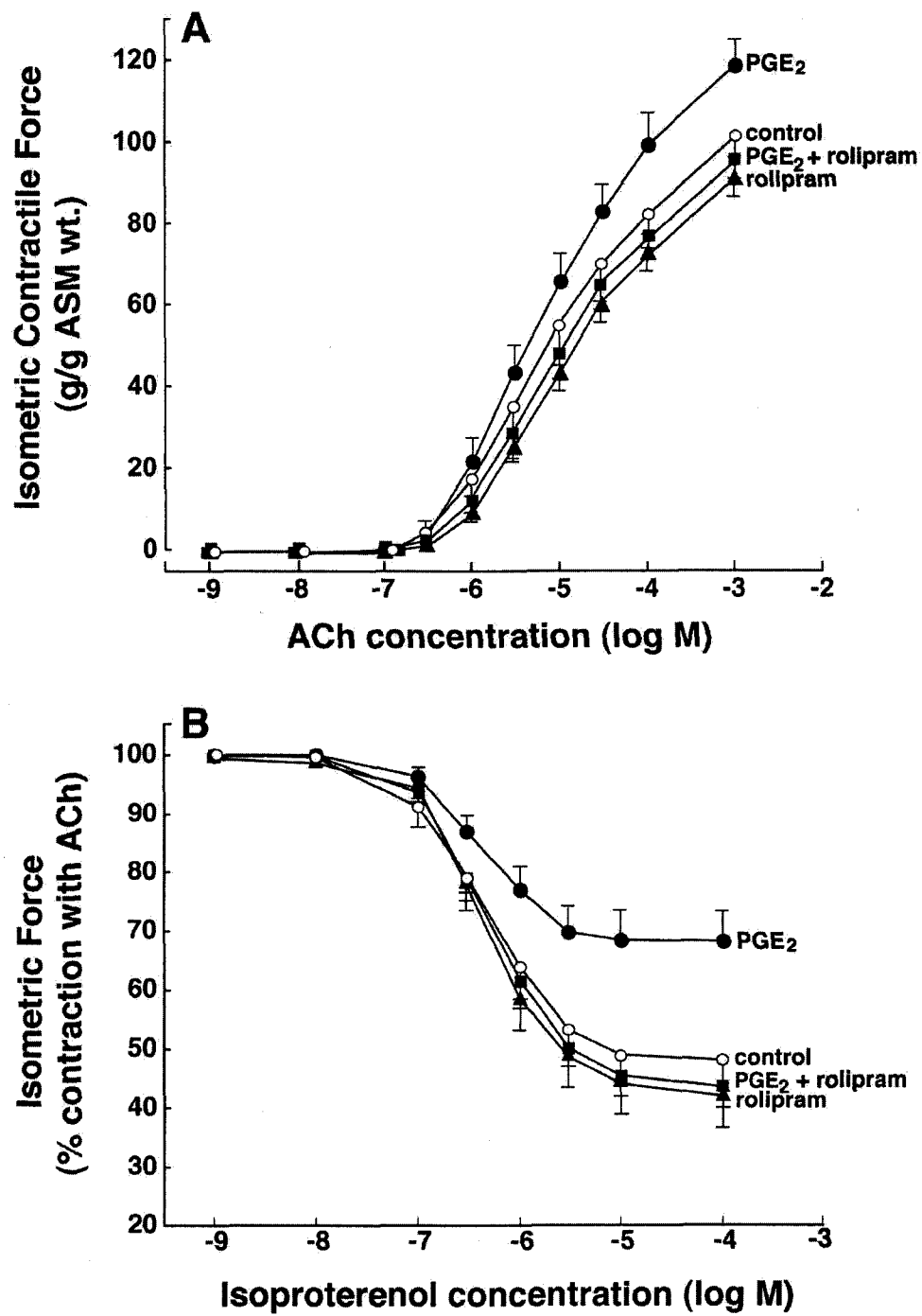
1. A method for the treatment of asthma in a patient in need thereof, comprising administration of an effective amount of an inhibitor of $G\beta\gamma$ signaling, said
5 administration being effective to reduce bronchoconstriction in said patient.
2. The method of claim 1, wherein asthma in said patient is exacerbated by chronic long term use of β_2 -adrenergic receptor agonists.
- 10 3. The method of claim 1 wherein said composition is delivered to a patient by a method selected from the group comprising, systemic, oral, intravenous, intramuscular, subcutaneous, intraorbital, intranasal, intracapsular, intraperitoneal, intracisternal, intratracheal, intraarticular administration, or by absorption through the skin to inhibit constriction of airway smooth muscle in the lung.
- 15 4. The method of claim 1, wherein said inhibitor of $G\beta\gamma$ signaling is formulated for aerosol delivery and is delivered via inhalation.
5. The method of claim 4, further comprising administration of at least one anti-
20 inflammatory agent selected from the group consisting of corticosteroids, sodium cromolyn, IgE inhibitors, phosphodiesterase inhibitors, methylxanthines, beta-adrenergic agents, and leukotriene modifiers.
6. The method of claim 5, wherein said agent is delivered simultaneously with said
25 inhibitor.
7. The method of claim 5, wherein said agent is delivered sequentially, before or after delivery of said inhibitor.
- 30 8. The method of claim 1, wherein said inhibitor of G_i - $\beta\gamma$ signaling is a membrane permeable $G\beta\gamma$ blocking peptide comprising SEQ ID NO: 1.

9. The composition of claim 4, wherein said aerosolized formulation comprises a propellant selected from the group consisting of halocarbons, hydrocarbons and esters.

- 5 10. The method of claim 1, wherein said inhibitor is selected from the group consisting of a membrane permeable G β γ blocking peptide, cyclohexanecarboxylic acid [2-(4,5,6-trihydroxy-3-oxo-3*H*-xanthen-9-yl)-(9Cl)], gallein, β ARKct, phosducin fragment containing amino acids 217-246 and phosducin fragment containing amino acids 213-233, said inhibitor optionally being linked to a membrane permeable carrier.

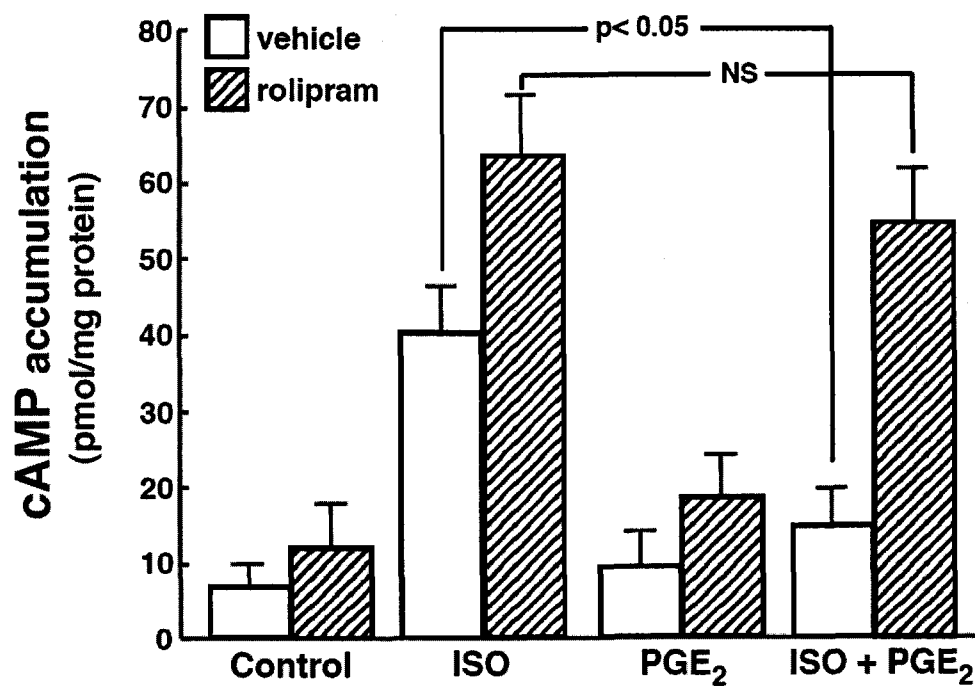
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Figure 1



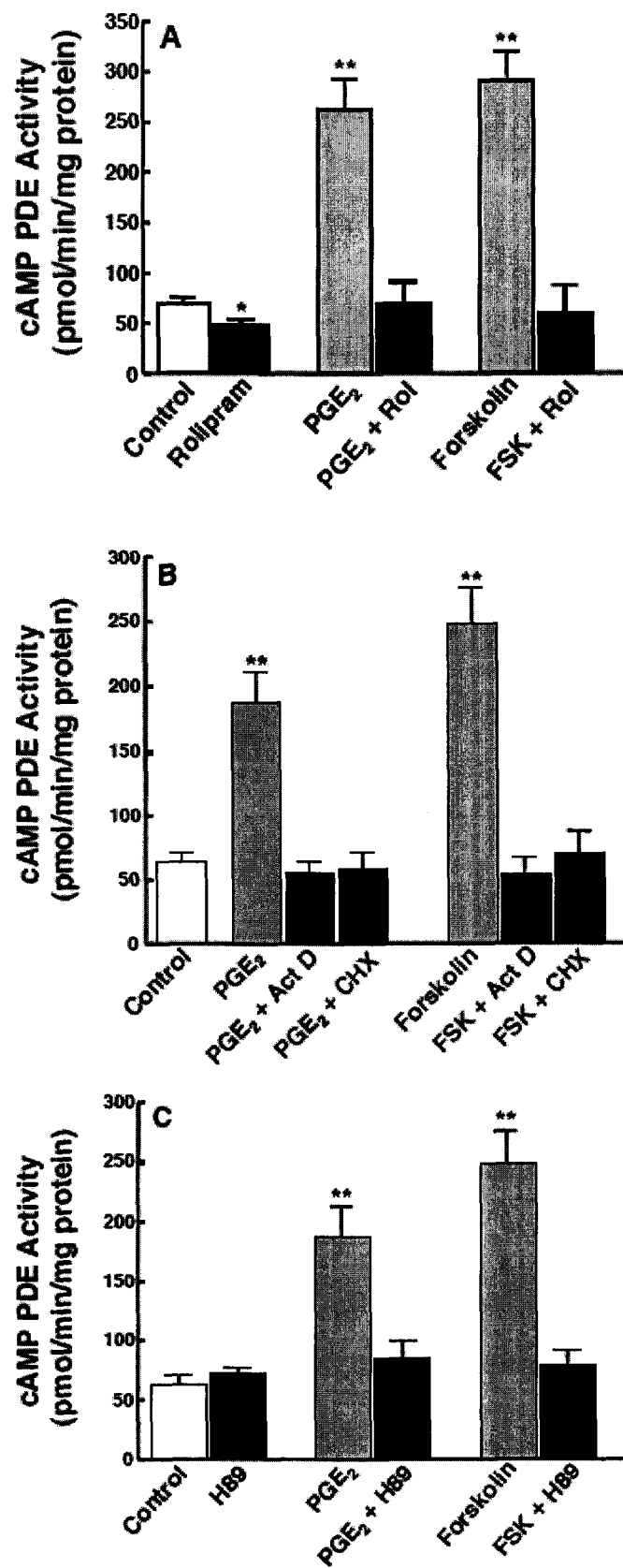
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Figure 2



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Figure 3



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Figure 4

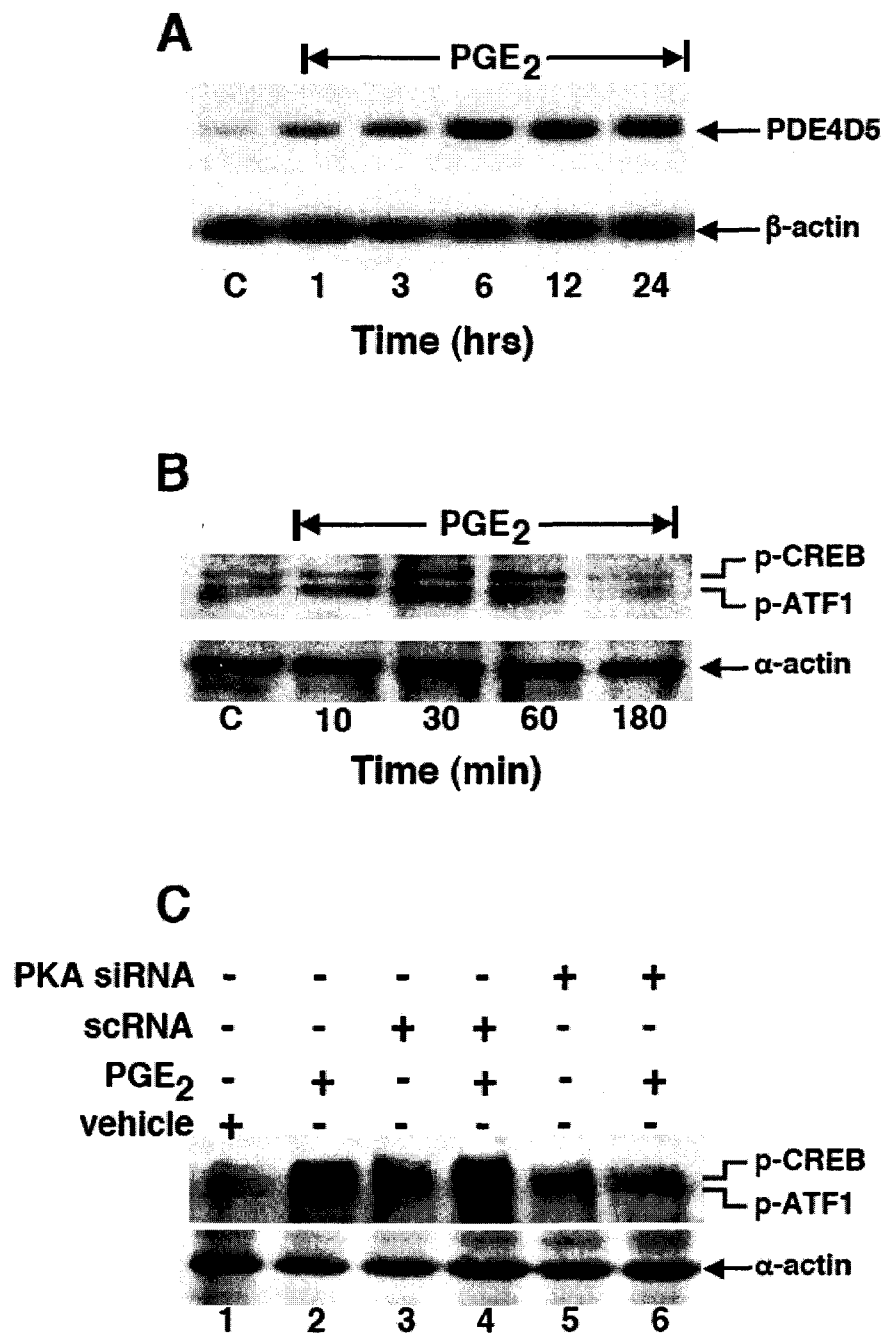
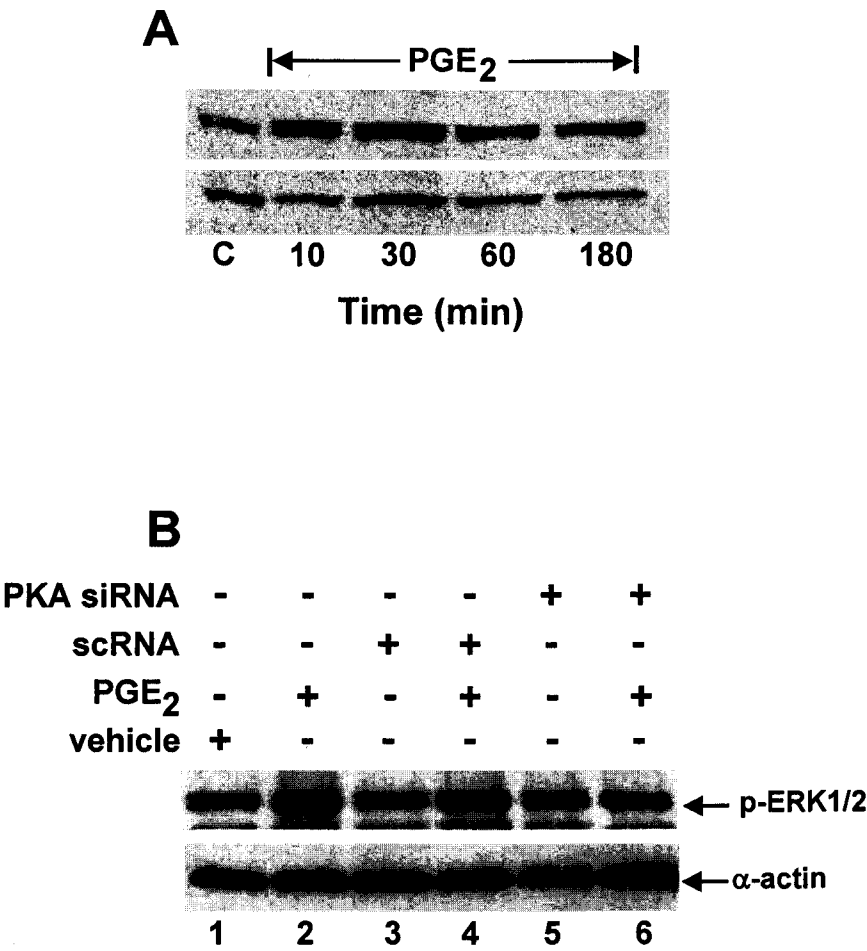
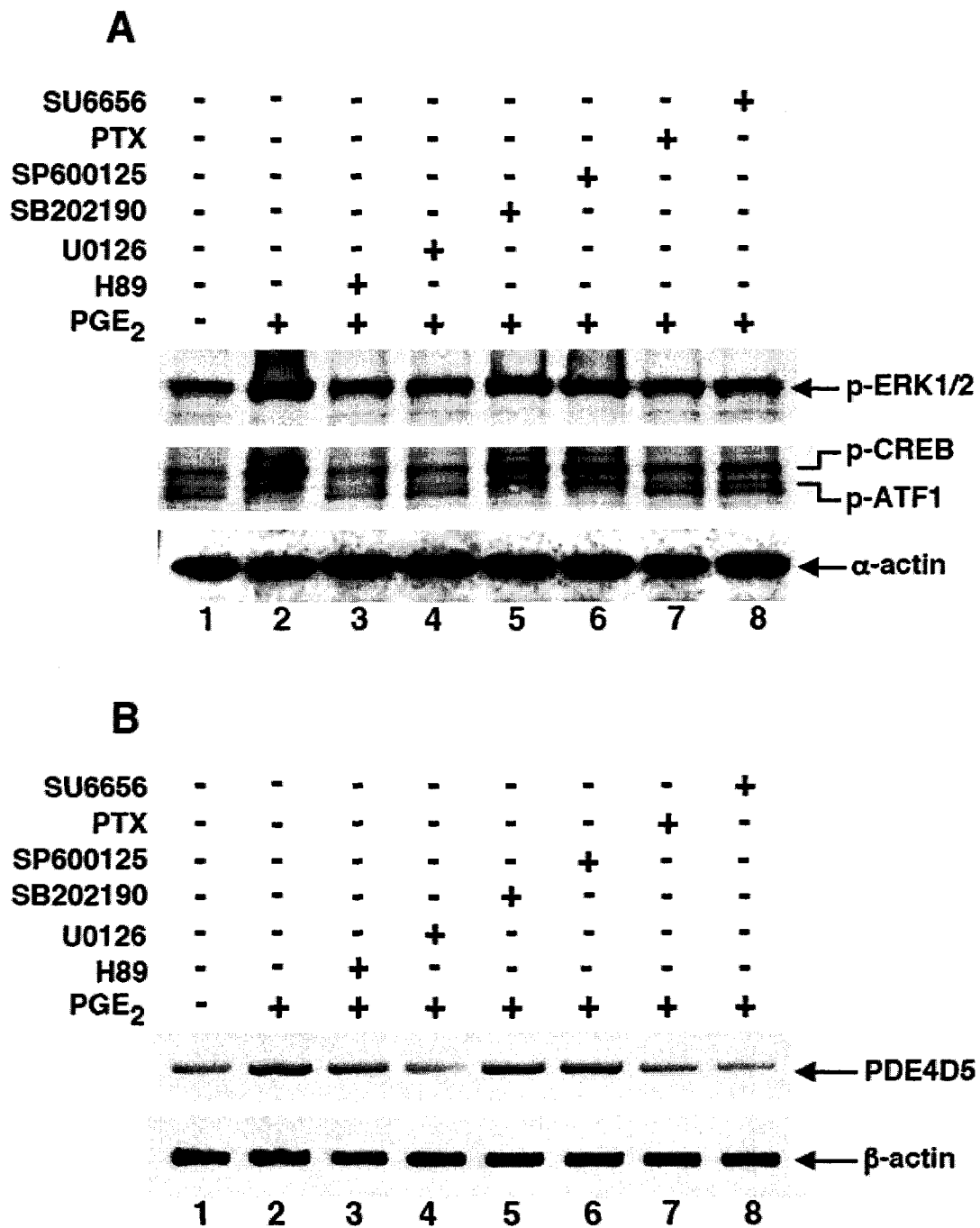


Figure 5



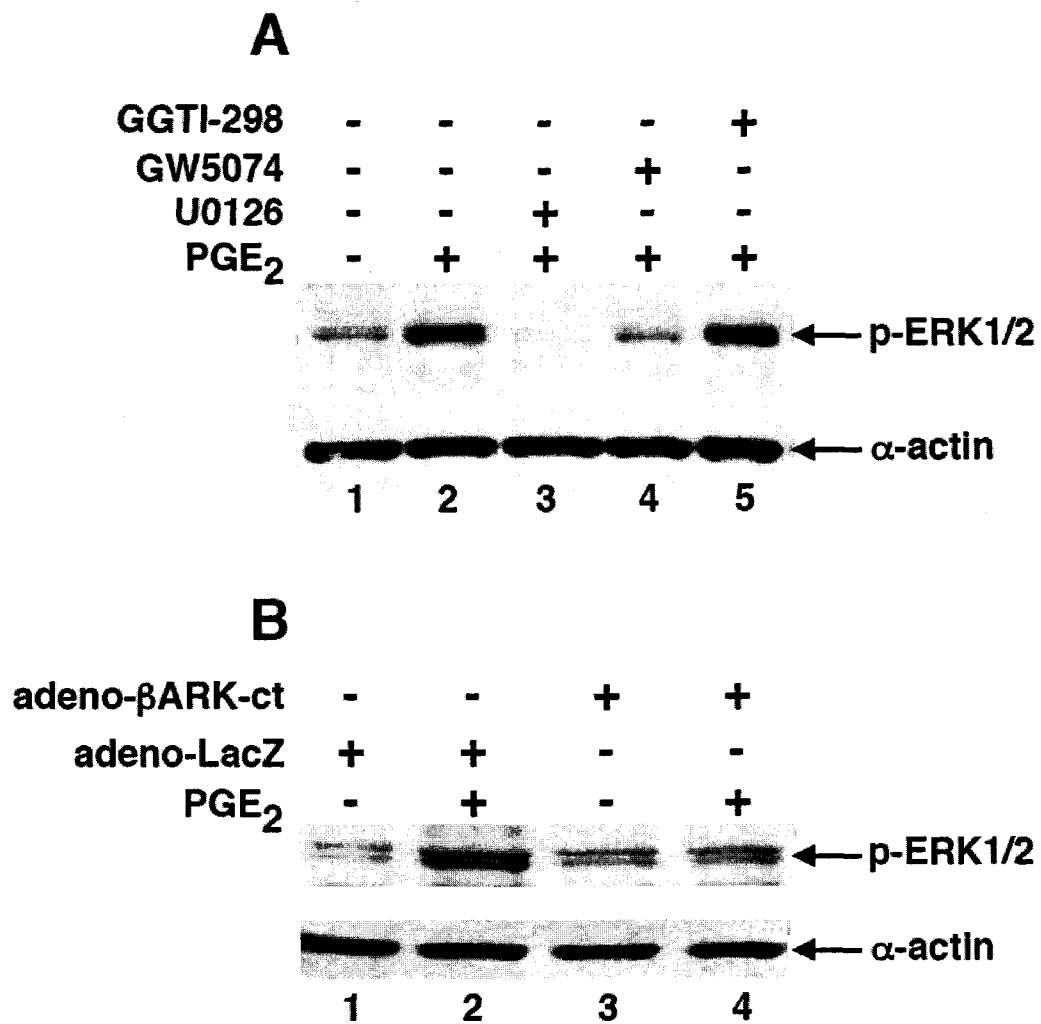
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Figure 6



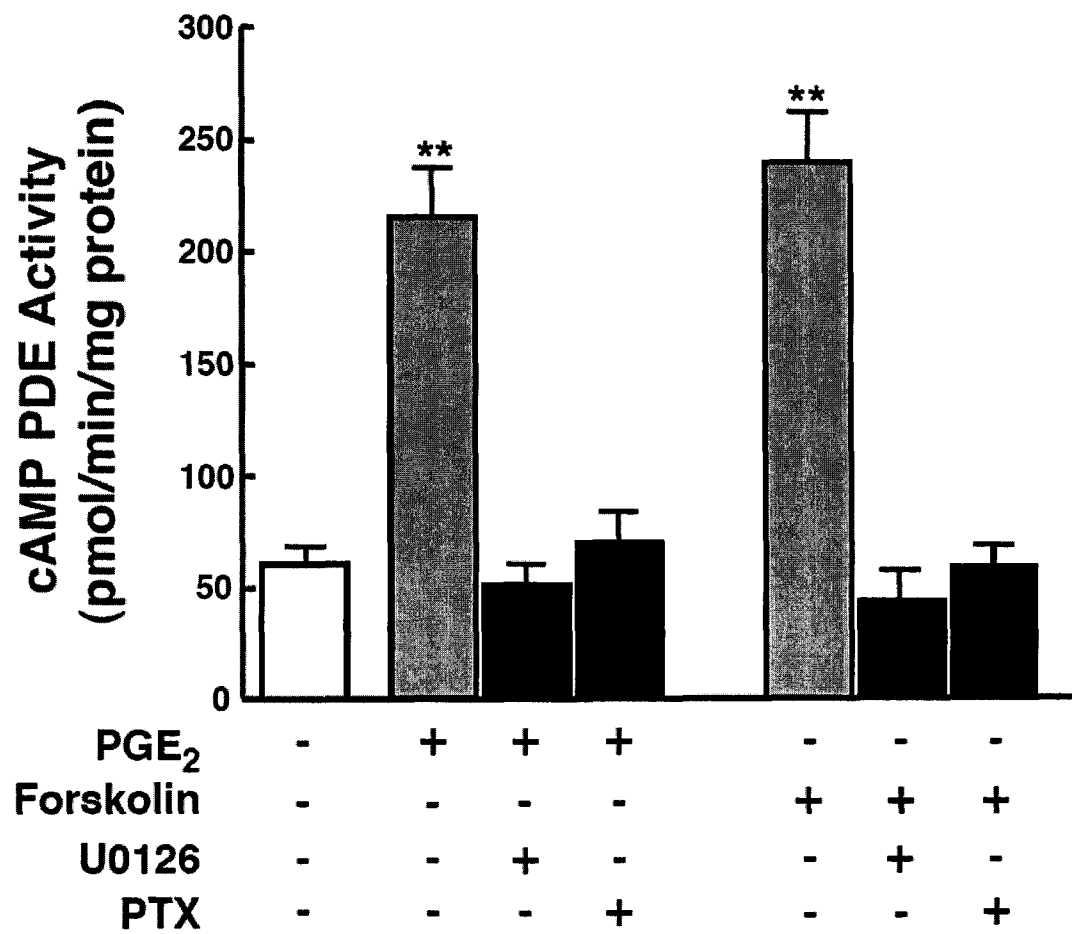
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Figure 7



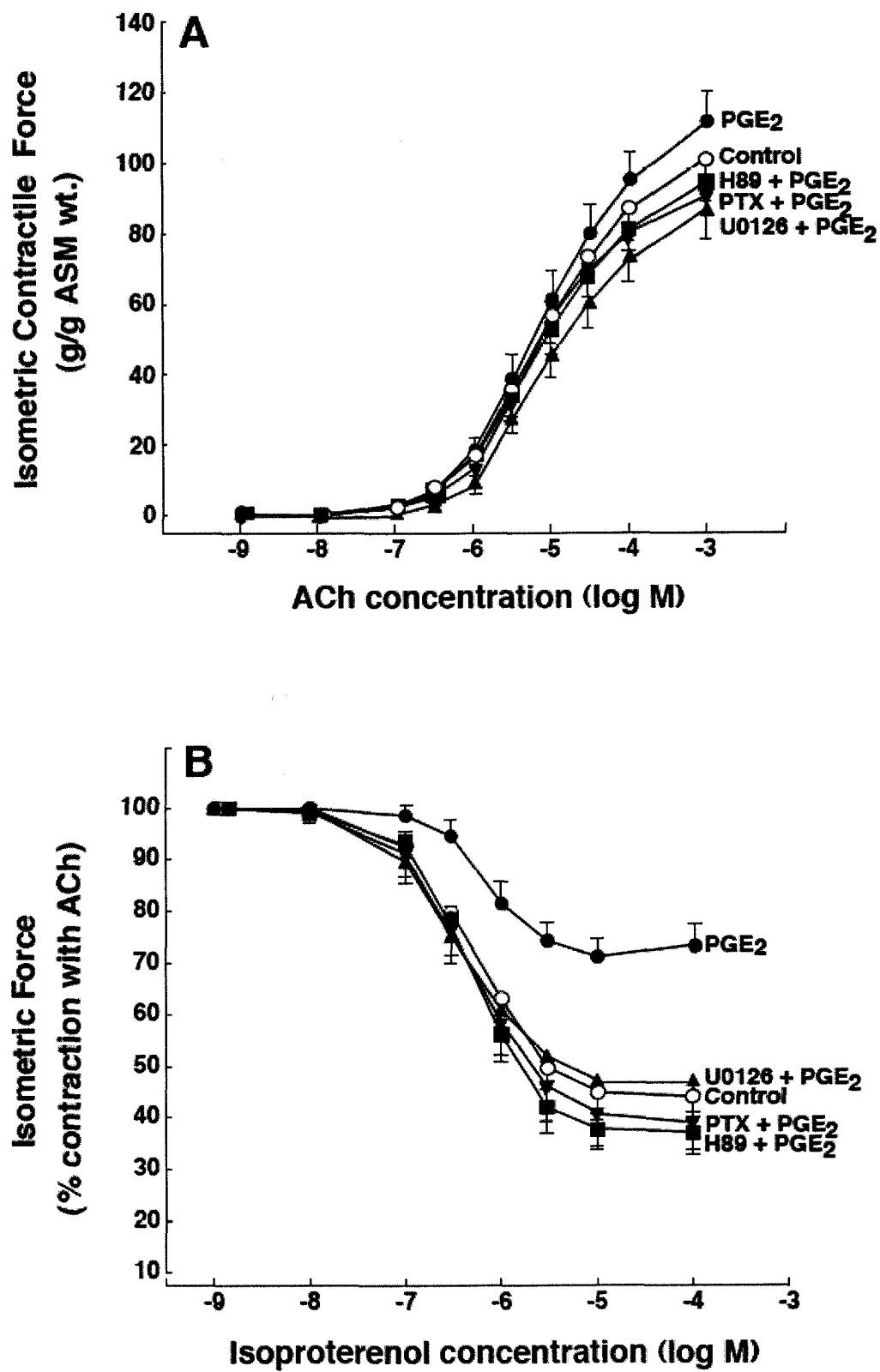
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Figure 8



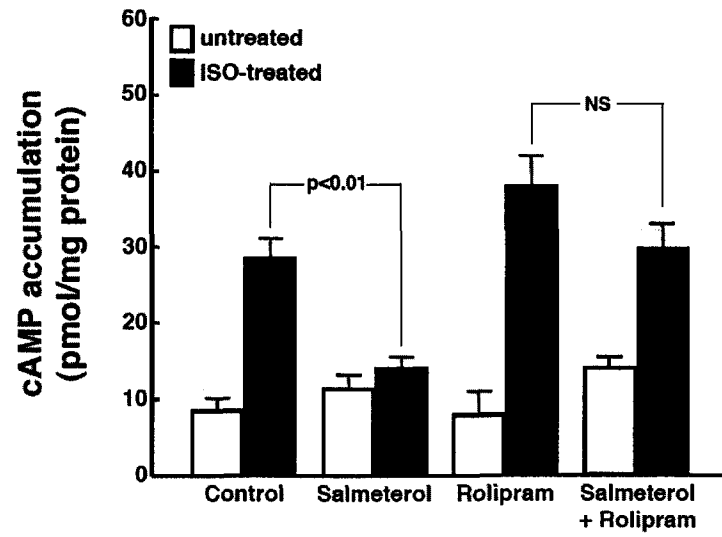
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Figure 9

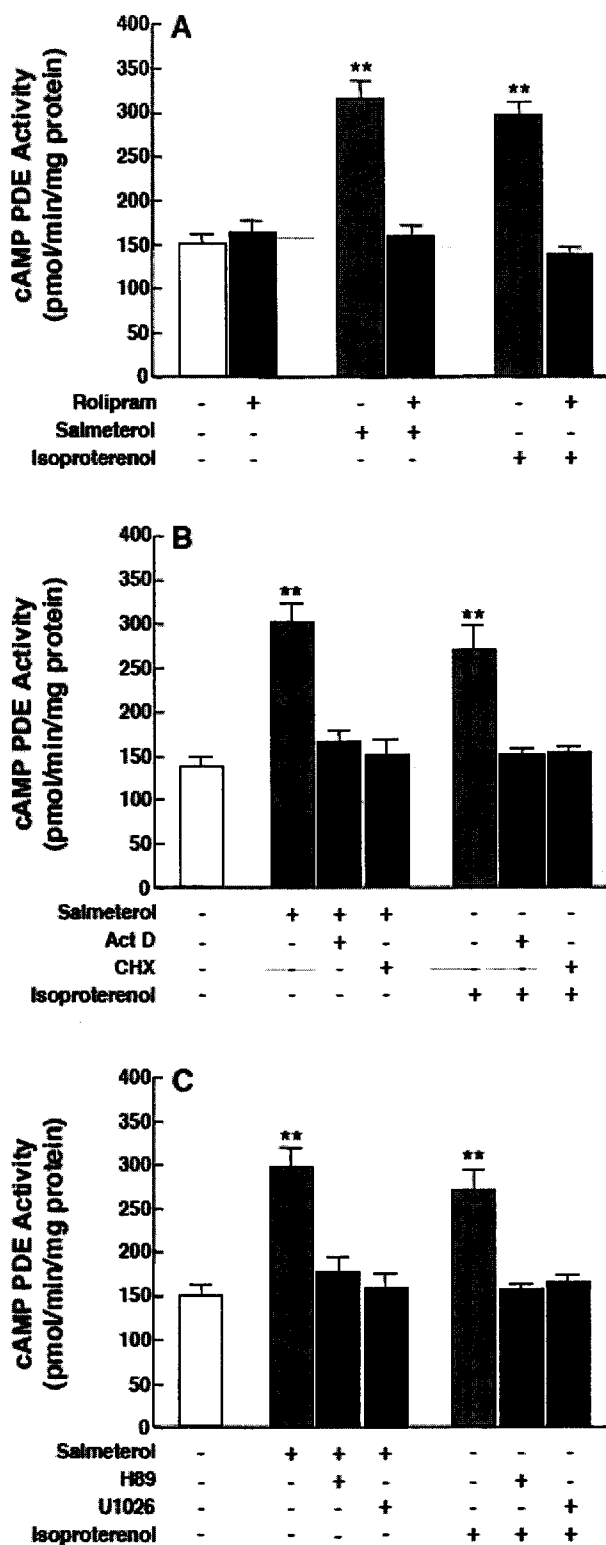


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Figure 10

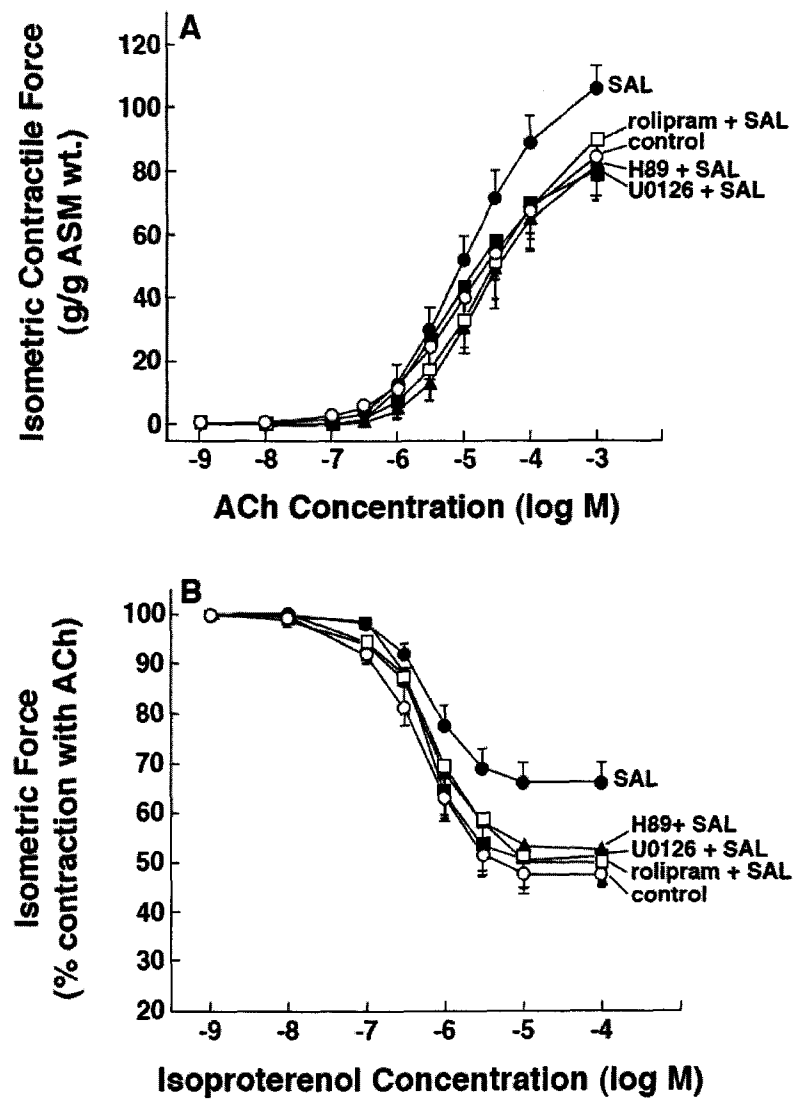


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Figure 11



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Figure 12



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Figure 13

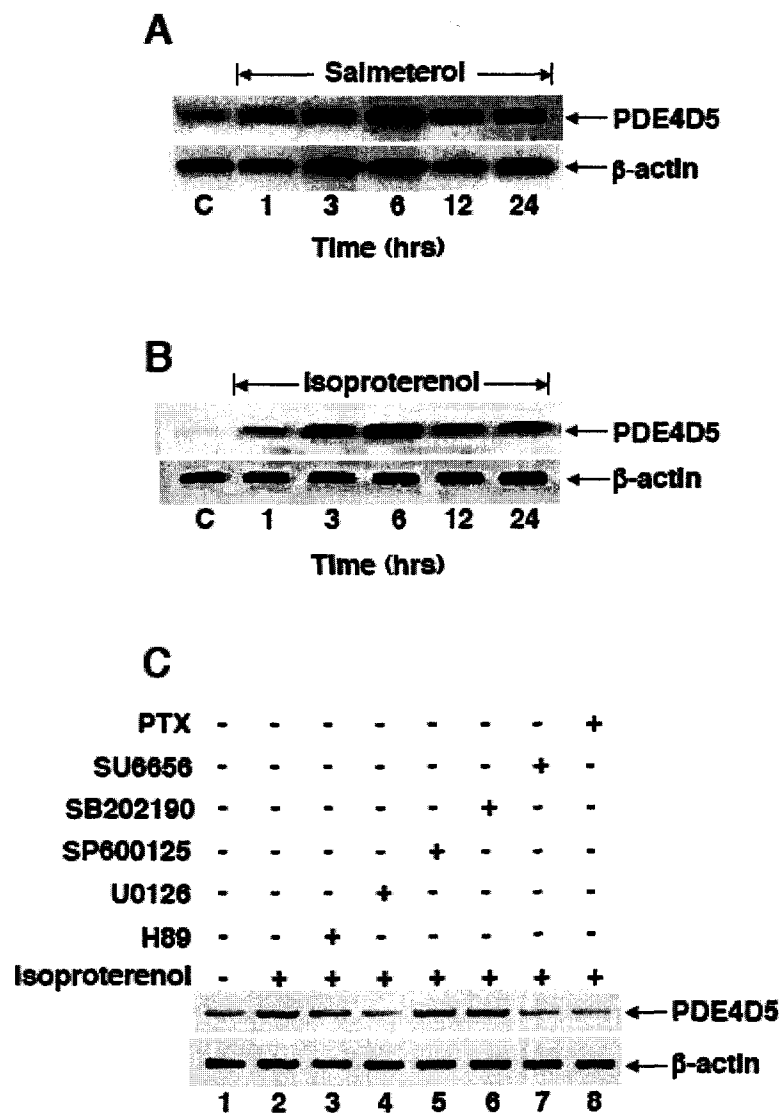
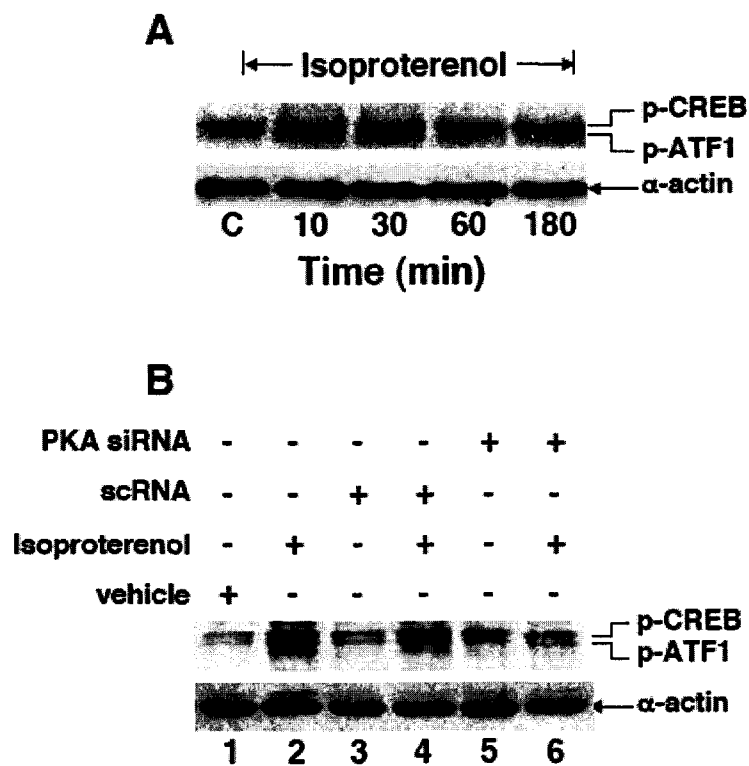


Figure 14



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Figure 15

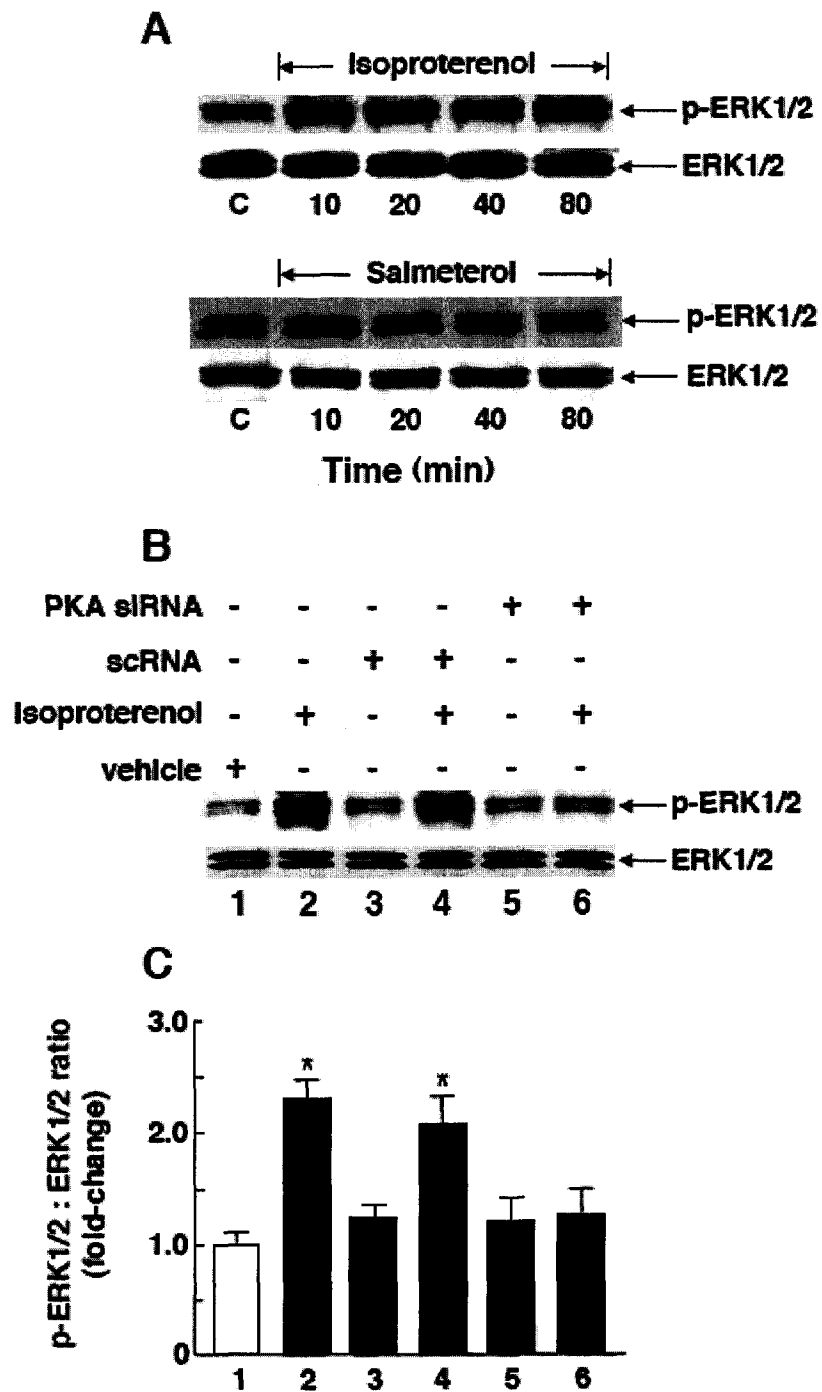
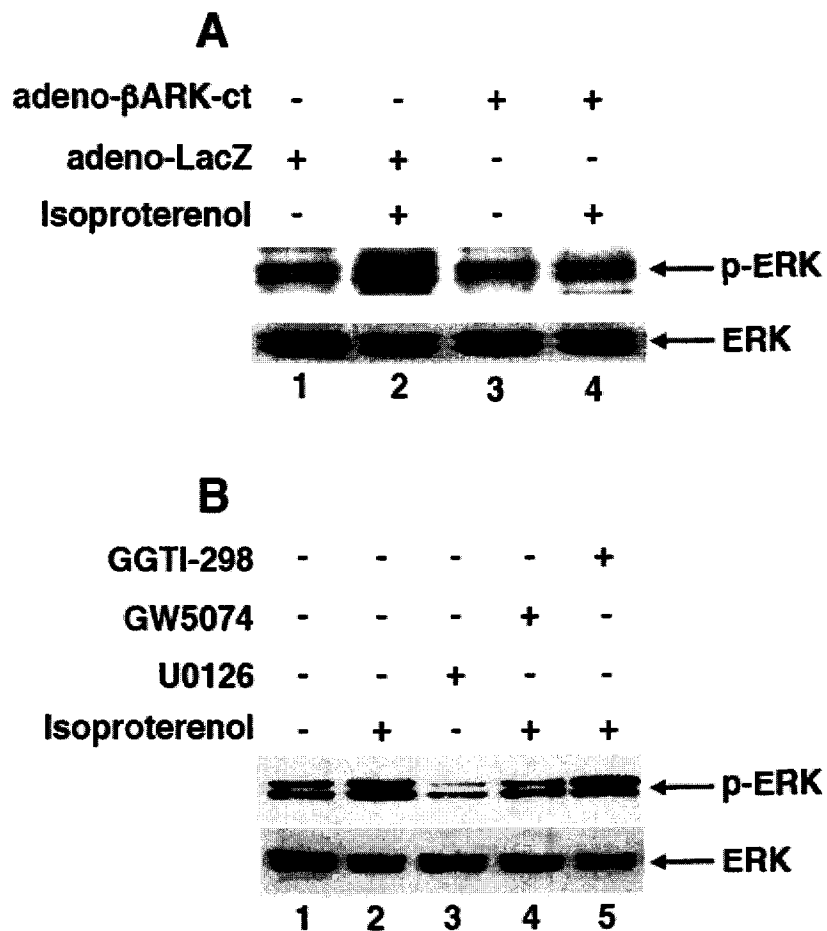
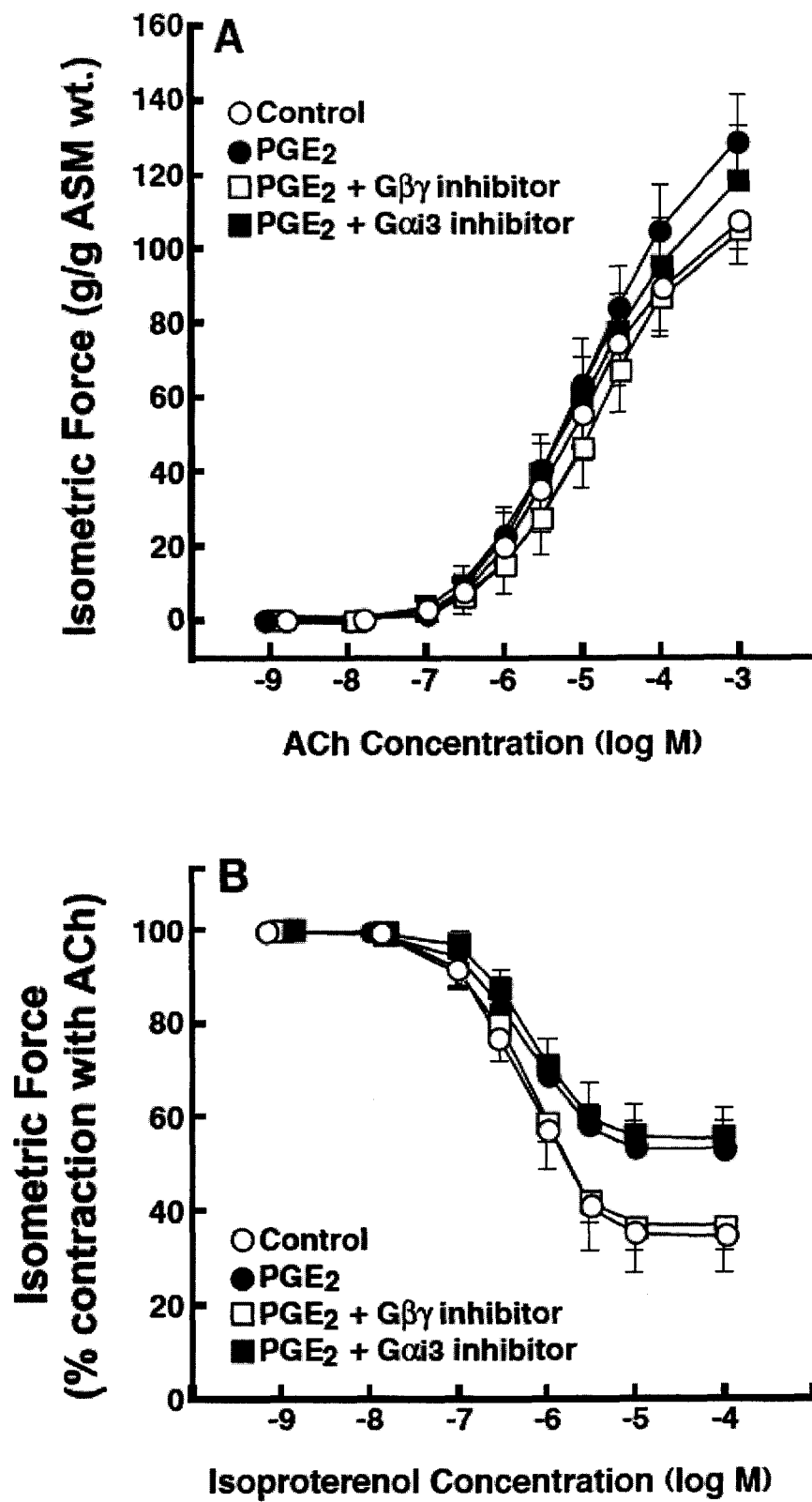


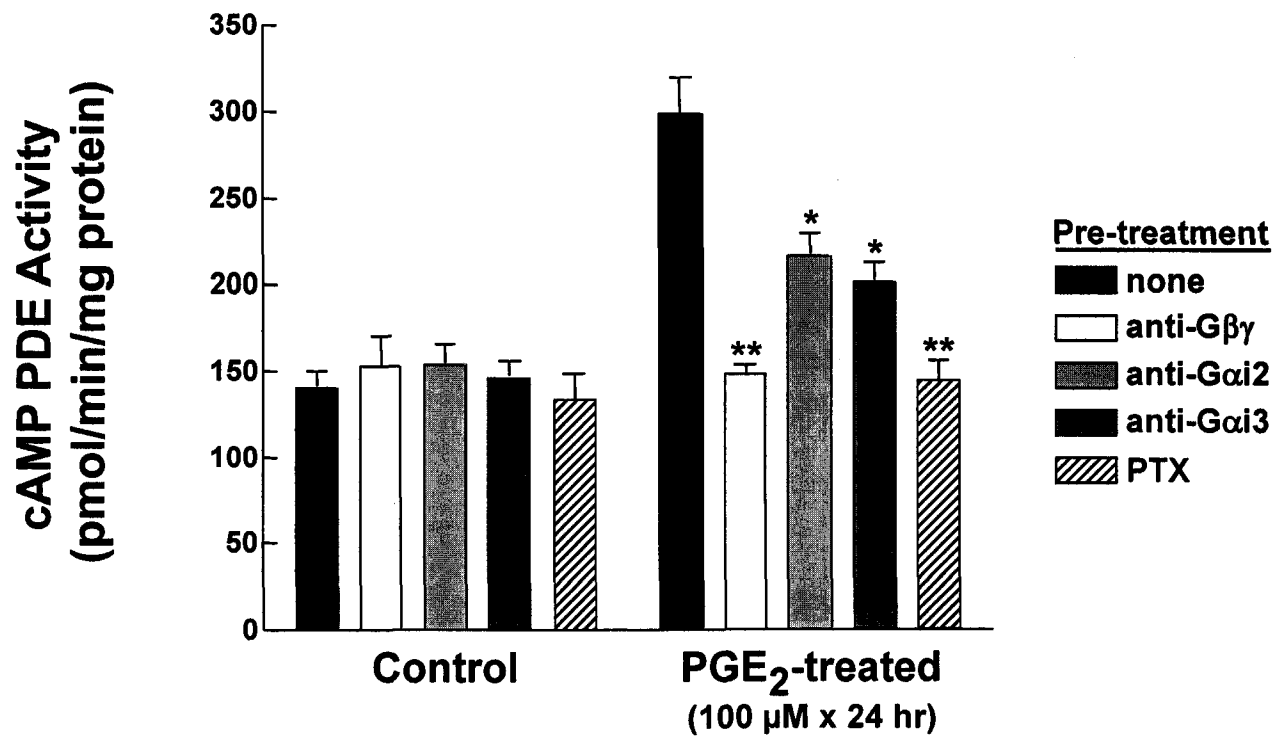
Figure 16



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Figure 17

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Figure 18

Human ASM cells



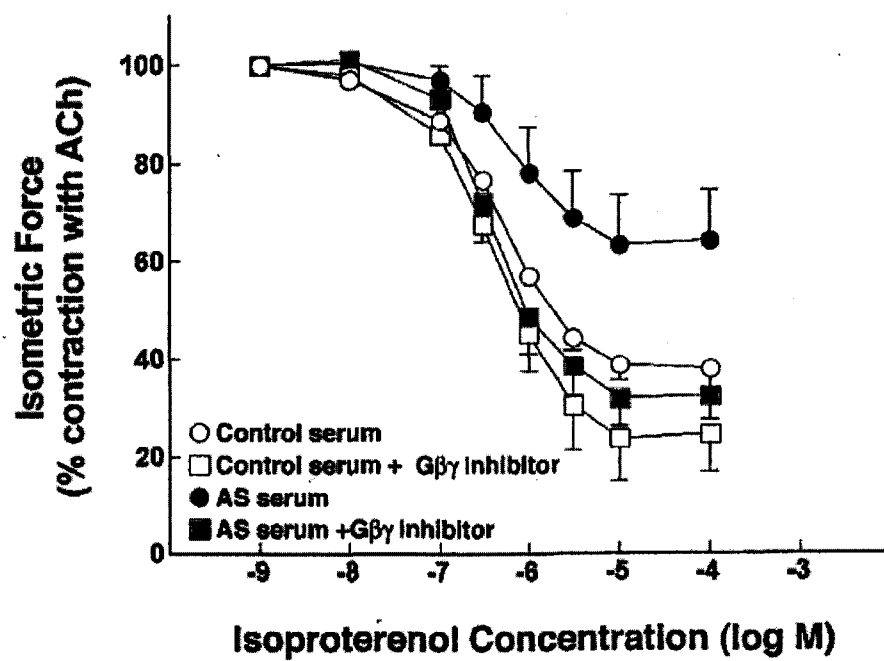
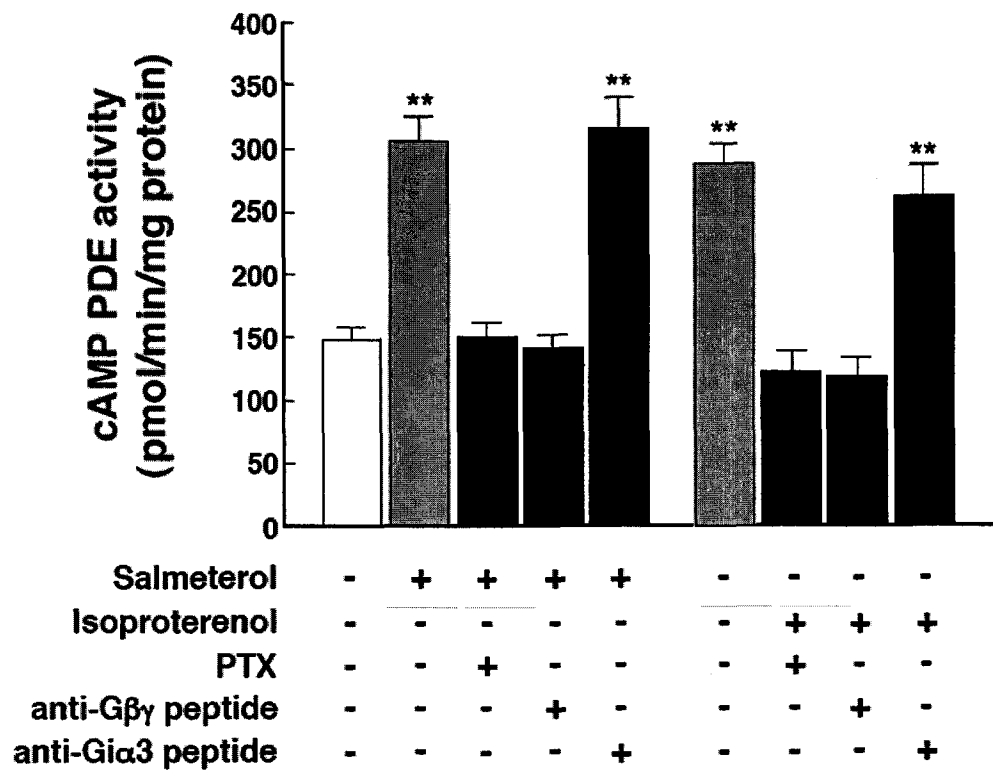
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Figure 19

Figure 20



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Figure 21

