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β_2 -Agonists upregulate PDE4 mRNA but not protein or activity in human airway smooth muscle cells from asthmatic and nonasthmatic volunteers

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Niimi K, Ge Q, Moir LM, Ammit AJ, Trian T, Burgess JK, Black JL, Oliver BG. β_2 -Agonists upregulate PDE4 mRNA but not protein or activity in human airway smooth muscle cells from asthmatic and nonasthmatic volunteers. *Am J Physiol Lung Cell Mol Physiol* 302: L334–L342, 2012. First published November 18, 2011; doi:10.1152/ajplung.00163.2011.— β_2 -Adrenergic receptor (β_2 AR) agonists induce airway relaxation via cAMP. Phosphodiesterase (PDE)s degrade and regulate cAMP, and in airway smooth muscle (ASM) cells PDE4D degrades cAMP. Long-acting β_2 -agonists are now contraindicated as monotherapy for asthma, and increased PDE4D has been speculated to contribute to this phenomenon. In this study we investigated the expression of PDE4D in asthmatic and nonasthmatic ASM cells and its regulation by formoterol and budesonide. Primary ASM cells from people with or without asthma were stimulated with transforming growth factor (TGF)- β_1 , formoterol, and/or budesonide. PDE4D mRNA was assessed by real-time PCR, or PCR to assess splice variant production. PDE4D protein was assessed by Western blotting, and we investigated the effect of formoterol on cAMP production and PDE activity. Interleukin (IL)-6 was assessed using ELISA. PDE4D mRNA was dose dependently upregulated by formoterol, with a single splice variant, PDE4D5, present. Formoterol did not induce PDE4D protein at time points between 3 to 72 h, whereas it did induce and increase IL-6 secretion. We pretreated cells with actinomycin D and a proteasome inhibitor, MG132, and found no evidence of alterations in mRNA, protein expression, or degradation of PDE4D. Finally PDE activity was not altered by formoterol. This study shows, for the first time, that PDE4D5 is predominantly expressed in human ASM cells from people with and without asthma and that formoterol does not upregulate PDE4D protein production. This leads us to speculate that continual therapy with β_2 AR agonists is unlikely to cause PDE4-mediated tachyphylaxis.

asthma; phosphodiesterase; β_2 -adrenergic receptor agonist

THE β_2 -ADRENERGIC RECEPTOR (β_2 AR) pathway is critically important in the management of asthma, as its activation results in airway smooth muscle (ASM) relaxation and therefore bronchodilation. β_2 AR agonists bind to and activate the β_2 AR, resulting in increased cAMP. In addition to relaxation, cAMP inhibits cellular proliferation and the secretion of inflammatory mediators and cytokines, all of which are important in the pathogenesis of asthma and chronic obstructive pulmonary disease (15, 18). ASM cells are the major target of β_2 AR agonists in the airways, and several studies have suggested that the β_2 AR-cAMP axis is abnormally regulated in asthma (10, 25, 37). Although the processes involved in the elevation of cAMP are well established, the mechanisms of cAMP turnover

are unclear. The only known mechanism is degradation through phosphodiesterase (PDE) activity, and PDEs thus constitute a vital regulatory component of cAMP signaling.

There are 11 known families of PDEs, consisting of at least 21 isoforms, with many isoforms having multiple splice variants. The different PDEs have differences in structure, substrate specificity, inhibitor selectivity, tissue and cell distribution, regulation by kinases, protein-protein interactions, and subcellular distribution (3, 17). It is well known that PDE activity plays a crucial role in regulating ASM contractility (26), mediating the constrictor hyperresponsiveness of the airways accompanying allergen in asthmatic subjects (39), and in animal models of allergic asthma (8, 14, 23, 34, 35). The majority of cAMP hydrolyzing activity in smooth muscle cells is attributed to isoforms of the PDE4 family, which are encoded by four distinct genes (PDE4A–D) that generate multiple PDE4 enzyme variants through activation of different promoters or alternative splicing (4, 9, 16). The dominant PDE4 expressed in ASM cells is PDE4D, which can encode six “long” isoforms (PDE4D3–5 and PDE4D7–9), one “short” isoform (PDE4D1), and two “super-short” isoforms (PDE4D2 and PDE4D6) (9, 16, 30). Increased PDE4 function attributable to either increased protein expression or activity might provide a feasible mechanism to account for some clinical features of asthma. Long-acting β_2 -agonists (LABAs) are now contraindicated as monotherapy for the treatment of asthma, and this is hypothesized to be attributable to decreased efficacy caused by increased PDE4. Nino et al. (19, 28) previously reported in their in vitro studies using rabbit tissue and isolated rabbit and human ASM cells that prolonged β_2 AR agonist exposure upregulated PDE4 activity as a result of enhanced expression of the PDE4D5 isoform (19, 28); however, this phenomenon has never previously been studied in human asthmatic ASM cells.

In our previous studies, we have shown that chronic treatment of ASM with β_2 AR agonists causes β_2 AR tachyphylaxis evidenced by reduced β_2 AR agonist-induced cAMP (37). Furthermore we have also shown that PDE4 exquisitely regulates β_2 AR agonist-induced cAMP, and moreover in airway smooth muscle cells from people with asthma there is increased PDE4D protein and PDE4 activity, which reduces β_2 AR agonist-induced cAMP compared with cells from people without asthma (38). Therefore, we hypothesized that PDE4-mediated β_2 AR tachyphylaxis would be greater in asthmatic ASM cells as a result of the intrinsically higher PDE4D levels. In this study we have examined the expression of PDE4D in asthmatic and nonasthmatic ASM cells and investigated whether treatment of human ASM cells with β_2 AR agonists increased PDE4D mRNA, protein, or activity.

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MATERIALS AND METHODS

Culture and treatment. We established primary cultures of ASM cells derived from human lung tissue (surgical resection, explanted lung or bronchial biopsies) as previously described (5, 20, 21) and in accordance with the approval of the ethics committees of Sydney South West Area Health Service and The University of Sydney. All volunteers provided written, informed consent. Cells from a minimum of three different people were used for each experiment. Table 1 shows the patient demographics. All experiments with ASM cells were carried out on 80% confluent cell cultures between passages 4 and 8. ASM cells were maintained in DMEM supplemented with 5% FBS. Twenty-four hours before stimulation of the cells, the growth medium was replaced with 0.1% BSA/DMEM. In separate experiments, the cells were treated with different concentrations, and for varying durations, of formoterol (10^{-12} to 10^{-6} M; Sigma, St. Louis, MO), salmeterol (10^{-7} M and 10^{-5} M; a gift from GlaxoSmithKline, Cambridge, UK), salbutamol (10^{-6} M; a gift from GlaxoSmithKline), isoprenaline (10^{-6} M; Sigma), budesonide (10^{-12} to 10^{-6} M; Sigma), and transforming growth factor (TGF)- β_1 (1 ng ml $^{-1}$; R&D Systems, Minneapolis MN).

Real-time PCR. The expression of PDE4D mRNA under basal and stimulated conditions was detected using real-time PCR (RT-PCR). Total RNA lysates were collected from nonasthmatic and asthmatic ASM cells at 0, 4, 8, 24 and 48 h after formoterol (10^{-8} M) or TGF- β_1 (1 ng/ml) stimulation. In subsequent experiments, nonasthmatic and asthmatic ASM cells were treated with formoterol (10^{-12} to 10^{-6} M), budesonide (10^{-12} to 10^{-6} M) or both (formoterol 10^{-10} to 10^{-6} M and budesonide 10^{-10} to 10^{-8} M, respectively), and total RNA lysates were collected 6 h after treatment. Then PDE4D mRNA was examined. RNA was extracted using a NucleoSpin RNA kit (Macherey-Nagel, Duren, Germany) and reverse transcribed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). RT-PCR was performed for detection of PDE4D mRNA using predeveloped specific primers (Hs01579625_m1, Applied Biosystems, Branchburg, NJ), TaqMan Universal PCR Master Mix (Applied Biosystems), and the StepOne Plus Real-Time RT-PCR System (Applied Biosystems). As described previously (22), predeveloped TaqMan reagent human 18S rRNA (Applied Biosystems) was included in the RT-PCR as an endogenous control. Data from the reaction were analyzed using StepOne Software v2.1 (Applied Biosystems).

PCR. mRNA expression of the PDE4D variants (PDE4D1, PDE4D2, PDE4D3, PDE4D5, PDE4D6) was assessed by gel based-PCR assay. Total RNA lysates were collected from nonasthmatic and asthmatic ASM cells at 6 h after formoterol (10^{-8} M) stimulation; RNA was then extracted, and reverse transcription was performed as described for RT-PCR (see above). We designed forward and reverse PCR primers according to primary sequence data relating to the variants of PDE4D1, PDE4D2, PDE4D3, PDE4D5, and PDE4D6 in the GenBank database. The sequence accession numbers used were PDE4D1: NM_001104631, PDE4D2: NM_006203, PDE4D3: NM_001165899, PDE4D5: AF012073, PDE4D6: AF536975. The primers (Geneworks, Thebarton, Australia) are shown in Table 2.

The amplifications were performed with 0.5U BIOTAQ Red DNA Pol (Bioline, London, UK). The cycle conditions commenced with an initial denaturation step at 95°C for 2 min. This was followed by denaturation at 95°C for 1 min, annealing at the temperature indicated in Table 2 for 1 min, and extension at 72°C for 2 min for the number of cycles indicated in Table 2. For all reactions, a final extension of 72°C for 5 min was performed in a Master cycler gradient (Eppendorf, Hamburg, Germany). PCR products were analyzed using 2% agarose in Tris-borate-EDTA gel electrophoresis, and the bands were visualized using SYBR safe DNA gel stain (Molecular Probes, Invitrogen, Eugene, OR).

Western blotting. Levels of PDE4D and GAPDH proteins were detected using Western blot, and analysis was conducted of lysates isolated from human ASM cells before and at various times following

treatment with formoterol (10^{-8} M) in the absence or presence of pretreatments with actinomycin D (5 μ g/ml) (Sigma) or a proteasome inhibitor, MG132, (Z-Leu-Leu-Leu-al) (10 μ M) (Sigma). Whole cell lysates were electrophoresed in buffer [50 mM Tris, 100 mM DTT, 2% (wt/vol) SDS, 0.1% (wt/vol) bromophenol blue, 10% (vol/vol) glycerol, pH 6.8]. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA), blocked in 3% milk in PBS-Tween20 and incubated with a 1:1,000 dilution of rabbit anti-human PDE4D (Santa Cruz Biotechnology, Santa Cruz, CA) for 14 h at 4°C. After being washed, membranes were incubated with a 1:1,300 dilution of horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG secondary antibody (DAKO, Glostrup, Denmark) for 2 h at room temperature. Levels of phosphorylated vasodilator-stimulated phosphoprotein (pVASP) were detected using mouse anti-human pVASP (5C6) (Santa Cruz Biotechnology). ASM cells were treated with formoterol (10^{-8} M) for 3 h or 24 h or stimulated with forskolin (10^{-5} M) for 5 or 15 min after pretreatment with formoterol (10^{-8} M) for 24 h. Cell lysates were isolated and electrophoresed as described as before. Immobilon Western chemiluminescence HRP Substrate (Millipore) was used to visualize the protein signals, and the bands were detected and analyzed using an Image Station 4000MM and software (Version 5.0) (Kodak Digital Science, New Haven, CT). The membranes were then stripped using stripping buffer (0.063 M Tris, pH 6.8, 2% SDS, 0.7% β -mercaptoethanol) at 50°C for 30 min and incubated with mouse anti-GAPDH monoclonal antibody (Millipore) for 1 h after blocking followed by incubation with an HRP-conjugated goat anti-mouse IgG secondary antibody (DAKO) for 1 h. The GAPDH bands served as control.

cAMP assay. cAMP production was assessed by stimulating ASM cells for 5 min with formoterol (10^{-8} M) or forskolin (10^{-5} M) after pretreatment with formoterol (10^{-8} M) for 3 or 24 h. Measurements of intracellular cAMP concentration were performed using the alpha screen cAMP kit (Perkin Elmer, Melbourne, Australia) as previously described (37). The pan-PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) was used at 0.5 mM.

PDE activity assay. Nonasthmatic ASM cells were treated with formoterol (10^{-8} M) for 5 min, 30 min, 3 h, or 24 h, and PDE activity was assayed in cell lysates using a cyclic nucleotide PDE assay kit (Enzo Life Science, Plymouth Meeting, PA) according to the manufacturer's protocol. The optical density at 620 nm was measured as PDE activity and normalized to the absorbance for the respective time point vehicle control. (Spectra Max M2, Molecular Devices, Downington, PA)

IL-6 ELISA. The amount of IL-6 in the cell culture medium was measured using an ELISA (with anti-IL-6 antibody pairs optimized for ELISA from BD Pharmingen, Franklin Lakes, NJ) as previously described (6). The limit of detection was 15.6 pg/ml.

Statistical analysis. Data were expressed as means $\pm$ SE and analyzed using GraphPad Prism version 5.00. The differences were assessed by *t*-test, one-way ANOVA, or two-way ANOVA using Bonferroni posttests as appropriate. A *P* value of <0.05 was considered statistically significant.

RESULTS

PDE4D mRNA was expressed in human ASM cells and upregulated by formoterol. There were no differences in the basal expression levels of PDE4D mRNA between nonasthmatic and asthmatic ASM cells (data not shown). In initial experiments formoterol (10^{-8} M) upregulated PDE4D transcripts at between 4–8 h in asthmatic and nonasthmatic ASM cells, both of which returned to basal levels by 24 h. TGF- β_1 (1 ng/ml) had no effect on PDE4D mRNA expression (Fig. 1, A and B).

Table 1. Patient demographics

Diagnosis	Age, yr	Sex	Sample	Smoking	Medication	FEV ₁ , % pred.	Experiment Where Used
Asthma	49	F	Bronch biopsy	NA	NA	NA	RNA
Asthma	20	M	Bronch biopsy	No	NA	NA	RNA (F+B)
Asthma	21	M	Bronch biopsy	No	NA	NA	RNA (F+B), IL-6 ELISA
Asthma	71	M	Resection for lymphoma	No	Bricanyl Pulmicort	57	RNA
Asthma	23	M	Bronch biopsy	No	NA	NA	RNA, RNA (F+B), PDE4D5 RNA, IL-6 ELISA
Asthma, Ca, asbestos exposure	69	M	Bronch biopsy	Ex-smoker	Seretide	NA	RNA
Asthma	45	M	Bronch biopsy	No	NA	99	RNA, PDE4D5 RNA
Asthma, COPD, large cell Ca	79	M	Resection	Ex-smoker	Seretide	49	RNA
Asthma	19	F	Bronch biopsy	NA	NA	NA	RNA, PDE4D5 RNA
Asthma	23	M	Bronch biopsy + brushing	NA	NA	82	RNA, RNA (F+B) IL-6 ELISA
Asthma, COPD, emphysema	51	F	Transplant	Ex-smoker	NA	NA	RNA (F+B), IL-6 ELISA
Asthma, Right middle lobe syndrome Seretide	59	F	Resection	No	Ventolin PRN	89	RNA
Asthma, spindle cell Ciclesonide	66	M	Resection	Ex-smoker	Ventolin PRN	NA	RNA
Asthma	39	M	Bronch biopsy + brushing	NA	NA	NA	RNA
Asthma	20	M	Bronch biopsy	No	NA	NA	RNA
Asthma	27	F	Bronch biopsy	NA	NA	88	IL-6 ELISA
Asthma	50	M	Bronch biopsy	NA	NA	NA	Western
Cystic fibrosis	28	M	Transplant	NA	NA	NA	Western
NSCC	70	F	Resection	No	NA	61	IL-6 ELISA
Mass	62	M	Resection	Yes	NA	88	IL-6 ELISA
Emphysema	49	M	Transplant	NA	NA	NA	RNA (F+B), IL-6 ELISA
Emphysema	52	F	Transplant	Ex-smoker	Salbutamol Ipratropium bromide	16	RNA (F+B), IL-6 ELISA
Pulmonary fibrosis	NA	M	Transplant	NA	NA	NA	RNA
NSCC	67	F	Resection	Ex smoker	NA	95	RNA
Bronchiectasis	60	M	Transplant	NA	NA	22	RNA
Emphysema	62	M	Transplant	NA	NA	36	RNA
BO post lung transplant	26	M	Transplant	NA	NA	NA	RNA
Emphysema	54	M	Transplant	NA	NA	62	Western, IL-6 ELISA
NSCC	56	M	Resection	Ex-smoker	NA	76	RNA, PDE4D5 RNA
Pulmonary fibrosis	62	M	Transplant	NA	NA	62	RNA, PDE4D5 RNA
Myelodysplastic syndrome	25	M	Transplant	NA	NA	NA	RNA, PDE4D5 RNA
NSCC	70	M	Resection	NA	NA	NA	RNA, IL-6 ELISA
Donor	45	F	Transplant	NA	NA	NA	IL-6 ELISA
Adeno Ca	70	M	Resection	Ex-smoker	NA	69	RNA, RNA (F+B), IL-6 ELISA
NSCC	63	M	Resection	Ex-smoker	NA	76	Western, IL-6 ELISA
Emphysema	64	M	Transplant	NA	NA	NA	Western
Adeno Ca	80	M	Resection	No	NA	73	Western (AD, pVASP), IL-6 ELISA
NSCC	75	M	Resection	NA	NA	NA	Western (MG132, pVASP), IL-6 ELISA
NSCC	71	M	Resection	No	NA	93	Western, Western (AD, MG132, SABA/LABA, pVASP)
Adeno Ca	75	M	Resection	Ex-smoker	NA	98	Western (SABA/LABA, pVASP), IL-6 ELISA
NSCC	72	M	Resection	Ex-smoker	NA	83	Western (AD, MG132, pVASP), IL-6 ELISA
IPF	65	M	Transplant	NA	NA	NA	PDE activity
Salcoidosis	57	M	Transplant	NA	NA	NA	Western, Western (pVASP), PDE activity, IL-6 ELISA
COPD	54	F	Transplant	NA	NA	NA	Western (SABA/LABA), PDE activity
Emphysema	53	F	Transplant	NA	NA	NA	Western, Western (pVASP)
Emphysema	43	F	Transplant	NA	NA	NA	Western, Western (pVASP)
NSCC	66	M	Resection	NA	NA	NA	Western, Western (pVASP), cAMP assay
Adeno Ca	65	M	Resection	Ex-smoker	NA	NA	cAMP assay
Healthy volunteer	20	M	Biopsy	No	NA	111	cAMP assay
Emphysema	44	F	Transplant	NA	NA	14	cAMP assay
Squamous cell Ca	66	M	Resection	Ex-smoker	NA	54	cAMP assay
NA	74	M	Resection	NA	NA	NA	cAMP assay
Pulmonary fibrosis	53	F	Transplant	NA	NA	NA	cAMP assay
NSCC	67	M	Resection	NA	NA	NA	PDE activity
Adeno Ca	61	M	Resection	NA	NA	NA	PDE activity

FEV₁, forced expiratory volume in 1 s; % pred., percent predicted; Bronch biopsy, bronchoscopic biopsy; NA, not available; RNA, phosphodiesterase (PDE)4D mRNA expression by formoterol; RNA (F+B), PDE4D mRNA expression by formoterol and budesonide; PDE4D5 RNA, PDE4D Splice variant expression; Western, PDE4D protein expression by formoterol; Western (AD), PDE4D protein expression by formoterol with actinomycin D; Western (MG132), PDE4D protein expression by formoterol with proteasome inhibitor; Western (LABA/SABA), PDE4D protein expression by β 2 agonists; PDE activity, PDE activity assay; IL6 ELISA, IL-6 release induced by β 2 agonists with and without actinomycin D/proteasome inhibitor; NSCC, Non-small cell carcinoma; Ca, carcinoma; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; BO, bronchiolitis obliterans; Western (pVASP); phosphorylated vasodilator-stimulated phosphoprotein expression by formoterol; cAMP assay, cAMP production induced by formoterol.

Table 2. Primers used in PCR experiments

Gene Product	Deoxynucleotide Sequence	Cycle Number	Product Size, bp	Annealing Temperature, °C
PDE4D1	sense 5'-TGG CGC CTT CAA GTG AGA AGC-3' antisense 5'-TCC CGC AGA TGT GCC ATT GTC C-3'	30, 36	643	48.5-64.1 (range)
PDE4D2	sense 5'-GCC CAC GAT AGC TGC TCA AAC A-3' antisense 5'-TGA CGG AGT GCC TGG TCT GTA G-3'	30, 36, 40	597	48.5-64.1 (range)
PDE4D3	sense 5'-GCG GAA TGG AAC CCT ATC TTG TCC-3' antisense 5'-TGA CGG AGT GCC TGG TCT GTA G-3'	28	627	61.5
PDE4D5	sense 5'-CGT TGC CCT GGA TTC TTG CCA-3' and antisense 5'-ACG CCG CTG TTT GGG GAT GT-3'	28	287	61.5
PDE4D6	sense 5'-TCT GCG TGG CTT GTG GAG GTT G-3' antisense 5'-TCC CTC TCT CGG TCT CCT TGG C-3'	30, 36	1192	48.5-64.1 (range)

PDE4D1, PDE4D2, and PDE4D6 primers did not detect specific products.

Budesonide did not reduce formoterol-induced PDE4D mRNA expression. PDE4D mRNA was dose dependently up-regulated by formoterol (10^{-12} M to 10^{-6} M) and budesonide (10^{-6} M) (Fig. 2, A and B). Combination treatment of budesonide and formoterol did not alter the formoterol effect at any concentration (Fig. 3, A and B).

PDE4D5 was the only splice variant of PDE4D expressed in ASM cells. We detected only PDE4D5 mRNA expression in both nonasthmatic and asthmatic ASM cells and formoterol-enhanced PDE4D5 mRNA expression (Fig. 4). PDE4D1, PDE4D2, PDE4D3, and PDE4D6 were not expressed in non-asthmatic and asthmatic ASM cells. PDE4D3 and PDE4D5 were, however, both expressed in a hepatic epithelial cell line and PDE4D5 in primary human airway epithelial cells (data not shown).

Formoterol did not alter PDE4D protein expression. Formoterol treatment did not alter PDE4D protein expression in nonasthmatic ASM cells at any of the time points examined (3–72 h, Fig. 5, 48- and 72-h time point data not shown), and asthmatic ASM cells exhibited the same pattern of expression ($n = 1$ data not shown). When nonasthmatic ASM cells were treated with the other β_2 AR agonists, salmeterol (10^{-7} M and 10^{-5} M), salbutamol (10^{-6} M), or isoprenaline (10^{-6} M), for 12 and 24 h, there was no effect on PDE4D protein levels (data not shown).

Pretreatment with actinomycin D (5 μ g/ml) or the proteasome inhibitor (MG132) (10 μ M) before and during formoterol (10^{-8} M) stimulation did not alter PDE4D protein in nonasthmatic ASM cells at 12 and 24 h (actinomycin D) and 3, 6, 9, and 24 h (MG132) (Fig. 6, 24-h time point data).

Formoterol changed phosphorylated VASP protein expression. We used the phosphorylation of VASP as a surrogate measure-

ment of cAMP (without inhibiting PDEs). First, we measured pVASP protein expression at 3 and 24 h after formoterol stimulation in ASM cells. We found that formoterol stimulation increased pVASP at 3 h but not at 24 h (Fig. 7, A and B). To ensure that the cAMP-VASP pathway was still capable of activation at 24 h we carried out the same experiment, but this time we stimulated the cells at the end of the 24-h treatment period with forskolin (a direct activator of cAMP) and found that pVASP was induced to a similar level in both β_2 -agonist treated and nontreated cells (Fig. 7B).

Formoterol induced β_2 AR desensitization. Following 24 h treatment with formoterol (10^{-8} M) cAMP was not induced; however, 5-min stimulation by formoterol (10^{-8} M) without any formoterol pretreatment increased cAMP production. In the cells with 24-h pretreatment of formoterol, decreased formoterol-induced (5 min, 10^{-8} M) cAMP occurred compared with vehicle. In contrast, desensitization of β_2 AR-cAMP axis did not occur following 3-h pretreatment with formoterol (10^{-8} M) (Fig. 8).

PDE activity in ASM cells was not changed by formoterol treatment. PDE activity did not change following formoterol (10^{-8} M) treatment for up to 24 h (Fig. 9).

IL-6 release from ASM cells. Formoterol (10^{-8} M), salmeterol (10^{-7} M, 10^{-5} M), salbutamol (10^{-6} M), and isoprenaline (10^{-6} M) treatment significantly increased IL-6 secretion in nonasthmatic ASM cells after the 6-h time point (data not shown). Budesonide inhibited the release of IL-6 induced by formoterol in nonasthmatic and asthmatic ASM cells. Actinomycin D inhibited IL-6 release following formoterol stimulation, whereas a proteasome inhibitor (MG132) upregulated IL-6 production induced by formoterol at 24 h (Fig. 10).

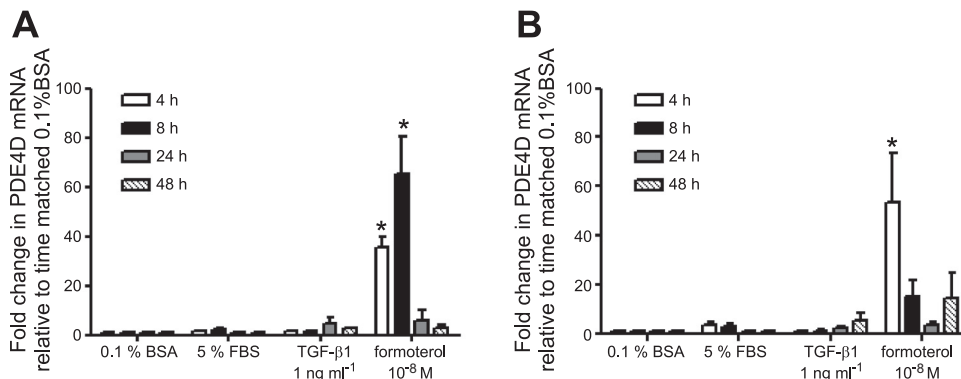
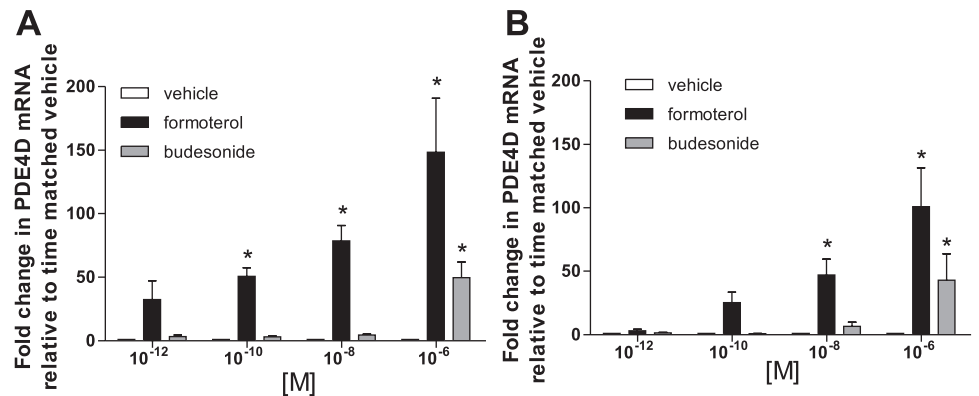


Fig. 1. Regulation of phosphodiesterase (PDE)4D mRNA expression by 5% FBS, formoterol, or transforming growth factor (TGF)- β 1 in airway smooth muscle (ASM) cells. ASM cells from nonasthmatic (A, $n = 3$) and asthmatic (B, $n = 5$) subjects treated with 5% FBS, formoterol (10^{-8} M), or TGF- β 1 (1 ng/ml) for 4, 8, 24, and 48 h. Data are expressed as means $\pm$ SE. * $P < 0.05$, significantly different from 0.1% BSA, 2-way ANOVA.

Fig. 2. Regulation of PDE4D mRNA expression by formoterol or budesonide in ASM cells. ASM cells from nonasthmatic (A, $n = 7$) and asthmatic (B, $n = 7$) subjects treated with formoterol (10^{-12} to 10^{-6} M) or budesonide (10^{-12} to 10^{-6} M) for 6 h. Data are expressed as means $\pm$ SE. * $P < 0.05$, significantly different from vehicle, 2-way ANOVA.



DISCUSSION

We found a rapid and transient increase in β 2AR agonist-induced PDE4D mRNA in both asthmatic and nonasthmatic ASM cells, and this was not affected by concomitant treatment of the cells with corticosteroids. This upregulation of the mRNA did not result in either increased PDE4D protein or increased enzyme activity. In contrast, IL-6 protein, which is known to be produced in response to elevated cAMP, was induced by β 2AR agonists, and the levels of production were reduced by concomitant treatment with budesonide. To investigate why new PDE4D protein was not detected, we pretreated the cells with actinomycin D or a proteasome inhibitor (MG132) before and during formoterol stimulation. No alteration in PDE4D protein was found, demonstrating that PDE4D protein is not rapidly degraded, and its presence in ASM is not dependent on continuous transcription or translation from mRNA. This leads us to speculate that continual therapy with β 2AR agonists is unlikely to cause PDE4D-mediated tachyphylaxis.

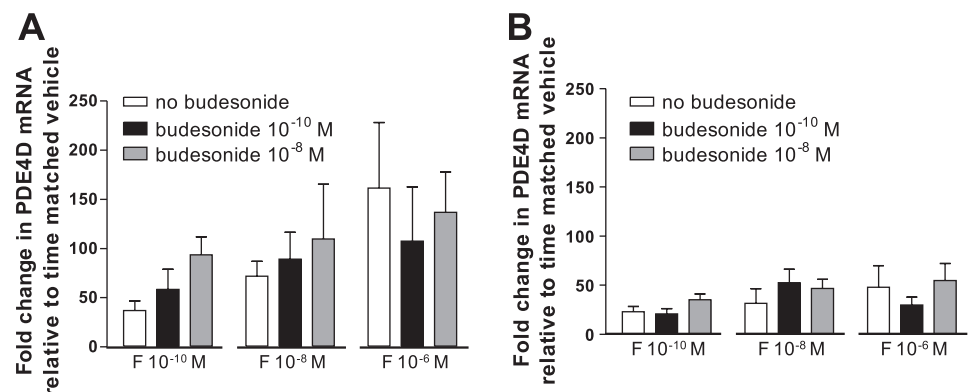
Initially we used RT-PCR to establish whether PDE4D was expressed in human ASM cells, and we found that mRNA encoding PDE4D was present. Because Hall et al. (4, 24, 26) have previously shown that PDE4, and in particular PDE4D5, is responsible for the vast majority of cAMP catalytic activity in human ASM cells, we focused our experimental approach on this particular isoform. We have also confirmed these findings and furthermore shown that PDE4 regulates β 2AR agonist-induced cAMP in ASM from people with asthma (38).

In keeping with findings from Grunstein's group (19, 28, 29) and Hall's group we found that β 2AR agonist dose dependently induced PDE4D mRNA in nonasthmatic ASM cells. We

further extended this observation by assessing β 2AR agonist-induced PDE4D mRNA in asthmatic cells, and found similar upregulation in these cells. To explore whether this upregulation was specific to PDE4D or a general feature of PDEs, we also measured two other PDEs, PDE3A and PDE7A. Although the mRNA for both was expressed in ASM cells, PDE3A was slightly upregulated by formoterol and PDE7A expression was not altered (data not shown).

Given that LABAs are seldom used as monotherapy, we next investigated whether concurrent treatment with steroids could inhibit the β 2AR agonist-induced PDE4D mRNA. There are some studies showing that steroids decrease PDE4D mRNA induced by β 2AR agonists in human ASM cells (29) and in the lungs of allergic rats (35). Surprisingly, in our experiments the corticosteroids did not affect the production of β 2AR agonist-induced PDE4D mRNA. Corticosteroids are potent anti-inflammatory agents that are known to inhibit the production of many mediators in human ASM cells. Because we and others have previously shown that IL-6 production can be induced by β 2AR agonists (1, 7) and IL-6 production is inhibited by corticosteroids (32), we also assessed IL-6 production in this study. As expected, we found that β 2AR agonist-induced IL-6 was inhibited by corticosteroids, demonstrating that these drugs were active and the concentrations examined in our study were able to affect responses in the ASM cells. This result also suggests that PDE4D mRNA production is steroid insensitive. Although the exact reason for the lack of effect of the steroid on PDE4D production is beyond the scope of this study, it is possible that the glucocorticoid response-binding element on the PDE4D promoter is

Fig. 3. Effect of concurrent β 2-adrenergic receptor (β 2AR) agonist and steroid on PDE4D mRNA in ASM cells. ASM cells from nonasthmatic (A, $n = 4$) and asthmatic (B, $n = 4$) subjects were treated with formoterol (F: 10^{-10} M, 10^{-8} M, 10^{-6} M) with and without budesonide (10^{-10} M, 10^{-8} M) for 6 h. Data are expressed as means $\pm$ SE, 1-way ANOVA.



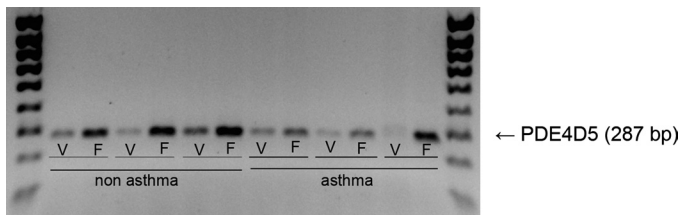


Fig. 4. PCR of transcripts for PDE4D subtypes in ASM cells. ASM cells from nonasthmatic and asthmatic subjects exposed to formoterol (10^{-8} M) for 6 h. After the 6-h treatment period, RNA was prepared, reverse transcribed into cDNA, and amplified using primer sets specific for PDE4D5. The image shows PCR product from 3 nonasthmatic cells and 3 asthmatic cells, which is representative of the result from nonasthmatic cells $n = 5$ and asthmatic cells $n = 7$. V, vehicle; F, formoterol.

not functional. An alternative explanation for the lack of effect of the corticosteroid may be that the overall production of the various PDE4D isoforms was not altered (as measured by the RT-PCR that we carried out), but an alteration in the levels of the different isoforms was occurring. Using PCR we initially investigated which PDE4D isoforms were present in the ASM cells and for the first time were able to show that human ASM cells only produce the PDE4D5 splice variant. In addition, we found using RT-PCR that it was this variant that was upregulated by the β 2AR agonist. Therefore it is highly unlikely that corticosteroids caused a change in the PDE4D splice variant produced.

We then measured the level of PDE4D protein using Western blotting and to our surprise found no increase in protein following treatment with β 2AR agonists or no change in PDE4D levels when the cells were treated with β 2AR agonists and corticosteroids.

The first report of β 2AR desensitization in human tracheal smooth muscle was made by Hall et al. (13). In their study they found that, when following direct stimulation of adenylate

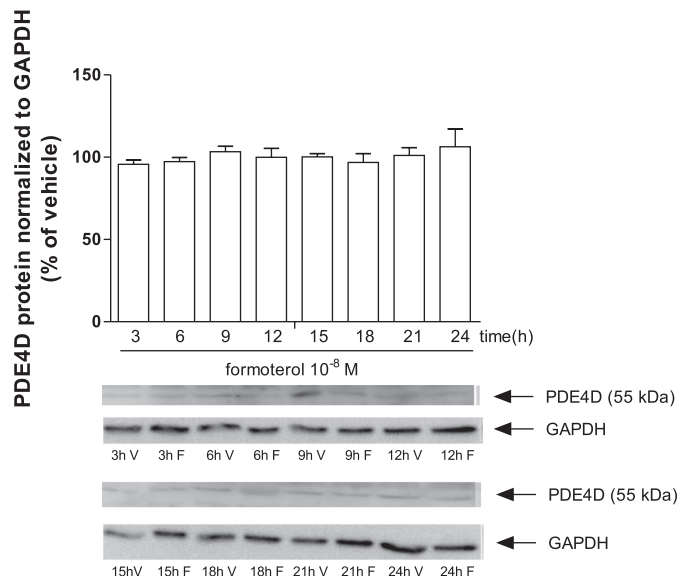


Fig. 5. Western blot analysis of PDE4D protein in ASM cells. ASM cells from nonasthmatic subjects were stimulated by formoterol (10^{-8} M) for 3, 6, 9, 12, 15, 18, 21, and 24 h and then lysed, and PDE4D protein was analyzed by Western blotting, using GAPDH as the loading control ($n = 4-6$). Data are expressed as percentage of time-matched vehicle and expressed as means $\pm$ SE, 1-way ANOVA. Representative image of Western blots of proteins from a nonasthmatic cell culture are shown under the graph.

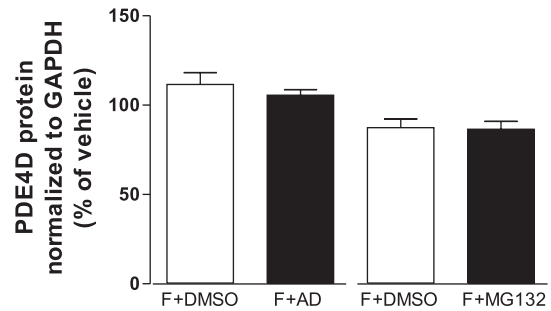


Fig. 6. Western blot analysis of PDE4D protein in ASM to elucidate evidence for alteration in protein expression or degradation of PDE4D. ASM cells from nonasthmatic subjects were treated for 30 min with actinomycin D (AD: 5 μ g/ml) or a proteasome inhibitor (MG132: 10 μ M) before and during formoterol (10^{-8} M) stimulation for 24 h. Cells were lysed, and PDE4D protein was analyzed by Western blotting, using GAPDH as the loading control. Data are expressed as percentage of time-matched vehicle and expressed as means $\pm$ SE ($n = 3$), 1-way ANOVA.

cyclase with forskolin, no impairment of cAMP occurred, leading the authors to suggest that β 2AR agonist-induced β 2AR desensitization did not involve altered PDE activity. Interestingly, in the same study, the authors also found that the β 2AR desensitization was not reversed by steroids (13). In a latter study by the same group, different results were obtained (24). They found that β 2AR agonists increased PDE4 mRNA, as we found. However, they also found that PDE4D5 activity was also increased. The increased PDE4D5 activity was due to increased intrinsic enzymatic activity and increased translation. We did not specifically measure PDE4D5 activity; rather we measured total PDE cAMP hydrolyzing activity. Hence it is possible that the increased PDE4D5 activity is a relatively minor change compared with total PDE activity. This notion is further supported by their own earlier findings (13) and our finding that similar VASP phosphorylation (indicating cAMP signaling) occurred when cells were first exposed to β 2AR agonists for 24h and were then stimulated with forskolin in the absence of a PDE inhibitor. It is also possible that the increased enzymatic activity of PDE4 following β 2AR agonist stimulation was not measurable in our study, as it has been shown to be rapid, occurring 1–2 min poststimulation and returning to basal activity following 4 min of stimulation (40).

These results are the opposite of those found by Nino et al. (28, 29). In their study β 2AR agonists increased PDE4D mRNA and protein assessed by immunoblot analysis. We also found increased mRNA but could not detect changes in either PDE4D protein or PDE activity. There are two possible reasons for this difference. The first is that the antibody that was used by Nino et al. specifically detects rabbit PDE4D; therefore their results might be derived from experiments in rabbit cells, and thus the difference in the two studies represents species variation. We have evaluated a different anti-rabbit PDE4D antibody and found no crossreactivity with human PDEs. The second possibility relates to the use of commercial cell lines (most likely from a single donor, although this is not specified in their methods). These commercial cells are known to have many differences compared with primary cells. For example, they can be passaged (and used) at least 15 times from the time of purchase. In comparison primary cells have a usable lifespan of up to and including eight passages from the initial isolation. It is possible that these commercial cells have a different

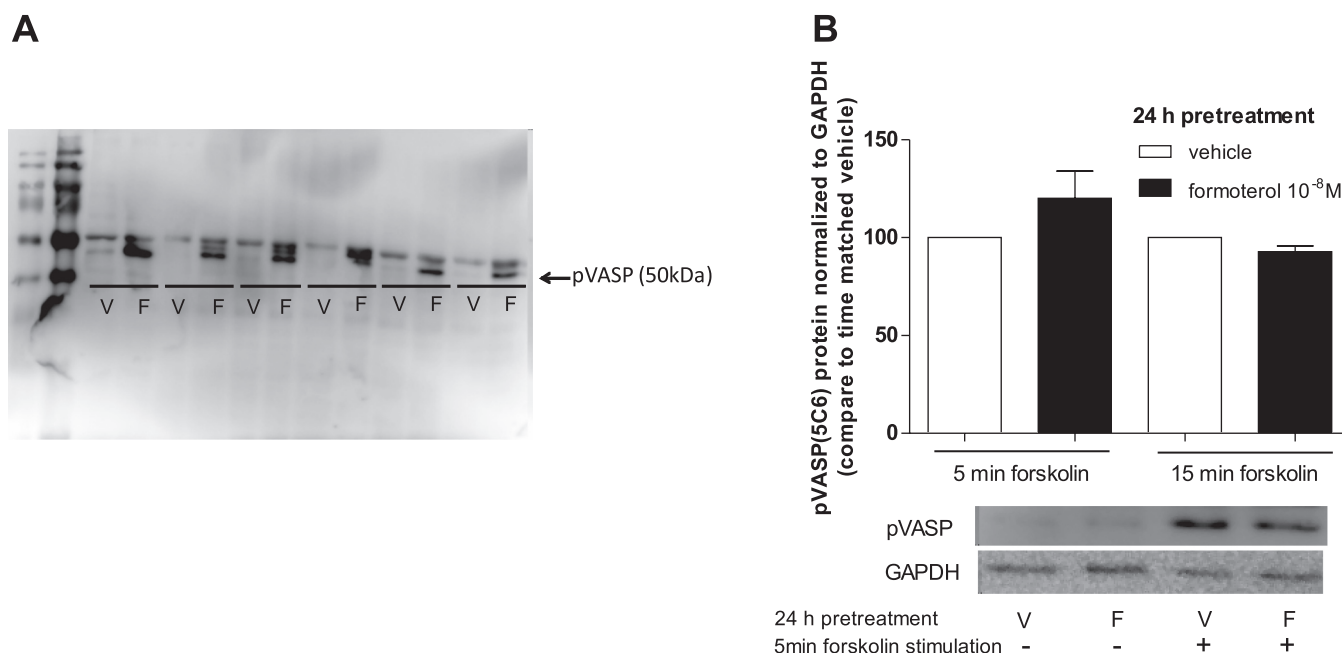


Fig. 7. Western blot analysis of phosphorylated vasodilator-stimulated phosphoprotein (pVASP) protein in ASM cells to investigate formoterol-induced cAMP. ASM cells from nonasthmatic subjects were treated with formoterol (10^{-8} M) for 3 h (A, $n = 6$). ASM cells from nonasthmatic subjects were treated with formoterol (10^{-8} M) for 24 h, and then stimulated with forskolin (10^{-5} M) for either 5 or 15 min (B $n = 3$). Cells were lysed, and pVASP protein was analyzed by Western blotting, using GAPDH as the loading control. Data are expressed as percentage of time-matched vehicle and expressed as means $\pm$ SE, 1-way ANOVA. Representative image of Western blots of proteins from a nonasthmatic cell culture are shown under the graph.

cellular physiology, or the original donor had abnormal physiology, hence the difference in the two studies. In our study we have looked at PDE protein levels in ASM cells isolated from over 10 individuals and have no evidence for upregulation in any of the cells tested. Furthermore we also confirmed our findings of no alteration in β 2AR-induced cAMP following chronic β 2AR agonist treatment by assessing the phosphorylation of VASP. We did observe β 2AR desensitization as others have also found; however, when we directly activated adenylyl cyclase using forskolin, cAMP production (assessed by phosphorylation of VASP) was not altered. As the importance of VASP in mediating β 2AR agonist effects has recently been illustrated by Goncharova et al. (12), we think that it is

highly likely that no functional alteration of PDE activity occurs following chronic stimulation with β 2AR agonists. In our experiments we did not have a positive control stimulus in the PDE activity assay, and therefore, and as alluded to above, our lack of change in PDE activity may reflect an inability of the assay to capture changes in specific PDE isoforms that regulate β 2AR agonist-induced cAMP.

Initially we hypothesized that the β 2AR agonist transiently upregulated the protein, so we then measured PDE4D levels at three hourly intervals up to 24 h and 48 and 72 h poststimulation. The peak induction of the mRNA occurred between 4 and 8 h and returned to baseline at 24 h, suggesting that the time course over which we measured PDE4D protein would capture

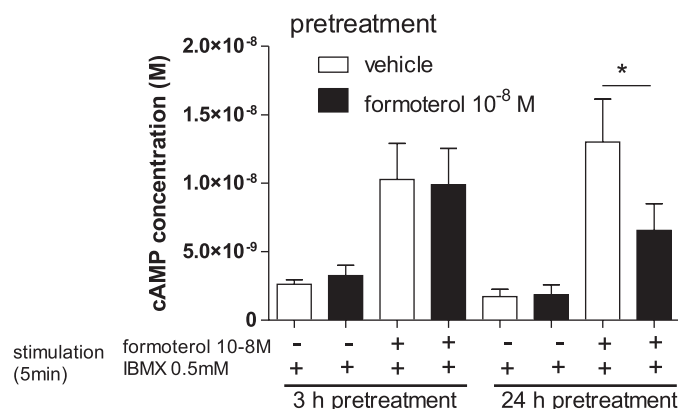


Fig. 8. cAMP regulation by formoterol in ASM cells. Cells were stimulated for 5 min with or without prior formoterol (10^{-8} M) pretreatment (10^{-8} M) for 3 or 24 h. cAMP degradation was inhibited by addition of a pan-PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX) 0.5 mM ($n = 8$). Data are expressed as means $\pm$ SE. * $P < 0.05$, significantly different from vehicle, t -test.

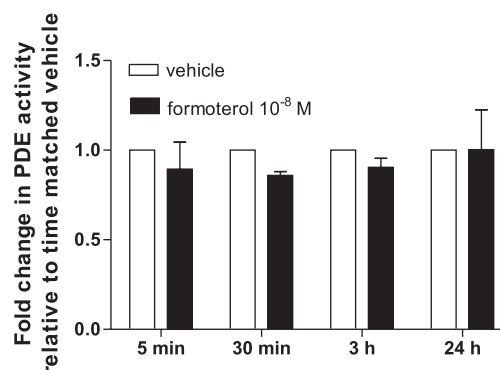


Fig. 9. The effect of β 2AR agonist on PDE activity in ASM cells. PDE activity was assayed in nonasthmatic human ASM cell lysates using a colorimetric assay kit. Cell lysates from nonasthmatic subjects were collected after treatment with formoterol (10^{-8} M) for 5 min, 30 min, 3 h, and 24 h, and outer diameter was measured ($n = 5$). Data are expressed as fold increase compared with time-matched vehicle and expressed as means $\pm$ SE, 1-way ANOVA.

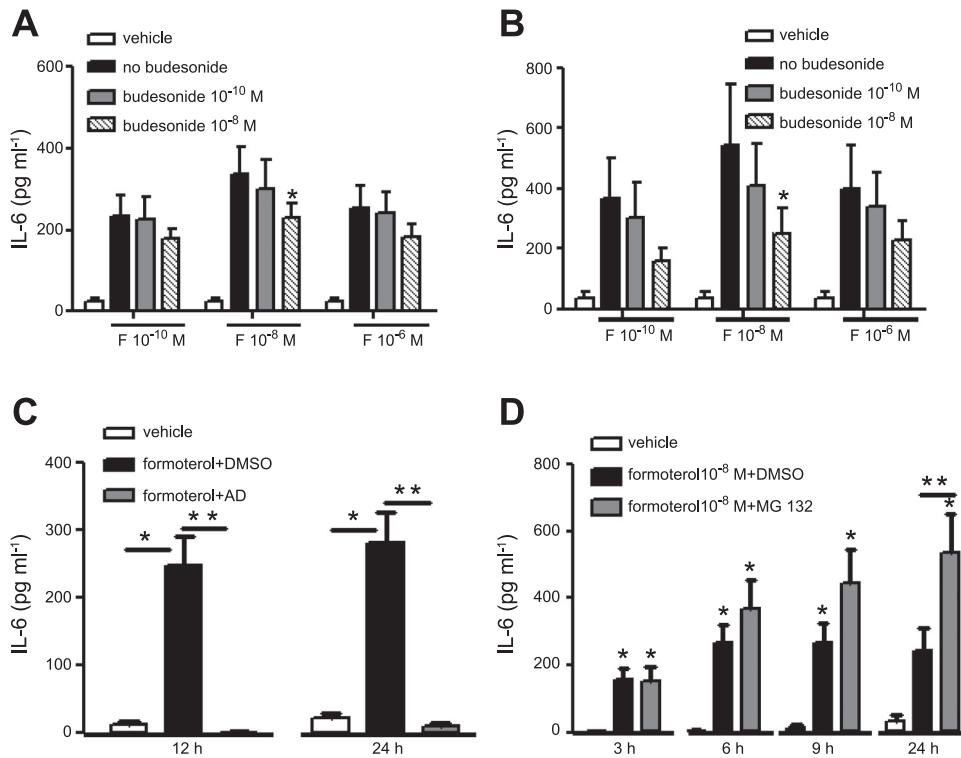


Fig. 10. Effect of formoterol and budesonide on IL-6 release. ASM cells from nonasthmatic and asthmatic subjects were treated with formoterol (10^{-10} M, 10^{-8} M, 10^{-6} M) with and without budesonide (10^{-10} M, 10^{-8} M) for 6 h, and supernatants were collected. (A: nonasthmatic cells, $n = 6$; B: asthmatic cells, $n = 4$). Data are means $\pm$ SE, $*P < 0.05$, significant difference from no budesonide, 2-way ANOVA. Effect of actinomycin D or MG132 on IL-6 release by formoterol. ASM cells from nonasthmatic subjects were treated with actinomycin D ($5 \mu\text{g ml}^{-1}$) or MG132 ($10 \mu\text{M}$) for 30 min before and during formoterol (10^{-8} M) stimulation, and then supernatants were collected at 12 and 24 h (actinomycin D) and 3, 6, 9, and 24 h (MG132). ($n = 4$) (C: actinomycin D; D: MG132). Data are means $\pm$ SE, $*P < 0.05$, significantly different from corresponding vehicle, $**P < 0.05$, significantly different from corresponding formoterol + DMSO, A: 1-way ANOVA, B: repeated-measures 1-way ANOVA.

any changes in production. We found no evidence of increased PDE4D protein and next hypothesized that we were unable to detect the changes in PDE4D protein, as it was being rapidly degraded by proteasomes. In similar experiments we inhibited proteasomic activity and measured PDE4D protein with and without β 2AR agonist stimulation. Inhibition of proteasomic activity did not result in altered PDE4D protein but did increase the amount of IL-6 protein, once again demonstrating that the treatment was active. To examine the transcriptional dependence, we also treated the ASM cells with actinomycin D and similarly found no alteration in PDE4D protein levels.

It is possible that PDE4D protein levels were changing, but the changes were too subtle to be detected using Western blotting; we therefore measured PDE hydrolytic activity. As we had found with the PDE4D protein measurements, we also found no changes in the hydrolytic activity following treatment with β 2AR agonist. Collectively these results suggest that β 2AR agonists do not alter the amount of PDE4D in human ASM cells.

These findings, while negative, are important in view of the recent Food and Drug Administration (FDA) boxed warning on the use of LABAs as monotherapy for the treatment of asthma. This warning is based on the reporting of a number of adverse outcomes, but several prominent asthma researchers have made some important observations highlighting the inconsistencies in the FDA ruling. This is a complex issue, the intricacies of which are discussed in recent review articles and editorials (2, 33, 36).

On a cellular level it was natural to hypothesize that prolonged use of β 2AR agonists was either desensitizing the β 2AR (11, 27) or upregulating PDEs, which would act to limit the effectiveness of β 2AR agonists (19, 28, 29). We and others have previously reported that β 2AR desensitization occurs following chronic β 2AR exposure (13). In this study we showed that formoterol treatment for 24 h decreased β 2AR

agonist-induced cAMP levels using cAMP assay, as a result of desensitization of the β 2AR. Because the measurement of cAMP in ASM cells is only possible in the presence of a PDE inhibitor, such as IBMX, that prevents cAMP degradation, in this experiment, IBMX was used at the standard concentration of 0.5 mM. Therefore to assess cAMP with intact PDE activity, we performed Western blot analysis of phosphorylated VASP protein. We found that formoterol treatment for 24 h did not affect forskolin-induced phosphorylation of VASP, and therefore it is reasonable to assume that cAMP was also not affected. In our experimental conditions, our data suggest that continual therapy with β 2AR agonists is unlikely to cause PDE4-mediated tachyphylaxis of the β 2AR. Furthermore, although the treatment of asthma is superior when corticosteroids and β 2AR agonists are used in combination, several studies have shown that people with asthma continue to respond to β 2AR agonists. However, there are circumstances in which β 2AR agonists are known to be ineffective in the treatment of asthma. Specifically, viral infections inhibit β 2AR agonist-induced bronchodilation in vivo (31) by causing β 2AR desensitization (37). It is also worth considering the possibility that PDE4 levels are modulated by other stimuli in ASM cells (19).

In conclusion, this study adds a substantial number of new findings pertinent to the treatment of human disease. We show, for the first time, that PDE4D5 is predominantly expressed in human ASM cells from people with and without asthma and that formoterol does not upregulate PDE4D protein production. This leads us to speculate that continual therapy with β 2AR agonists is unlikely to cause PDE4-mediated tachyphylaxis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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